The Trypanosomiases

Edited by
I. Maudlin,
P.H. Holmes and
M.A. Miles

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Contents

PART 7. VECTOR CONTROL

Colour plate section after p. 302

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Preface

It is over 30 years since the publication of *The African Trypanosomiases* (edited by H.W. Mulligan), which reviewed both state-of-the-art laboratory science and measures in place for disease control in Africa. The editors of the present work have tried to maintain this broad perspective and extend it to the problems of trypanosomiasis outside Africa, with sections devoted to the trypanosomiases of America and Asia. Given the advances made in science in the intervening years, an encyclopaedic coverage of the subject would now be almost impossible and so we have been forced to be selective in the topics covered. However, we are delighted to have assembled so many of world's leading experts to contribute to this update of 'Mulligan'. We hope it will have a similar impact on scientific advancement and disease control.

Enormous progress has been made in those 30 years in a number of completely unrelated areas, many of which have impacted on the study and control of trypanosomiasis, including molecular biology, insect behaviour, geographical information systems (GIS) and disease impact evaluation. Molecular biology was in its infancy in 1970 and did not rate a mention in Mulligan's text. This subject has mushroomed to the extent that it now affects all branches of biology and medicine and has had no less an impact on studies of the trypanosome. Indeed the trypanosome, because of its molecular idiosyncrasies and the ease with which it can be grown in culture, has become a model organism. Genome projects are under way to sequence *Trypanosoma brucei* and *T. cruzi* and others are in the pipeline. Molecular studies have laid bare the evolutionary history of the trypanosome, given insight into the relationships between species, and revealed genetic recombination and hybridization. We now know much of the way the trypanosome works at the molecular level but, so far, little in the way of practical benefit has been gained from this enormous effort. It is easy to belittle such 'blue skies' research but we must be patient – we have only recently celebrated the 50th anniversary of the firing of the Watson–Crick starting gun. We are beginning to see the first fruits of this work; the gene that confers human infectivity to *T. brucei rhodesiense* has recently been identified and we are, at last, able to distinguish it from its non-human infective relative, *T. brucei brucei*. The next 10 years should see enormous progress in this area.

By contrast, studies of vector behaviour have brought very practical benefits in a relatively short period of time. As a result of ground-breaking studies on tsetse behaviour we are now able to attract tsetse from long distances and, with a variety of devices, reduce their populations to levels that are not threatening. In one case (admittedly an island), the population has

been eradicated. Such achievements have stimulated renewed interest in the possible elimination of tsetse from large areas of Africa and there is now unprecedented international interest in developing such initiatives through PAAT (Programme Against African Trypanosomiasis) and more recently PATTEC (Pan-African Tsetse and Trypanosomiasis Eradication Campaign). In comparison, major advances in vector elimination have already been made in South America. Research on the vectors of *T. cruzi* led to the control strategies adopted by the Southern Cone Initiative, which has dramatically reduced the transmission of disease in South America. The Southern Cone Initiative provides a model for the role of research collaboration in stimulating regional cooperation, in mobilizing political will, and in driving international control programmes.

GIS combined with satellite imagery is beginning to provide useful tools for combating disease. In the case of tsetse, the likely distribution of the fly can now be predicted with some accuracy. This science is also moving at a rate that should enable us, before too long, to predict outbreaks of disease.

Nevertheless, much remains to be done, especially in the field of chemotherapy. New drugs for the treatment of trypanosomiasis both in humans and in domestic livestock remain elusive. Highly toxic arsenicals still dominate the treatment of human African trypanosomiasis. It is even difficult to persuade manufacturers to continue supplying the limited range of drugs currently available. The World Health Organization (WHO) and Medicins Sans Frontieres (MSF), through the Drugs for Neglected Diseases initiative (DNDi), are addressing this problem by appealing to the pharmaceutical industry to maintain supplies and involving the industry, with its enormous resources for screening, in the search for new, less toxic compounds. Although drugs remain readily available for the treatment of animal African trypanosomiasis, they are all based on molecules developed over 40 years ago. Reflecting the situation in Africa, little progress has been made in the treatment of Chagas disease, which is still reliant on two very old compounds (nifurtimox and benznidazole).

However many scientific breakthroughs are made, in the end disease control is dependent on resources. In the case of the Southern Cone Initiative, a simple cost–benefit case was made that produced funding from the countries directly involved. Control of trypanosomiasis in Africa presents a much greater economic problem for the affected countries, which are struggling to provide resources to deal with a variety of problems including AIDS, malaria and tuberculosis. Economic analysis reveals that when someone requires treatment for human African trypanosomiasis this may cost their family a quarter of a year's income. Among health interventions, controlling human trypanosomiasis ranks very highly in terms of cost-effectiveness and is good value for money, whatever the source of the funds – donor or affected country.

Time is of the essence, especially in sub-Saharan Africa. Whereas in the 1970s we could consider human African trypanosomiasis a disease on the retreat, we appear to have taken a step backwards with epidemics of Gambian sleeping sickness sweeping central Africa. The causes of these outbreaks are essentially bound up with societal problems, which are not open to the reductionist approach that molecular biology offers. These diseases primarily affect people and their domestic livestock in rural settings in developing countries; in other words the poorest of the poor. Major international efforts supported by substantial funding to both monitor and treat infections could make huge improvements to the lives of millions of Africans. However, a glance at the area affected by human African trypanosomiasis shows that this disease thrives where conflict holds sway. Good governance would go some way towards alleviating and even preventing the problems this disease brings in its wake; political changes are afoot in the affected regions of Africa that offer hope for the future.

We hope that this new textbook will be of value both to bring the established scientist up to date with ongoing research across the field of trypanosomiasis and to provide food for thought for the new student of the subject – on whom we rely for scientific advances in the

future. Finally we hope that it will help to attract the attention of the decision makers to the plight of millions of people who suffer in their daily lives from the constraints of trypanosomiasis and who depend on the commitment of governments and donors to allocate sufficient resources to bring an end to the worldwide misery these organisms wreak.

Editorial note

We have used the established term 'trypanosomiasis' throughout this book, despite the recent use of the term 'trypanosomosis' by some veterinary parasitologists. Our reason for using the established terminology was to reflect the wishes of the vast majority of the authors and to achieve consistency.

Ian Maudlin,
Peter Holmes,
Michael Miles
August 2003

Acknowledgements

We wish to express our most sincere thanks to the contributors to this book for the high standard of their contributions and kind cooperation. The scientists who have prepared the various chapters are all extremely busy people and the work involved was considerable.

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Ian Maudlin,
Peter Holmes,
Michael Miles

The Technical Centre for Agricultural and Rural Cooperation (CTA) was established in 1983 under the Lomé Convention between the ACP (African, Caribbean and Pacific) Group of States and the European Union Member States. Since 2000, it has operated within the framework of the ACP-EC Cotonou Agreement.

CTA's tasks are to develop and provide services that improve access to information for agricultural and rural development, and to strengthen the capacity of ACP countries to produce, acquire, exchange and utilise information in this area. CTA's programmes are designed to: provide a wide range of information products and services and enhance awareness of relevant information sources; promote the integrated use of appropriate communication channels and intensify contacts and information exchange (particularly intra-ACP); and develop ACP capacity to generate and manage agricultural information and to formulate ICM strategies, including those relevant to science and technology. CTA's work incorporates new developments in methodologies and cross-cutting issues such as gender and social capital.

PART 1.
BIOLOGY OF TRYPANOSOMES

1 Systematics of Trypanosomes of Medical and Veterinary Importance

Jamie R. Stevens and Sylvain Brisse

Introduction

Since the publication of the comprehensive 'pre-molecular' texts on trypanosomes and trypanosomiasis by Mulligan (1970) and Hoare (1972), much new biochemical and molecular data relating to the systematics and phylogeny of trypanosomes has become available. Accordingly, this chapter will focus primarily on species and taxonomic groups of medical and veterinary interest for which new evidence is available to confirm or readdress their systematic position and evolutionary relationships.

Species of trypanosome infecting mammals fall into two distinct groups and, accordingly, have been divided into two sections (Hoare, 1972): (A) the Stercoraria (subgenera *Schizotrypanum*, *Megatrypanum* and *Herpetosoma*), in which trypanosomes are typically produced in the hindgut and are then passed on by contaminative transmission from the posterior; and (B) the Salivaria (subgenera *Duttonella*, *Nannomonas*, *Trypanozoon*), in which transmission occurs by the anterior station and is inoculative; characteristically, salivarian species, by virtue of variant surface glycoprotein (VSG) genes, are the only trypanosomes to exhibit antigenic variation.

The systematic position of *Trypanosoma* among the protozoa and the revised classification of the mammalian trypanosomes according to Levine *et al.* (1980) (but, see also Corliss, 1994) is as follows:

Subkingdom PROTOZOA Goldfuss, 1818; emend. Levine *et al.* (1980).
Phylum SARCOMASTIGOPHORA Honigberg and Balaniuth, 1963; emend. Levine *et al.* (1980).
Subphylum MASTIGOPHORA Diesing, 1866; emend. Levine *et al.* (1980).
Class ZOOMASTIGOPHOREA Calkins, 1909.
Order KINETOPLASTIDA Honigberg, 1963.
Family TRYPANOSOMATIDAE Doflein, 1901; emend. Grobben, 1905.
Genus *Trypanosoma* Gruby, 1843.

Section A: Stercoraria

Subgenus *Megatrypanum* Hoare, 1964

Trypanosoma *(*Megatrypanum*)* theileri *Laveran, 1902*

Synonyms: *T. transvaaliense* Laveran, 1902; *T. tragelaphi* Kinghorn *et al.*, 1913 (*p.p.*); *T. cephalophi* Bruce *et al.*, 1915 (*p.p.*).

Trypanosoma (*M.*) *theileri* is a common parasite of domestic and wild ruminants worldwide. This very large trypanosome was discovered by Theiler in 1903 in South

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Africa in the blood of cattle and is the type species of subgenus *Megatrypanum*.

MORPHOLOGY *T. theileri* is one of the largest mammalian trypanosomes (mean length 60–70 μm, occasionally up to 100 μm). The posterior end of the body is pointed; the large kinetoplast is typically near the nucleus, which occupies a more or less central position; the undulating membrane is well developed and there is a long free flagellum.

HOST RANGE AND PATHOGENICITY *T. theileri* has a cosmopolitan distribution among domestic cattle, while in Africa it is also found in various antelopes. Generally, infection is cryptic and is usually detected by blood culture; there is no evidence of any specific pathological changes attributable to *T. theileri* and its effect on the bovine host is controversial (Hoare, 1972). Tabanid flies have been shown to be the major vector (Hoare, 1972).

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION Few biochemical and molecular characterization studies have been undertaken with *T. theileri*.

Isoenzymes All ten *T. theileri* isolates from European cattle (*Bos taurus*) characterized by Bose *et al.* (1993) showed identical isoenzyme patterns, which differed from those of *Megatrypanum* species orginating from deer. By contrast, *Megatrypanum* spp. from deer grouped into at least three distinct clusters, one of which was specific to roe deer (*Capreolus capreolus*).

Karyotype The chromosomes of *Megatrypanum* species range in size from 300 kilobase pairs (kb) to 2.2 megabase pairs (Mb); also in contrast to salivarian trypanosomes, mini-chromosomes (40–200 kb) were not observed (Bose *et al.*, 1993). In overall size range, the molecular karyotype of *Megatrypanum* spp. is more similar to that of *Leishmania* than *Trypanosoma*, although the high level of karyotype polymorphism is similar to that of *Trypanosoma* (Bose *et al.*, 1993).

DNA sequence analysis The 18S ribosomal RNA (rRNA) gene sequences (Stevens *et al.*, 1999a) of *T. theileri* and a deer-derived *Megatrypanum* sp. (Bose *et al.*, 1993) were all but identical, varying by only a single nucleotide. Phylogenetic analysis of the 18S rRNA gene (Stevens *et al.*, 1999a) (Fig. 1.1) clustered these isolates tightly together (100% bootstrap support) in a clade showing no affinity with any other *Megatrypanum* species, but close to *T. cyclops*, a trypanosome isolated from a Malaysian primate.

Subgenus *Herpetosoma* Doflein, 1901

The great majority of trypanosomes of subgenus *Herpetosoma* parasitize rodents and are not frequently implicated as agents of trypanosomiasis of medical and veterinary importance (see also *T.* (*S.*) *rangeli* below).

Trypanosoma (Herpetosoma) lewisi (Kent, 1880) Laveran and Mesnil, 1901

T. (*H.*) *lewisi* is the type species of subgenus *Herpetosoma* and is parasitic in rats throughout the world. It is monomorphic, with a characteristically curved body drawn out to a point at the posterior end (length 21–37 μm); the undulating membrane is slightly developed, with a well-developed free flagellum. *T. lewisi* develops in and is transmitted by rat fleas.

A case of human infection with trypanosomes identified as *T. lewisi* was reported by Johnson in 1933, who identified the parasite in a sick Malaysian baby. The parasites were morphologically identical to *T. lewisi* infecting rats that infested the child's dwelling. The details of this report have been much discussed (Hoare, 1972).

DNA sequence analysis Phylogenetic analysis of the *T. lewisi* 18S rRNA gene (Haag *et al.*, 1998; Stevens *et al.* 1999a, 2001) (Fig. 1.1) clustered this species tightly together (94% bootstrap support) with other rodent-derived *Herpetosoma* species, in a clade showing no affinity with other supposedly *Herpetosoma* species.

Fig. 1.1. Phylogenetic tree based on bootstrapped maximum parsimony analysis of 18S SSU ribosomal RNA gene sequences. The tree represents an extended analysis of Stevens *et al.* (1999a, 2001) and is based on an alignment of 1809 nucleotide positions, being one of three alignments tried (Morrison and Ellis, 1997; Stevens *et al.*, 1999a). It contains 61 *Trypanosoma* taxa and shows the genus to be monophyletic. *Nannomonas* subgroups are denoted by (s) = savannah, (r-f) = riverine-forest, (Kc) = Kenya coast and (T) = Tsavo; sequence accession numbers are given in Haag *et al.* (1998) and Stevens *et al.* (1999a,b, 2001). Analysis performed using PAUP* 4 (Swofford, 2001).

Subgenus *Schizotrypanum* Chagas, 1909

The subgenus *Schizotrypanum* comprises small trypanosomes that are very difficult to distinguish morphologically, with a mean size range of bloodstream forms, including flagellum, from 14 to 24 μm (Hoare, 1972). A voluminous kinetoplast and typically curved bloodstream forms constitute distinctive morphological characters of this homogeneous subgenus (Hoare, 1972). Recognized *Schizotrypanum* taxa include *T.* (*S.*) *cruzi*, the agent of Chagas disease, *T.* (*S.*) *rangeli* (see below) and species (*T. vespertilionis*, *T. dionisii*, *T. hedricki* and *T. myoti*) which appear to be restricted to bats (Chiroptera).

The structure and life cycle of *Schizotrypanum* species conform to the type species *T. cruzi* (Hoare, 1972), though several aspects of the life cycle of bat species are not completely known (Molyneux, 1991). Few data have been gathered on bat trypanosomes since the reviews of Marinkelle (1976) and Molyneux (1991), and the description of the subgenus *Schizotrypanum* remains unsettled in several respects. Firstly, *T. cruzi*-like trypanosomes from non-chiropteran South American wild mammals may represent species distinct from *T. cruzi*, since their infectivity to humans is unknown except for a few accidental infections (Hoare, 1972; Miles, 1979). However, biochemical and molecular characterizations of trypanosomes from a variety of silvatic mammals, mainly using isoenzyme electrophoresis, have always indicated their close similarity with *T. cruzi* strains. Secondly, the species *T. phyllostomae* Cartaya, 1910 was proposed for South American bat trypanosomes, but this species was not considered to be restricted to Chiroptera and should probably be considered as synonymous with *T. cruzi*. Thirdly, recent molecular phylogenetic analysis based on small subunit (SSU) rDNA suggested that some other stercorarian species, including *T. rangeli*, may be more appropriately classified as subgenus *Schizotrypanum* (Stevens *et al.*, 1999b). Finally, the presence of *Schizotrypanum* in Australia is indicated by the description of *T. pteropi* Breinl, 1912 and *T. hipposideri* Mackerras, 1959 from Australian bats, but the validity of these species is uncertain since they are only known from blood smears (Marinkelle, 1976). More recently, a trypanosome isolated from an Australian kangaroo showed an 18S rDNA sequence similar to those of representatives of the subgenus *Schizotrypanum* (Stevens *et al.*, 1999a,b).

Trypanosoma (Schizotrypanum) cruzi *Chagas, 1909*

Synonyms: *Schizotrypanum cruzi* Chagas, 1909; *Trypanosoma vickersae* Brumpt, 1909; *T. rhesii* Terry, 1911; *T. triatomae* Kofoid and McCulloch, 1916; *Schizotrypanum mexicanii* Perrin, 1950; *T. deanei* Coutinho and Pattolli, 1964; *T. phyllostomae* Cartaya, 1910.

MORPHOLOGY The morphology of *T. cruzi* has been covered in detail by Hoare (1972). In mammalian blood *T. cruzi* is described as a small (mean length including flagellum 16.3–21.8 μm), typically 'C'-shaped trypanosome with a large kinetoplast (diameter approximately 1.2 μm) near the posterior end, a free flagellum, and an undulating membrane with two to three shallow convolutions. Slender and broad trypomastigote forms can be distinguished in the blood, possibly reflecting the complex relationship between the parasite and host tissues (Miles, 1979). The amastigote, found in pseudocysts within cells of the mammalian host, represents the intracellular reproductive form of *T. cruzi*, whereas the epimastigote form corresponds to the reproductive stage in the insect vector and in axenic culture media. Finally, metacyclic trypomastigotes develop in the hindgut of the vectors.

HOST RANGE AND PATHOGENICITY The cycle in the vector, which takes place entirely in the lumen of the gut and with contaminative transmission through the faeces, is typical for the section Stercoraria. Trypomastigotes ingested with the blood meal differentiate into epimastigotes, which readily multiply in the midgut and ultimately differentiate into infective metacyclic trypomastigotes in the hindgut. In contrast to *T. rangeli*, *T. cruzi* is considered harmless to its vectors and infec-

tion rates of the Triatominae can often exceed 50%. *T. cruzi* seems to be able to develop, at least partially, in a wide range of other invertebrates including leeches, *Cimex lectularius* (bed bug) and ticks (Hoare, 1972).

In contrast with the other species of the subgenus *Schizotrypanum*, *T. cruzi* has a wide host range. More than 150 species of mammals, including bats, have been reported to be infected with *T. cruzi*, and all mammals are considered to be susceptible. Distinct phylogenetic lineages of *T. cruzi*, however, appear to have preferential associations with different groups of hosts (see below). Birds and reptiles are apparently not susceptible to *T. cruzi* infection, although the vectors often feed on them.

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION

Early methods developed to characterize *T. cruzi* and distinguish it from bat trypanosomes or from *T. rangeli* include DNA buoyant density, lectin-binding properties, sialidase/transsialidase activity detection methods, and complement-lysis and antigenic properties (Baker *et al.*, 1978; Molyneux, 1991; D'Alessandro and Saravia, 1992; Medina-Acosta *et al.*, 1994). The findings of more recent characterization methods follow.

Isoenzymes and randomly amplified polymorphic DNA (RAPD) characterization Multilocus isoenzyme electrophoresis can be described as the gold standard of *T. cruzi* characterization. It has shown considerable heterogeneity within this species and revealed the predominantly clonal structure of natural populations of *T. cruzi*. Three principal groups of zymodemes (sets of stocks exhibiting the same electrophoretic patterns for a given set of enzymatic loci) were first described and called Z1, Z2 and Z3 (Miles *et al.*, 1978). The degree of genetic differentiation among the major zymodeme groups Z1, Z2 and Z3 was higher, based on isoenzymes, than that between some species of *Leishmania*. Further studies identified 43 zymodemes, equated to clonal genotypes or 'clonets' (Tibayrenc *et al.*, 1986) (see Chapter 5). Clustering analysis of isoenzymes and RAPD data from a representative set of these subclones later led to the definition of two major phylogenetic lineages within *T. cruzi* (Tibayrenc, 1995). Miles's Z1 group fell within lineage 1, while both Z2 and Z3 were grouped into lineage 2; both lineages are highly heterogeneous. Precise characterization of representative stocks of each lineage with 20 RAPD primers and 22 isoenzyme loci revealed the existence of five discrete phylogenetic subdivisions, designated IIa–IIe, within the second major isoenzyme/RAPD lineage, whereas no clear subdivision was found within the first lineage (Brisse *et al.*, 2000a). The distinction among the five lower subdivisions of major lineage 2 was confirmed by analysis of 434 *T. cruzi* stocks with broad geographical and ecological origins by isoenzymes (Barnabé *et al.*, 2000). The *T. cruzi* subdivisions are each identified by several isoenzyme and RAPD markers, as well as specific sequence-characterized amplified region (SCAR) markers (Brisse *et al.*, 2000b). Two of these lineages (IId and IIe) show a fixed heterozygosity at several loci and composite RAPD profiles, which first suggested that they originated by hybridization between parental stocks belonging to lineages IIb and IIc (Brisse *et al.*, 2000a). This hybridization hypothesis was fully demonstrated by nucleotide sequence analysis (Machado and Ayala, 2001; Brisse *et al.*, 2003; see below). The reference clone CL Brener chosen for the *T. cruzi* genome project belongs to the hybrid lineage IIe (Brisse *et al.*, 2000a).

Mini-exon genes The tandemly repeated mini-exon (also called spliced leader RNA) genes have been used to differentiate *T. cruzi* and *T. rangeli* by the size difference of the repeat unit, 582 and 858 nucleotides long, respectively, revealed by PCR targeting of conserved regions. Moreover, based on the sequence diversity of the intergenic region of the mini-exon repeat, PCR assays can differentiate between groups of stocks that appear to correspond to isoenzyme/RAPD lineages 1 (PCR product of 350 bp), IIb/IId/IIe (300 bp), and IIa and IIc (no amplification product). Sequence variability and functional specificity of the mini-exon gene promoter also revealed a dimorphic pattern which distinguished stocks of lineage 1 and 2 (Nunes *et al.*, 1997; Fernandes *et al.*, 1998; Brisse *et al.*, 2001).

Ribosomal RNA genes Sequencing of the SSU rRNA has confirmed the close relationship of *T. cruzi* with *Schizotrypanum* bat trypanosomes, in particular with *T. cruzi marinkellei* (Stevens *et al.*, 1999b). Using SSU and LSU nucleotide sequences from a diverse collection of *T. cruzi* strains, Kawashita *et al.* (2001) have found four 'riboclades'. However, confidence in 18S rDNA sequence data to infer phylogenetic relationships has been challenged by the observation that *T. cruzi* and other trypanosomatids contain two distinct copies of these genes (Stothard *et al.*, 1998). Digestion of PCR amplified SSU rDNA (riboprinting) appears useful for *T. cruzi* lineage characterization, and a PCR assay based on the SSU rRNA gene is useful to distinguish *T. cruzi* lineages (Brisse *et al.*, 2001).

Hybridization with specific probes and multiplex PCR of the large subunit (LSU) rRNA (Souto *et al.*, 1999) has been used to differentiate *T. cruzi* from other trypanosome species, including *T. rangeli*. Moreover, 24Sα LSU rRNA proved very valuable in differentiating strains and lineages within *T. cruzi* and, in combination with mini-exon characterization, led to the description of two *T. cruzi* lineages (Souto *et al.*, 1996), which corresponded mostly with isoenzyme and RAPD groupings. Indeed, PCR amplification of a sequence from the D7 divergent domain resulted in two amplification products (110 bp and 125 bp), alone or in combination. Additional size variation of the LSU rRNA D7 domain was later found and PCR assays could distinguish, in combination with characterization of the SSU rRNA, the six *T. cruzi* isoenzyme/RAPD lineages (Brisse *et al.*, 2001). Consistently, the sequence and functional specificity of the rRNA gene promoter clearly distinguished stocks of isoenzyme/RAPD lineage 1 from representatives of lineage 2 (Nunes *et al.*, 1997). Using a more diverse strain collection, four groups of sequences were subsequently found within *T. cruzi* based on the sequence of the D7 domain (Kawashita *et al.*, 2001), similar to results found with the SSU rRNA and other genes (see below).

Nucleotide sequencing of single genes Contrasting with the initial definition of two *T. cruzi* major lineages, nucleotide sequencing of individual nuclear and mitochondrial genes revealed the presence of three to four allelic groups within *T. cruzi*, depending on the strain sampled and the genes sequenced (Robello *et al.*, 2000; Kawashita *et al.*, 2001; Machado and Ayala, 2001; Brisse *et al.*, 2003). Machado and Ayala (2001) showed that sequence data from two nuclear genes (dihydrofolate reductase–thymidylate synthase and trypanothione reductase) and a portion of the maxicircle DNA provide convincing evidence of genetic exchange and hybridization in *T. cruzi*. Single gene sequence diversity studies converge in suggesting that *T. cruzi* is composed of at least four primary lineages and two hybrid lineages (Machado and Ayala, 2001; Brisse *et al.*, 2003). The initial finding of only two major lineages using multilocus approaches such as isoenzyme and RAPD can be explained by their insufficient resolution at the higher clustering levels, due to their overly fast molecular clock. Alternatively (and not exclusively), the recent ancestor of *T. cruzi* perhaps consisted of three or four isolated lineages, and genetic exchange caused some lineage 2 strains to carry alleles from distinct ancestral lineages. In this case, multilocus approaches would represent the genome-wide genetic relationships between *T. cruzi* strains, and single gene genealogies would only represent the relationships between alleles from one locus.

Other characterization methods Many other DNA-based characterization methods have revealed polymorphism in *T. cruzi*; most of them show correlation with isoenzyme and RAPD classification of *T. cruzi* stocks, including molecular karyotype, microsatellites and kinetoplast DNA variability (Macedo and Pena, 1998).

THE NOMENCLATURE OF *T. CRUZI* LINEAGES In a welcome effort to harmonize *T. cruzi* strain designation (Anonymous, 1999), it was proposed to designate strains corresponding to Z1 (Miles *et al.*, 1978), isoenzyme/RAPD lineage 1 (Tibayrenc, 1995; Brisse *et al.*, 2000a) and 24Sα rDNA/mini-exon lineage 2 (Souto *et al.*,

1996) as group *T. cruzi* I, whereas group *T. cruzi* II would comprise strains belonging to zymodeme Z2 group, which corresponds to isoenzyme/RAPD lineage IIb (and not to the whole isoenzyme/RAPD major lineage 2; see Brisse *et al.*, 2001) and (*p.p.*) to rDNA/mini-exon lineage 1. Awaiting further clarification of their phylogenetic status, it was proposed simply to refer to heterozygous genotypes (isoenzyme/RAPD lineages IId and IIe) and to Miles's Z3 group and alike – mini-exon 2 group (Fernandes *et al.*, 1998), isoenzyme/RAPD lineages IIa and IIc – as *T. cruzi*. *T. cruzi* lineages differ with respect to their geographical distribution and ecological associations (Miles *et al.*, 1978; Miles, 1983; Zingales *et al.*, 1998; Barnabé *et al.*, 2000), which underlines the epidemiological, and possibly taxonomic, relevance of their distinction.

Subspecies: Trypanosoma *(*Schizotrypanum*)* cruzi marinkellei *Baker* et al.*, 1978*

This subspecific rank denomination was proposed for South and Central American bat-restricted trypanosomes mainly based on isoenzyme and DNA buoyant density analyses (Baker *et al.*, 1978). *T. cruzi* sensu stricto, the agent of Chagas disease, should accordingly become the nominate subspecies *T. cruzi cruzi* Chagas, 1909, but usage has retained the binominal denomination *T. cruzi*. Three groups of *T. cruzi marinkellei* strains have been distinguished (Baker *et al.*, 1978; Taylor *et al.*, 1982; Barnabé *et al.*, 2003). The name *Trypanosoma hastatus* was recently used for bat trypanosomes from Brazil (Steindel *et al.*, 1998), but distinction from *T. cruzi marinkellei* was not shown.

Trypanosoma *(*Schizotrypanum*)* rangeli *Tejera, 1920*

Synonyms: *Trypanosoma escomeli* Yorke, 1920; *T. guatemalense* de Leon, 1946; *Schizotrypanum rangeli* Tejera, 1920, de Leon, 1949; *Trypanosoma cebus* Floch and Abonnenc, 1949; *T. ariarii* Groot, Renjifo and Uribe, 1951.

HISTORY OF *T. RANGELI* SYSTEMATICS *T.* (*S.*) *rangeli* presents the exceptional characteristic, in the Stercoraria section, of being capable of transmission both through the faeces and through the salivary glands (although anterior transmission is much more efficient than posterior transmission). This has stimulated much debate about the taxonomic position of this parasite. It has been considered as a possible evolutionary transition form between Stercoraria and Salivaria, or as belonging to a new subgenus, or as remaining within the subgenus *Herpetosoma* (D'Alessandro and Saravia, 1992). Recent molecular data derived from SSU rDNA sequence indicate the close relationship of *T. rangeli* with the subgenus *Schizotrypanum* species (Stevens *et al.*, 1999a,b) (Fig. 1.1), which leads us to confirm its classification in the subgenus *Schizotrypanum* (see below).

MORPHOLOGY Morphometric measurements of *T. rangeli* have been summarized previously (Hoare, 1972; D'Alessandro and Saravia, 1992). *T. rangeli* blood trypomastigotes are large and slender (26–34 µm including the flagellum), with a subterminal kinetoplast characteristically smaller than that of *T. cruzi*. Both in the vector and in culture, *T. rangeli* can be very polymorphic, with epimastigotes, amastigotes and trypomastigote forms of various sizes (de Sousa, 1999). Epimastigotes have been reported to range from 28 to 67 µm in length in the lumen of the insect midgut. Morphological distinction from *T. cruzi* is readily achievable but, due to size variability, morphology alone is not sufficient to exclude the presence of one or the other flagellate in samples.

HOST RANGE AND DISTRIBUTION *T. rangeli* is infective to humans and to a wide range of domestic and silvatic animals. However, it is not pathogenic to its hosts and self cure in humans occurs within 3 years of infection. Treatment of people infected with this parasite is considered unnecessary and, so far, we are not aware of any reported opportunistic pathogenic behaviour of *T. rangeli* in HIV-infected persons. *T. rangeli* in humans has been reported from Guatemala, Honduras, El Salvador, Costa Rica, Panama, Colombia, Venezuela, Peru and Brazil (D'Alessandro and Saravia, 1992).

VECTORS Natural and experimental vectors of *T. rangeli* have been detailed previously (D'Alessandro and Saravia, 1992). Importantly, among the major vectors of *T. cruzi*, the agent of Chagas disease, naturally infected *Rhodnius prolixus* and *Triatoma dimidiata* with *T. rangeli* in their salivary glands have been demonstrated, whereas *T. infestans* was experimentally shown to be an anterior station vector of *T. rangeli*. Nine of the 12 nominal species of *Rhodnius* are known vectors of *T. rangeli*. In contrast to *T. cruzi*, *T. rangeli* is pathogenic to its invertebrate hosts, as detailed in D'Alessandro and Saravia (1992).

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION Isoenzymes readily differentiate *T. rangeli* from *T. cruzi*. However, little isoenzyme variability has been found among *T. rangeli* strains isolated in different localities of Colombia, though strains isolated in southern Brazil could be differentiated from those of Colombia and Central America, in particular in the non-transcribed spacers of the mini-exon gene tandem repeats (Steindel *et al.*, 1994; Grisard *et al.*, 1999). Exploring the genetic heterogeneity of *T. rangeli* strains from more diverse geographical and ecological regions will most probably reveal more heterogeneity than currently described.

DNA sequence analysis SSU rRNA sequence data (Stevens *et al.*, 1999b) indicate that *T. rangeli* and *T.* (*M.*) *conorhini*, as well as *T.* (*H.*) *leeuwenhoeki* and *T.* (*M.*) *minasense*, are more closely related to the subgenus *Schizotrypanum* than to the two type species of their respective subgenera, *T.* (*H.*) *lewisi* or *T.* (*M.*) *theileri*. Concordant with these results, it is interesting that *T. rangeli* and *T. leeuwenhoeki* were reported to express exclusively sialidase enzymatic activity, and *T. cruzi* and *T. conorhini* trans-sialidase activity, in contrast to *T. lewisi* which showed both activities (Medina-Acosta *et al.*, 1994). These data tend to indicate that the taxonomic classification of the section Stercoraria, and in particular the distinction of the three subgenera, may yet need further consideration.

Section B: Salivaria

Subgenus *Duttonella* Chalmers, 1918

The principal and type species of the subgenus *Duttonella* is *Trypanosoma* (*D.*) *vivax* Ziemann, 1905; it has one recognized subspecies, *T.* (*D.*) *vivax viennei* Lavier, 1921. The subgenus also contains a morphologically similar but smaller species, *T.* (*D.*) *uniforme* Bruce *et al.*, 1911, which was last reported from a giraffe in Tanzania.

Classically, the subgenus is represented by monomorphic (but see *T. vivax* below) trypanosomes which exhibit a highly characteristic combination of morphological and subcellular features. The typical bloodstream form has a somewhat expanded or club-shaped posterior end, which tapers towards the anterior. Movement in wet blood films is rapid and distinctive. The large, generally terminally placed kinetoplast is distinctive and is often sufficient to separate *Duttonella* from other Salivarian trypanosomes. Trypanosomes of this subgenus are parasitic predominantly in wild and domestic ungulates in Africa and Latin America; significantly, they do not readily infect laboratory rodents. The development of these trypanosomes in *Glossina* spp. is confined to the proboscis (Table 1.1).

Trypanosoma (Duttonella) vivax *Ziemann, 1905*

Synonyms: *Trypanosoma cazalboui* Laveran, 1906; *T. congolense* Broden & Rodhain, 1910; *T. bovis* Kleine, 1910; *T. caprae* Kleine, 1910; *T. viennei* Lavier, 1921.

MORPHOLOGY In addition to the general characteristics of *Duttonella* noted above, *T. vivax* differs from other salivarian trypanosomes by its length (means from 21 μm to 25.4 μm). However, more recent research on strains that naturally infect rodents or that have been adapted to laboratory rodents has revealed the existence of longer, more granular bloodstream forms of *T. vivax* with a clearly subterminal kinetoplast (Gardiner, 1989), which appear at the peak of parasitaemia; these are termed 'late' forms (Gardiner, 1989)

Table 1.1. Characterization of trypanosomes according to their site of development in *Glossina* spp. (after Hoare, 1970).

		Site of development in tsetse fly				
Subgenus	Species	Proboscis	Salivary glands	Midgut	Hindgut	Characteristics
Duttonella	*T. vivax*	Trypomastigotes, epimastigotes and metatrypanosomes	No development	No development	No development	Kinetoplast large, free flagellum present in all stages
Nannomonas	*T. congolense* *T. simiae*	Trypomastigotes, epimastigotes and metatrypanosomes	No development	Trypomastigotes	No development	Kinetoplast medium, free flagellum absent in all stages
Trypanozoon	*T. b. brucei* *T. b. rhodesiense* *T. b. gambiense*	No development	Trypomastigotes, epimastigotes and metatrypanosomes	Trypomastigotes	No development	Kinetoplast small, free flagellum present in all stages except metatrypanosomes, in which it is typically absent
Pycnomonas	*T. suis*	Metacyclics	Trypomastigotes, epimastigotes and metatrypanosomes	Trypomastigotes	No development	Kinetoplast small, free flagellum present in all stages, including metatrypanosomes
Crocodile parasite	*T. grayi*	No development	No development	Epimastigotes (including filamental forms) and trypomastigotes	Epimastigotes and metatrypanosomes	Kinetoplast large, free flagellum present in all stages

and appear to be pre-adapted to continuation of the life cycle in the tsetse vector. Overall, it appears evident that length is not a reliable taxonomic character.

CYCLE IN VECTOR The development of *T. vivax* in *Glossina* spp. is confined entirely to the proboscis; subgenus *Duttonella* trypanosomes are unique in this respect (Table 1.1). In general, the high incidence of naturally infected tsetse flies suggests that *T. vivax* is better adapted to development in its host than other salivarian species (Hoare, 1972).

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION Of the species of subgenus *Duttonella* described, only *T. vivax* and *T. v. viennei* have been subjected to study by modern biochemical and molecular methods.

Isoenzymes The first clear evidence of genetically distinct forms of *T. vivax* was provided by Allsopp and Newton (1985), who showed that rodent infectivity and the ability to induce a haemorrhagic syndrome could be correlated with isoenzyme profile. Later work has associated some isoenzyme patterns with geographical origin.

Karyotype and kinetoplast DNA *T. vivax* has a similar molecular karyotype to other salivarian trypanosomes, with chromosomal DNAs ranging in size from approximately 50 to 6000 kb. However, unlike other salivarian trypanosomes, which have an estimated 100 mini-chromosomes of 50–100 kb, *T. vivax* has only one or two mini-chromosomes which are characterized by the presence of a highly repetitive guanine/cytosine-rich satellite DNA of 177 bp in size (Dickin and Gibson, 1989).

T. vivax kinetoplast DNA minicircles are approximately half the size of other salivarian trypanosomes while the relative amount of maxicircle DNA is high – at least twice that of *T. brucei*.

DNA sequence analysis Molecular-based phylogenetic analysis of *T. vivax* to date (Lukes *et al.*, 1997; Haag *et al.*, 1998; Stevens *et al.*, 1999a) is based on 18S SSU and 28S LSU RNA sequences (Acc. No. U22316; T. Urakawa and P.A. Majiwa, unpublished) from a single isolate, the much studied rodent-adapted Y486 stock, which was originally isolated in Nigeria from a zebu cow (*Bos indicus*) in 1973. However, in all analyses, a similar pattern of results is observed.

Firstly, the *T. vivax* lineage shows a greatly elevated substitution rate within its rRNA sequences. Analyses suggest that it may have undergone three to eight times as many subsitutions as the lineages of many non-salivarian trypanosomes (Haag *et al.*, 1998; Stevens *et al.*, 1999a) and may be evolving more than twice as fast as other salivarian trypanosomes (Stevens and Rambaut, 2001).

Secondly, *T. vivax* is the first taxon to diverge from the monophyletic clade of salivarian trypanosomes in phylogenetic analyses (Lukes *et al.*, 1997; Stevens *et al.*, 1999a). The well-supported early separation of the *T. vivax* lineage (Fig. 1.1) is consistent with the view that *T. vivax* represents the most ancient of the salivarian trypanosomes (Haag *et al.*, 1998) and that *T. vivax* represents an early stage of adaptation to transmission by tsetse flies (Lukes *et al.*, 1997), both hypotheses having originally been proposed by Hoare (1972).

T. vivax is alone in having an 18S rRNA guanine/cytosine content of 55.4%, a figure some 3% higher than any other trypanosomatid included in the study of Haag *et al.* (1998).

Subspecies: Trypanosoma (Duttonella) vivax viennei *Lavier, 1921*

T. (*D.*) *v. viennei* is morphologically identical to African *T. vivax* and its inability to undergo cyclical development in testse appears to be the only clear behavioural attribute of the subspecies, but, to our knowledge, no investigations have been carried out to determine the basis of this trait.

T. vivax is thought to have been introduced to South America (French Guiana) and the Caribbean (Guadeloupe and Martinique) with zebu cattle imported from Senegal, West Africa. Certainly, the results of comparative isoenzyme studies and DNA probe analysis of *T. v. viennei* from Colombia support a West African origin for *T. v. viennei*.

Subgenus *Nannomonas* Hoare, 1964

The subgenus *Nannomonas* contains three recognized species: *Trypanosoma* (*N.*) *congolense* Broden, 1904, *T.* (*N.*) *simiae* Bruce *et al.*, 1912 and *T.* (*N.*) *godfreyi* (McNamara *et al.*, 1994). The type species, and the most frequently encountered pathogen of African livestock, is *T. congolense*, while *T. simiae* and *T. godfreyi* are primarily confined to suids. Within both *T. congolense* and *T. simiae* a number of types are recognized, each of which is more or less readily identified by a range of behavioural/ecological and molecular characteristics.

The subgenus *Nannomonas* is represented by small trypanosomes (measuring 8–24 μm in length). Typically, they lack a free flagellum and, in most cases, the undulating membrane is inconspicuous; they possess a medium-sized kinetoplast which is generally marginally positioned. The posterior end of the body is either rounded (in the smaller forms) or pointed (in the longer ones); the degree of polymorphism is especially pronounced in *T. simiae.* Nevertheless, it is difficult to distinguish species of *Nannomonas* apart since they resemble each other morphologically and have identical cycles of development in the tsetse midgut and proboscis (Table 1.1). Indeed, as Hoare (1972) noted, at a time when *T.* (*N.*) *godfreyi* had not been described, in view of the gradation of characters both within and between species it might be justifiable to treat *Nannomonas* as a monotypic subgenus represented by a single species subdivided into subspecies. As discussed below, however, the levels of genetic diversity observed within this subgenus (and, indeed, within species within the subgenus) are not concurrent with such taxonomic reductionism.

Molecular-based phylogenetic analyses of *Trypanosoma*, based on 18S SSU and 28S LSU rRNA sequences (Lukes *et al.*, 1997; Haag *et al.*, 1998; Stevens *et al.*, 1999a), confirm the monophyly and phylogenetic validity of this subgenus.

Trypanosoma (Nannomonas) congolense *Broden, 1904*

Synonyms: *T. dimorphon* Laveran & Mesnil, 1904; *T. nanum* Laveran, 1905; *T. confusum* Montgomery & Kinghorn, 1909; *T. pecorum* Bruce *et al.*, 1910; *T. montgomeryi* Laveran, 1909 (*p.p.*).

MORPHOLOGY While morphological differences between populations of *T. congolense* have no taxonomic validity, those researching this parasite should be aware that at least three strains or races, apparently separable by their mean length and percentage of short and long forms, have been documented. Significantly, no morphological differences have been associated with the strain groups recognized latterly on the basis of biochemical and molecular characteristics.

HOST RANGE *T. congolense* infects a broad range of domestic mammals, including bovines, equines, sheep, goats, camels, pigs and dogs. Unlike other species within the subgenus, natural infections of *T. congolense* are generally easy to cultivate in laboratory rodents, while infections in pigs are characteristically mild.

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION *T. congolense* has been extensively characterized with a range of biochemical and molecular tools. Indeed, the three groups or 'types' of *T. congolense* currently recognized (savannah, riverine/forest, Kenya coast) have been defined primarily on the basis of such analyses. Recent work by Stevens *et al.* (1999a) and Gibson *et al.* (2001) indicates that another type, *T. congolense* Tsavo (Majiwa *et al.*, 1993), is more appropriately classified as a strain of *T. simiae*.

Isoenzymes The degree of genetic diversity within *T. congolense* was first demonstrated with isoenzymes. Two main groups with characteristic isoenzyme profiles, which grouped together according to the ecological or geographical origins of the stocks, were noted. The 'Humid' division included isolates from humid regions of West Africa, while the 'Dry' division included isolates originating from the savannah zone. A third genetically distinct 'Kenya coast' group was later identified (Majiwa *et al.*, 1985) on the basis of isoenzymes.

Subsequently, the groups were formally defined on the basis of cladistic analysis of

zymodemes from an extended set of stocks (Gashumba *et al.*, 1988). The names of the groups are: savannah, which includes isolates originating from both East and West African savannah (= 'Dry' division); Riverine/forest (= 'Humid' division); and Kenya coast (also named 'Kilifi'). More recently the savannah type has been subdivided into east and west groups.

DNA probes Ongoing with the broad-scale epidemiological and evolutionary isoenzyme-based studies, species-specific DNA probes for each of the three strain groups identified by isoenzymes have been developed. The probes, which consist of one repeat unit (350–370 bp in length) of the non-conserved repetitive genomic satellite DNA, are highly specific and have proved invaluable as detection tools in epidemiological and characterization studies (McNamara and Snow, 1991). Their use for evolutionary analyses is, however, limited.

RAPDs RAPD analysis has been used to corroborate the results of isoenzyme analysis. The results confirm the distinct genetic basis of the strain groups recognized within *T. congolense*, including the subdivision of savannah type into east and west forms. However, the level of resolution of the RAPD markers used appears to be saturated, making evolutionary interpretation of these data unreliable at the interspecies level.

DNA sequence analysis Phylogenetic analysis of *T. congolense* based on 18S SSU rRNA sequences (Haag *et al.*, 1998) confirms the monophyly of *T. congolense*, placing various *T. congolense* types in a clade adjacent to *T. simiae* sequences, and apart from *T. brucei* and *T. vivax*. Subsequent work based on 18S sequences, and including representatives of all recognized *T. congolense* strain groups (Stevens *et al.*, 1999a) (Fig. 1.1), confirms the genetically distinct nature of the three strain groups. The results also provide some support for the distinct nature of Kenya coast-type trypanosomes, which appear to have undergone considerably faster evolution than isolates in either the riverine/forest or savannah groups, despite Kenya coast and savannah-type trypanosomes apparently sharing a common ancestor. Significantly, the *T. congolense* clade does not include *T. congolense* Tsavo, which clusters with *T. simiae* and *T. godfreyi*.

Trypanosoma (Nannomonas) simiae *Bruce* et al., *1912*

Synonyms: *T. ignotum* Kinghorn & Yorke, 1912; *T. rodhaini* Walravens, 1924; *T. porci* Schwetz, 1932; *T. congolense porci* Schwetz, 1934; *T. montgomeryi* Laveran, 1909 (*p.p.*).

MORPHOLOGY As noted, the greatest degree of polymorphism within the subgenus *Nannomonas* is observed in *T. simiae*. Characteristically, three forms may be observed within the same vertebrate host: elongated-stout forms with a well-developed undulating membrane; long-slender forms with a poorly developed undulating membrane; and short forms.

HOST RANGE AND PATHOGENICITY Apart from isolated reports of *T. simiae* infecting camels, a horse and an ox (Hoare, 1972), this parasite is naturally confined to suids. It is the only species of trypanosome that is extremely pathogenic to domestic pigs, in which it causes acute and fatal disease outbreaks of short duration. Its name derives from its description from experimentally infected monkeys; David Bruce himself later expressed regret at having given this parasite such a misleading name (Hoare, 1972). Recently, a new type of *Nannomonas* from Tsavo West, Kenya, has also been shown to be infective only to pigs. Unlike *T. congolense*, *T. simiae* generally fails to infect laboratory rodents (Dukes *et al.*, 1991).

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION Due in large part to the inability to amplify *T. simiae* infections in laboratory rodents, this parasite has not been as extensively characterized with biochemical and molecular markers as has *T. congolense*.

Isoenzymes Limited isoenzyme studies, which confirm the genetically distinct nature of *T. simiae* in relation to other

Salivarian species, have been performed (Gashumba *et al.*, 1988; McNamara *et al.*, 1994). However, neither study included sufficient stocks to allow evaluation of intraspecific genetic variation.

DNA probes Species-specific probes for *T. simiae* show that the satellite DNA of *T. simiae* is distinct from that of *T. congolense* and other Salivarian trypanosomes (Majiwa and Webster, 1987). Another type-specific probe, based on a 150 bp satellite DNA repeat, for the characterization and identification of a genetically distinct *Nannomonas* isolated from *Glossina pallidipes* caught in Tsavo West National Park, Kenya, was used to type a *T. congolense* Tsavo-type (Majiwa *et al.*, 1993). However, recent phylogenetic (Haag *et al.*, 1998; Stevens *et al.*, 1999a; Gibson *et al.*, 2001) and infectivity studies suggest that this isolate is more appropriately classified as *T. simiae* Tsavo.

Additional molecular characterization In order to characterize *T. simiae* Tsavo, a range of additional comparative molecular analyses on *T. simiae* and *T. congolense* were used. These included restriction fragment length polymorphism (RFLP) and kinetoplast DNA digests, Southern blotting using a minicircle-based probe, and RAPD analysis which showed the Tsavo-type *Nannomonas* to be distinct and dissimilar to all previously recognized species and types of *Nannomonas* (Majiwa *et al.*, 1993).

DNA sequence analysis Phylogenetic analyses of 18S rRNA sequences, which include both *T. simiae* and *T. simiae* Tsavo, together with a range of *T. congolense* types and other Salivaria have recently been conducted (Haag *et al.*, 1998; Stevens *et al.*, 1999a; Gibson *et al.*, 2001) (Fig. 1.1). These studies indicate a distinct but close and well-supported relationship between *T. simiae* and *T. simiae* Tsavo, and latterly (Stevens *et al.*, 1999a; Gibson *et al.*, 2001) between all species of *Nannomonas* pathogenic to pigs, i.e. *T. simiae*, *T. simiae* Tsavo and *T. godfreyi* (see below). These studies also reinforce the idea of a very distinct evolutionary division between *T. congolense* and the pig pathogen group (*T. simiae*/*T. godfreyi*).

Trypanosoma (Nannomonas) godfreyi *McNamara* et al., *1994*

Trypanosoma (*Nannomonas*) *godfreyi* was formally described by McNamara *et al.* (1994), though the existence of a previously unrecognized *Nannomonas* trypanosome had been known for several years from studies of infected tsetse in The Gambia, in which it produced a characteristic *Nannomonas* infection (midgut and proboscis). Following its discovery in West Africa, it has since been identified in Zimbabwe (Woolhouse *et al.*, 1993), suggesting a wide distribution associated with woodland/savannah areas where *morsitans*-group tsetse and suids are present. The discovery of *T. godfreyi* may go some way to explaining the variations in behaviour and infectivity observed in *Nannomonas*.

MORPHOLOGY

T. godfreyi is a small (length 9–22 μm, mean 13.7 μm) trypanosome, being significantly shorter than either *T. simiae* or *T. congolense*. There is no free flagellum and the size and position of the kinetoplast (marginal) are typical of the subgenus *Nannomonas*. Biometric analysis of over 700 bloodstream-form trypanosomes from stained slides indicated that *T. godfreyi* is not polymorphic in pigs.

HOST RANGE AND PATHOGENICITY

To date, *T. godfreyi* has only been isolated naturally from tsetse (*Glossina morsitans submorsitans* and *G. pallidipes*). Experimentally, however, it readily infects pigs, in which it produces a chronic, occasionally lethal infection, somewhat different from the acute rapidly fatal disease associated with *T. simiae* and the mild infection characteristic of *T. congolense* (McNamara *et al.*, 1994). It does not infect domestic cattle, sheep or rodents, which probably accounts for it remaining undiscovered until relatively recently (Dukes *et al.*, 1991). In nature, it is presumed to infect wild suids, e.g. *Phacochoerus aethiopicus*.

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION

DNA probes *T. godfreyi* was first reported as a 'new' *Nannomonas* which did not

hybridize with available *Nannomonas* probes. To aid in the formal identification of *T. godfreyi*, McNamara *et al*. (1994) developed a repeat element-based probe which confirmed the distinct genetic identity of the parasite compared with all other *Nannomonas* species/types, including Tsavo-type. Subsequently, specific primers designed to amplify *T. godfreyi* satellite DNA were identified (Masiga *et al*., 1996), the repeated monomer being 149 bp long.

Isoenzymes Isoenzyme analysis of *T. godfreyi* was performed by McNamara *et al.* (1994). Comparisons were made with isolates representative of all other *Nannomonas* types (except Tsavo-type). Numerical analysis of the isoenzyme data confirmed the unique identity of *T. godfreyi* and, moreover, indicated it to be the most distinct of all *Nannomonas* types; *T. simiae* and *T. congolense* stocks grouped loosely together and apart from *T. godfreyi* isolates, which clustered tightly together.

DNA sequence analysis Phylogenetic analysis of the 18S rRNA sequence (Stevens *et al*., 1999a; Gibson *et al*., 2001) (Fig. 1.1) identifies *T. godfreyi* as distinct from, but clustered with (84% bootstrap support), *T. simiae* Tsavo; in turn, this couplet forms a well-supported cluster (97% bootstrap support) with several *T. simiae* isolates. Such a result suggests a divergent evolutionary lineage for *T. godfreyi* (and *T. simiae* Tsavo), separate from *T. simiae* and very distinct from all *T. congolense*.

Subgenus Trypanozoon *Lühe, 1906*

The subgenus *Trypanozoon* is the most homogeneous group of salivarian trypanosomes. It contains three recognized species (*T. brucei*, *T. evansi* and *T. equiperdum*) which are morphologically indistinguishable, but which exhibit distinct epidemiological, pathological and genetic characteristics. In Africa the most important species is *T. brucei*, of which two subspecies, *T. brucei rhodesiense* and *T. brucei gambiense*, are responsible for human sleeping sickness. A third subspecies, *T. brucei brucei*, infects a range of mammalian hosts; in domestic livestock, e.g. cattle, the condition is generally mild, while in equines the disease is severe. *T. brucei* is tsetse-borne and characteristically develops in the midgut and salivary glands of the vector. The other two recognized species within the subgenus are transmitted directly, *T. evansi* by mechanical inoculators (e.g. tabanid flies) and *T. equiperdum* by direct contact between equine hosts.

Trypanosoma (Trypanozoon) brucei *Plimmer and Bradford, 1899*

Synonyms: *T. pecaudi* Laveran, 1907; *T. togolense* Mesnil & Brimont, 1909; *T. elephantis* Bruce *et al*., 1909; *T. anceps* Bruce *et al*., 1914; *T. ugandae* Stephens & Blacklock, 1913; *T. dukei* Knuth & du Toit, 1921.

Subspecies include:

- ***Trypanosoma* (*Trypanozoon*) *brucei brucei* Plimmer and Bradford, 1899**
  Synonyms: *Trypanozoon brucei* Lühe, 1906; *T. pecaudi* Laveran, 1907; *T. togolense* Mesnil & Brimont, 1909; *T. ugandae* Stephens & Blacklock, 1913; *T. multiforme* Kinghorn *et al*., 1913 (*p.p.*); *T. anceps* Bruce *et al*., 1914; *Castellanella brucei* Chalmers, 1918; *T. dukei* Knuth & du Toit, 1921.
- ***Trypanosoma* (*Trypanozoon*) *brucei gambiense* Dutton, 1902**
  Synonyms: *T. ugandense* Castellani, 1903; *T. castellanii* Kruse, 1903; *T. hominis* Manson, 1903; *T. fordii* Maxwell-Adams, 1903; *T. nepveui* Sambon, 1903; *Trypanozoon gambiense* Lühe, 1906; *Trypanosoma tullochii* Minchin, 1907; *T. rovumense* Beck & Weck, 1913; *T. nigeriense* Macfie, 1913; *T. gambiense* var. *longum* Da Costa *et al*., 1915; *Castellanella gambiensis* Chalmers, 1918; *C. castellanii* Kruse, 1903; Chalmers, 1918.
- ***Trypanosoma* (*Trypanozoon*) *brucei rhodesiense* Stephens and Fantham, 1910**
  Synonym: *T. gambiense* var. *rhodesiense* Swellengrebel, 1911; *T. brucei* vel *rhodesiense* Bruce *et al*., 1915.

MORPHOLOGY Bloodstream forms of *T. brucei* (and *Trypanozoon*) measure from 11–42 μm in length. They are typically poly-

morphic, being represented by three forms: (i) long slender forms (mean length 23–30 μm), which possess a long free flagellum, a well-developed undulating membrane, a subterminal kinetoplast and a narrow drawn-out posterior end; (ii) short stumpy forms (mean length 17–22 μm), which are stout, usually lacking a free flagellum, possess a well-developed undulating membrane and have the kinetoplast near the broadly rounded posterior end; and (iii) intermediate forms (mean length 20–25 μm) in which the flagellum is shorter and the posterior end blunter than in the slender forms.

It is now generally recognized that no consistant morphological or morphometric differences can reliably distinguish the subspecies of *T. brucei* or, indeed, the species within *Trypanozoon*.

HOST RANGE AND PATHOGENICITY The over-enthusiastic use of host range in the early development of *Trypanozoon* taxonomy was responsible for a proliferation of new 'species' in the early part of the 20th century.

Today, host range is not generally used as a basis for taxonomy, but more as a guideline. Biochemical and molecular taxonomic studies show that trypanosomes classified as *T. b. brucei* can occasionally be isolated from humans, while others that appear to be *T. b. gambiense* occur in pigs and sheep (see Chapter 5 for full details of *T. brucei* population genetics). Nevertheless, some general trends are apparent: *T. b. brucei* infects all domestic mammals, camels, a range of antelopes and some carnivores; *T. b. rhodesiense* infects humans, domestic livestock (including cattle, pigs, goats) and a variety of wild animals including antelopes; *T. b. gambiense* has only been isolated from humans, pigs and sheep. *T. b. brucei* and *T. b. rhodesiense* are easily grown in laboratory rodents, whereas *T. b. gambiense* is not readily amplified in rodents without immunosuppression and lengthy adaptation. In humans and rodents *T. b. gambiense* characteristically produces a chronic infection, while *T. b. rhodesiense* produces an acute infection (see Chapter 10) but the classic course of disease is by no means clear cut.

Van Xong *et al.* (1998) recently identified a single gene from *T. b. rhodesiense* that is capable of transforming the phenotype of a *T. b. brucei* clone from sensitive to resistant to the lytic factor in human serum. Expression of this gene could not be detected in *T. b. gambiense*, suggesting that the trait for human infectivity in this subspecies may have a different basis.

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION Such is the need to identify and track trypanosomes of medical and veterinary importance that virtually every biochemical and molecular technique developed has at some time been used to analyse *T. brucei* sensu lato. Consequently, the section below will seek only to highlight those techniques and studies that have contributed directly to a better understanding of the systematics and evolution of the species. Research aimed primarily at epidemiological and population genetics studies is dealt with in subsequent chapters.

Isoenzymes Taxonomic studies of *T. brucei* s.l. using a range of 11 enzymes succeeded in identifying seven distinct strain groups within *T. brucei* (Gibson *et al.*, 1980, 1985) that broadly corresponded to one or other of the three recognized subspecies. The strain groups represented genetically distinct types and formed a framework for subsequent epidemiological studies. However, early isoenzyme-based characterization studies were essentially phenetic and it was not until the study by Godfrey *et al.* (1990), which presented the first major cladistic analysis of *T. brucei* trypanosomes, that a truly phylogenetic interpretation of relationships within *Trypanozoon* was advanced.

More recently, the utility of isoenzyme studies for phylogenetic analysis has been called into question by Hide *et al.* (1996), who focused on the degree of homoplasy associated with isoenzymes and phylogenetically diverse taxa. Certainly, of the many isoenzyme studies conducted to date, none – with the exception of the sparsely sampled *T. b. brucei* strain group *kiboko* (Gibson *et al.*, 1980) – have identified isoenzymic variants that would differentiate consistently between *T. b. rhodesiense* and *T. b. brucei*

stocks from East Africa, although, by contrast, *T. b. gambiense* was readily defined.

Karyotype The distinct genetic identity of *T. b. gambiense* and the close association of *T. b. rhodesiense* and *T. b. brucei* suggested by isoenzyme analysis also finds support in karyotype analyses. Gibson and Borst (1986) showed that the mini-chromosomes of *T. b. gambiense* were remarkably small, being only 25–50 kb, compared with 50–150 kb in other *Trypanozoon* isolates. Similarly, microfluorometry analysis of nuclear DNA content shows that the genome of *T. b. gambiense* contains about 30% less than that of *T. b. brucei* and *T. b. rhodesiense*, providing further support for the distinct genetic identity of *T. b. gambiense*.

RFLP analysis Analysis of restriction fragment length polymorphisms (RFLPs) derived from variant surface glycoprotein (VSG) genes and ribosomal RNA genes have proved particularly useful for taxonomic studies of *T. brucei*. The limited number of groups defined in initial studies corresponded broadly with the major groups defined by isoenzymes and the genetically homogeneous *T. b. gambiense* was again readily identified; perhaps significantly, numerical analysis of RFLP data is also capable of differentiating *T. brucei* taxa from other *Trypanozoon* species, i.e. *T. evansi* and *T. equiperdum*. Latterly, the groups defined by RFLP analysis in an extended study (which used both RFLPs and isoenzymes) were shown to accord with the results of human serum resistance tests, highlighting the degree of convergent evolution (homoplasy) present in isoenzymes (Hide *et al*., 1996), but confirming the existence of certain strain groups within *T. b. rhodesiense* previously recognized with isoenzymes. (See Chapter 5 for a more detailed discussion of the complexities of *T. brucei* population genetics.)

RAPD analysis Phylogenetic analysis of *T. brucei* RAPD fragment data suffers from a high level of homoplasy (Mathieu-Daudé *et al*., 1995) and few studies have been undertaken. Mathieu-Daudé *et al.* (1995) succeeded only in reliably defining a clade (100% bootstrap support) corresponding to *T. b. gambiense*, with limited support for an east/west divide between *T. brucei* stocks. As with isoenzymes, RAPD analysis is unable to distinguish *T. evansi* from *T. brucei*.

Additional molecular characterization The 177 bp satellite DNA-based repetitive probes developed for *T. brucei* (Gibson and Borst, 1986) detect all three subspecies, suggesting that they consitute a closely related group of organisms (cf. *T. congolense*). PCR probes for classical Group 1 (Gibson, 1986) *T. b. gambiense* have been developed by Bromidge *et al.* (1993). While these probes serve to confirm the distinct genetic nature of classical *T. b. gambiense*, as compared with other *T. brucei* types, including so-called atypical, '*rhodesiense*-like' Group 2 (Gibson, 1986) *T. b. gambiense,* they are of limited use for evolutionary analysis.

DNA sequence analysis Early rRNA sequence-based phylogenetic studies of *T. brucei* used one or at most two (Haag *et al*., 1998) *Trypanozoon* taxa and thus provided little information about the evolution of *T. brucei* and subgenus *Trypanozoon*. Moreover, with hindsight (Lukes *et al*., 1997), it is apparent that this under-representation and taxon imbalance severely affected the topology of the phylogenetic trees produced and frequently resulted in an incorrect placement of *T. brucei* and a paraphyletic representation of the genus *Trypanosoma* (see below). Conversely, studies based on protein coding genes, e.g. glyceraldehyde-3-phosphate dehydrogenase (GAPDH), have consistently shown *T. brucei* to be contained within a monophyletic genus *Trypanosoma*.

Due to limited numbers of taxa, however, none of these studies provided information on evolutionary relationships between *T. brucei* subspecies or within the subgenus *Trypanozoon*. More recently, a broad study of *Trypanosoma* spp. based on 18S rRNA sequences (Stevens *et al*., 1999a) (Fig. 1.1) showed some support for a grouping of *T. b. rhodesiense* with *T. b. brucei* (66% bootstrap support), within a tight (100% bootstrap support) but undifferentiated clade of *Trypanozoon* taxa. Such a result serves to underline further the genetic homogeneity of subgenus *Trypanozoon*.

Trypanosoma (Trypanozoon) evansi (Steel, 1885) Balbiani 1888

Synonyms: *T. equinum*, Voges, 1901; *T. evansi* var. *mborii* Laveran, 1903; *T. soudanense* Laveran, 1907; *T. soudanense* var. *berberum* Sergent *et al.*, 1912; *T. marocanum* Sergent *et al.*, 1915; *T. aegyptum* Nattan-Larrier and Noyer, 1932.

T. evansi, the type species of subgenus *Trypanozoon*, is found worldwide (exceptions being North America and Australia). It is mechanically transmitted by biting flies, chiefly tabanids, and causes an often severe wasting disease (surra) of livestock and indigenous wildlife. A dyskinetoplastic variant was previously described as *T. equinum*.

MORPHOLOGY In the mammalian host, *T. evansi* is typically monomorphic and occurs almost exclusively as long-slender forms (trypomastigotes) indistinguishable from the slender and intermediate forms of *T. brucei* (see above); its overall length ranges between 15 and 36 μm. Naturally dyskinetoplastic strains, in which the normally highly condensed DNA within the kinetoplast is reduced or absent, are not uncommon and are characteristic of mechanically transmitted *Trypanozoon* that no longer undergo a cycle of development in tsetse flies; akinetoplastic strains have also been reported.

HOST RANGE *T. evansi* infects a wide range of domestic animals (e.g. camels, equines, bovines, goats, dogs) and wild animals with varying degrees of pathogenicity. In China, *T. evansi* is an important pathogen of draught buffalo (Lun *et al.*, 1992a), while in South America horses and farmed capybaras are severely affected. In both sub-Saharan Africa and in North Africa beyond the tsetse belt it is a major pathogen of camels and this association has been suggested as a possible route for its transportation beyond Africa and its evolution from *T. brucei* (Hoare, 1972). It readily infects laboratory mammals and thus has been much studied.

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION

Isoenzymes *T. evansi* has been thoroughly characterized by isoenzymes and has proved to be remarkably genetically homogeneous (Godfrey *et al.*, 1990; Lun *et al.*, 1992a), with isolates from Africa, Kuwait, South America and China showing minimal variation compared with that detected even within strain groups of other *Trypanozoon* species. Exceptionally, some genetically distinct camel-derived isolates from Kenya have been characterized. Significantly, *T. evansi* cannot be unequivocally distinguished from *T. b. brucei* by isoenzymes.

kDNA characteristics *T. evansi* is distinguished from tsetse-transmitted *T. brucei* by its distinctive kDNA, which shows a lack of minicircle sequence heterogeneity and an absence of maxicircles (Borst *et al.*, 1987). Its kDNA characteristics (Borst *et al.*, 1987) also support a division of the species into two strain groups, typical type and a camel type, as first isolated at Ngurunit, Kenya.

Additional molecular characterization Isolates of *T. evansi* have frequently been included in large-scale characterization studies of *T. brucei*. While RFLP characterization was capable of distinguishing *T. evansi* from other *Trypanozoon*, RAPD analysis was not.

DNA sequence analysis Few studies to date have analysed DNA sequences from *T. evansi*. Phylogenetic analysis of the 18S rRNA of *T. evansi* does not differentiate *T. evansi* from *T. equiperdum* or *T. b. gambiense*. Such a result supports the hypothesis that *T. evansi* has only recently evolved from *T. brucei* (Hoare, 1972), but highlights the inappropriate use of such a slowly evolving marker as a ribosomal gene for studying genetic variation at the level of *Trypanozoon*.

Trypanosoma (Trypanozoon) equiperdum Doflein, 1901

Synonyms: *T. rougeti* Laveran and Mesnil, 1901; *T. equi* Blacklock and Yorke, 1913.

T. (*T.*) *equiperdum* Doflein, 1901, is a venereally transmitted parasite of equines that is responsible for dourine, a usually fatal chronic disease of equines. In the past, it was common worldwide in open areas where horses are free to roam and mate,

e.g. the Russian steppes, North Africa, the American plains and the Canadian prairies. The disease has now largely been eradicated, mainly by legislation of animal movement, drug treatment and the destruction of sick animals. Over the last few decades the disease has been reported only occasionally from Mediterranean Africa, Italy, Afghanistan, Iran and, more recently, China.

MORPHOLOGY Predominantly confined to the disease lesions, *T. equiperdum* is monomorphic and is indistinguishable from *T. evansi*.

HOST RANGE AND PATHOGENICITY *T. equiperdum*, which has only ever been isolated from equines, is an exception to the lack of host specificity shown by most groupings within *Trypanozoon*. It is initially highly refractory to laboratory animals and can only be isolated into splenectomized or otherwise immunosuppressed animals; however, once established, it is readily passaged through laboratory rodents. Additionally, it has been isolated and maintained by inoculation in rabbit testicles.

In the past, *T. equiperdum* was widely employed for general chemotherapeutic and immunological studies on trypanosomes and, due to the increasing rarity of the parasite and because of the difficulty of isolating it, some existing laboratory strains have been passaged over the course of decades. Accordingly, it is to be expected that some changes in the genetic make-up and physiology of such parasites may have occurred.

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION

Isoenzymes A *T. equiperdum* strain (NIMR 1) of 'doubtful valdity' was found to have a similar isoenzyme profile to typical *T. evansi*. More recently, Lun *et al.* (1992a) characterized a stock of known origin and demonstrated that it had a distinctive combination of isoenzyme patterns; nevertheless, its isoenzyme profile was not sufficiently different as to separate it unambiguously from a variety of *T. evansi* isolates characterized in the same study.

Karyotype and kDNA characteristics Pulsed-field gradient gel electrophoresis (PFGE) analysis (Lun *et al.*, 1992b) shows *T. equiperdum* to possess a distinctive karyotype, again against a similar background of variability in *T. evansi*. The kDNA of *T. equiperdum* is organized like that of *T. brucei*, although the maxicircles are smaller than those of *T. brucei*; the minicircles, like those of *T. evansi* which has also lost the ability to undergo cyclic transmission, are homogeneous in sequence (Lun *et al.*, 1992b).

DNA sequence analysis Phylogenetic analyses of *T. equiperdum* are limited and studies to date have been based on the 18S rRNA gene (Haag *et al.*, 1998; Stevens *et al.*, 1999a) (Fig. 1.1). Results show that *T. equiperdum* clusters tightly with other *Trypanozoon* species (see *T. evansi*).

Subgenus *Pycnomonas* Hoare, 1964

The subgenus *Pycnomonas* contains the single type-species, *Trypanosoma* (*P.*) *suis* Ochmann, 1905, which was reported on only three occasions between 1905 and 1954 (Hoare, 1972). *T. suis* was placed in a separate subgenus on the basis of its life cycle in the fly and was considered to be an intermediate evolutionary stage between development in the proboscis and salivary glands (Hoare, 1972).

Trypanosoma (Pycnomonas) suis *Ochmann, 1905*

T. suis was first described in 1905 by Ochmann, who found it in a herd of sick pigs in Tanzania, but it was not until the 1950s that its life cycle and morphology were fully documented. *T. suis* is a short, stout monomorphic trypanosome with a free flagellum and a small subterminal kinetoplast. It develops in *Glossina* spp. like the tsetse-borne species of *Trypanozoon*, i.e. in the midgut and salivary-glands; however, salivary gland forms are reputedly not infective, full maturation taking place in the hypopharynx of the fly. Pigs are the only domestic mammalian hosts.

More recently, *T. suis* was isolated from *G. pallidipes* caught near Mombasa, Kenya. This trypanosome isolate produced a chronic infection in pigs and allegedly showed many of the characteristics of *T. suis*; stained specimens were unequivocally identified as *T. suis*. However, while recent examination of this isolate (Gibson *et al.*, 2001) from cryopreserved material showed morphology characteristic of *T. suis* (i.e. short trypanosomes with a pronounced undulating membrane and an obvious free flagellum), experimental fly transmissions of the isolate failed to demonstrate salivary gland infections, as would be expected for *T. suis*. Moreover, species-specific PCR analysis produced a positive identification for *T. congolense* Tsavo-type (Majiwa *et al.*, 1993), a result confirmed by phylogenetic analysis of the 18S rRNA sequence (Gibson *et al.*, 2001). While it may be that *T. suis* and *T. congolense* Tsavo-type (renamed *T. simiae* Tsavo-type; see subgenus *Nannomonas* above) are the same organism, the lack of salivary gland infections would suggest otherwise and thus, at the time of writing, it appears that *T. suis* remains as elusive as ever.

Review of Contemporary Taxonomy

Genus *Schizotrypanum*

Of all *Trypanosoma* subgenera currently recognized, the species complement of *Schizotrypanum* is perhaps that most affected by the findings of recent phylogenetic studies (Stevens *et al.*, 1999b). Several isolates/species previously classified as *T.* (*Megatrypanum*) spp., together with five isolates of *T. rangeli* and a single stock of *T. leeuwenhoeki* (both previously classified as *T.* (*Herpetosoma*) spp.) have been shown to cluster unambiguously in a clade including all available *T.* (*Schizotrypanum*) spp. 18S rRNA sequenced to date. Given the majority subgenus *Schizotrypanum* species complement of this clade and the arrangement of taxa within Fig. 1.1, it is proposed that this clade and its extended complement of species/isolates be defined hereafter as subgenus *Schizotrypanum*. However, the structure of subclades within the *Schizotrypanum* clade (Fig. 1.1), together with the findings of published and ongoing phylogenetic studies (e.g. Stevens *et al.*, 2001; Barnabé *et al.*, 2003), suggest that the systematics of subgenus *Schizotrypanum* may require further revision and possible subdivision in the near future.

Salivarian trypanosomes

The results of phylogenetic analysis of the salivarian trypanosomes differ somewhat from those relating to many stercorarian species (Stevens *et al.*, 2001) (Fig. 1.1). Apart from the reclassification of *T. simiae* Tsavo (previously *T. congolense* Tsavo), the wholesale 'jumping' of species between subgenera (cf. Stercoraria) has not occurred (Fig. 1.1) and the major finding concerns *levels* of phylogenetic resolution within salivarian subgenera and how this relates to the definition of species within subgenera.

In particular, the relationship between phylogenetic variability and the recognition of species status, which is most at odds in subgenus *Trypanozoon* and *T.* (*N.*) *congolense*, needs to be resolved. *T.* (*Trypanozoon*) spp. sequences, which include representatives of the three species *T. brucei*, *T. evansi* and *T. equiperdum*, differ by at most two nucleotides across the whole 18S rRNA gene, while *T. evansi*, *T. equiperdum* and *T. b. gambiense* are identical. This minimal level of phylogenetic variation contrasts starkly with that observed within *T. congolense*, where strain groups/'types' are separated by differences of more than 100 nucleotides and even intra-strain group variation ranges between five and 11 nucleotides across the 18S rRNA gene. Of course, it could be argued that such differences in resolution simply highlight the inadequacy of the 18S rRNA gene and the use of phylogenetics generally as a basis for studying systematics and constructing taxonomies. Nevertheless, the relationships described by the 18S rRNA-based phylogeny are also supported by a broad range of additional biological and molecular evidence that has proved beneficial in arriving at a better understanding of the systematics of *T. simiae* and related pig-pathogenic *T.* (*Nannomonas*) spp. (McNamara *et al.*, 1994). Accordingly, it is suggested that the intraspecific taxonomy of *T. congolense* also requires review.

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2 Antigenic Variation

David Barry and Mark Carrington

Biology of Antigenic Variation

There are two main strategies used by parasites that allow them to evade the immune responses of their mammalian hosts. Protozoan parasites use population growth to avoid eradication by specific, potent immune responses. This is in contrast to helminth parasites, which are large parasites that do not undergo repeated bursts of proliferative growth within the host and actively disarm or divert the immune system, for example by producing molecules mimicking regulators of immunity. Those protozoan parasites that live in the vasculature are challenged and killed by specific responses, but yield a steady trickle of variants unrecognized by the current response. Proliferation of these variants produces a new parasite population, which in turn is subject to a new, specific immune response. This is the basis of antigenic variation, which was discovered a century ago in African trypanosomes. Regardless of whether they are mainly tissue-fluid dwelling, as is the case for *Trypanosoma brucei*, or blood dwelling, such as for *T. vivax* and *T. congolense*, the level of trypanosomes in the blood fluctuates with time, due to antigenic variation (Fig. 2.1). This survival process shares a number of features with evasion systems in many other micropathogens, including viruses and bacteria. There are general logistic similarities among these systems, which collectively are known as contingency gene systems (Moxon *et al*., 1994).

The basis of the trypanosome system of antigenic variation is the protective coat on the parasite (reviewed by Cross, 1996; Barry and McCulloch, 2001; Borst, 2002). The entire cell surface of bloodstream and metacyclic form trypanosomes, including the flagellum, is covered with a coat that is thought to provide general protection against non-specific host resistance mechanisms. The coat is a prominent immunogen and elicits high titres of antibodies that are lytic to the parasite. Through antigenic variation, which operates simply by rare individuals changing to another coat, some parasites survive and can produce a new wave of growth. Each variant is termed a distinct variable antigen type (VAT). The different VATs retain the general protectiveness of the coat, while providing the variation enabling avoidance of specific antibodies. As antigenic variation is centrally linked to the growth and transmission of parasites, it is important to understand its underlying organismal, molecular and genetic mechanisms.

Antigenic variation is clonal. Individual trypanosomes spontaneously undergo the switch to a new coat and continue to proliferate. As with other contingency systems, the switch is not induced by the selecting

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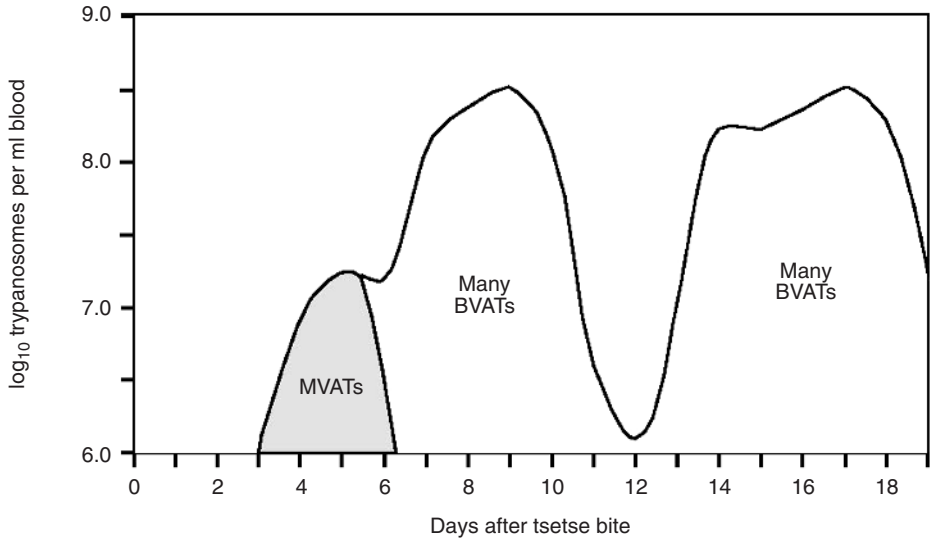


Fig. 2.1. Trypanosome growth and antigenic variation in a mouse following introduction of a metacyclic population via tsetse transmission. The parasitaemia fluctuates due to alternating antibody assault and trypanosome growth, which is also inhibited by differentiation to the non-proliferative stumpy stage. In the first wave, the initial VATs are those expressed by the metacyclic population, which continue to be expressed after differentiation to the bloodstream stages and are removed by a combination of antibody-mediated lethality and switching to bloodstream VATs (BVATs). As indicated, the major peaks comprise a mixture of different VATs. MVATs = metacyclic VATs. Data are derived from Hajduk and Vickerman (1981).

agent, which in this case is antibodies. Thus, switching is pre-emptive, generating a minority of new VATs before the antibody assault on trypanosomes of the old VAT. Antigen switching is rapid, at an average rate of one switch in every 100 trypanosome doublings, which is typical of contingency systems and is several orders of magnitude faster than fortuitous changes arising from background mutation. That the rate is similar amongst different pathogens probably reflects a general balance between the replication of pathogens and the kinetics of onset of immunity against them. Although the overall antigenic variation rate is probably constant throughout the infection, the whole process of antigenic variation is imprecise when examined at the level of the infecting trypanosome population, probably as a consequence of the complex relationships between the rates of parasite growth and differentiation, and of immune response onset, in a given host species. Rather than there being a simple sequence of one VAT at a time, most parasitaemia peaks are composed of a mixture of VATs and the whole process is sequential, in the sense that VATs do not all appear together, but rather are spread out throughout infection (Gray, 1965; Van Meirvenne *et al.*, 1975). Despite being sequential, VAT expression appears not to run according to a specific order. Instead, as has long been recognized for *T. brucei* and the related *T. equiperdum*, as well as *T. vivax*, there is a hierarchy in timing, some VATs tending to appear early and others later (Capbern *et al.*, 1977; Barry, 1986). It should be noted, however, that short-term infections have revealed some more specific patterns of expression and that there has been little study of the sequence of expression later in chronic infections. Hierarchical expression may well have considerable benefit for the parasite, by prolonging infection and thus increasing the probability of transmission to tsetse. As it leads to unpredictability in VAT expression sequence, it probably has a major advantage over a specific order of expression, which is more likely to be vulnerable to disruption by antibody action.

For a long time now, it has been held that there is little prospect for vaccination against the surface of African trypanosomes. The coat appears to be the only clear candidate as an exposed antigen, and the extent of its antigenic variation during individual infections is enormous and unknown. Direct cloning of trypanosomes from a cloned line of *T. equiperdum* shows that there is a minimum of 101 distinct VATs (Capbern *et al.*, 1977) and indirect estimation of the gene family involved placed the maximum at about 1000 distinct genes. However, there are complications with such estimates, as described later. Thanks to antigenic variation, infections can run for months or even years. In experimentally induced infections of cattle, which support the growth of large numbers of trypanosomes and hence extensive antigenic variation, there is some preliminary evidence that self-cure occurs and that this is associated with anti-VAT responses (Nantulya *et al.*, 1986). One interpretation is that the trypanosome has exhausted its repertoire of VATs. When different trypanosomes from the same, or different, geographical areas are compared, their VAT repertoires are different from each other and, although some VATs are shared (Table 2.1), this diversity must result from a rapid rate of evolution. Repertoires can become mixed, creating novel combinations of the repertoire, following mating of different trypanosome strains in tsetse. Nevertheless, as mating is not essential for transmission through flies, trypanosomes can be transmitted clonally, such as in epidemics of acute sleeping sickness, resulting in persistence of a single repertoire. There is evidence that *T. gambiense* has a smaller repertoire than other *T. brucei* (Pays *et al.*, 1983) and it would be interesting, for the purpose of understanding the relationship between antigenic variation and the course of chronic infection, to obtain comparison of respective repertoire sizes through genome sequencing. Similarly, the taxonomic separation between the *brucei* group and *T. vivax* and the *T. congolense* group means that there are likely to be informative differences between their antigenic variation systems.

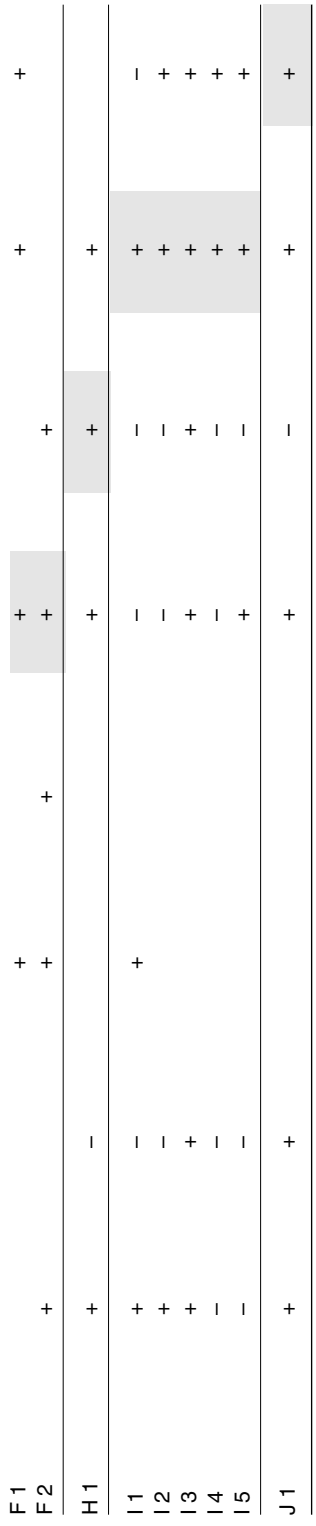
As the surface coat has a general protective role for trypanosomes in their mammalian hosts, it is essential and has to appear on the metacyclic stage in the salivary glands of the tsetse, which is the first stage to be introduced into the mammalian host. The metacyclic population is diverse in these coats (reviewed by Barry and McCulloch, 2001), expressing up to 27 metacyclic VATs (MVATs) in *T. brucei* and 12 in *T. congolense*; the figure for *T. vivax* is unknown. An intriguing feature of the MVAT repertoire is that, in the short term, it is invariant. No matter what VAT was expressed by the bloodstream trypanosomes that infected the fly, the same MVAT set becomes displayed as the metacyclic population differentiates. The combination of invariance and relatively small repertoire used to raise hopes for the development of a vaccine, but the invariance is not strict over a number of fly passages, leading to the loss of some MVATs from the set and the appearance of others. It is likely that this turnover would thwart field vaccination based on the MVAT coats.

Variant Surface Glycoprotein Coat

With the demonstration of the surface coat on bloodstream and metacyclic form trypanosomes (Vickerman, 1969) came the impetus to seek an understanding of antigenic variation through molecular analysis of the coat. Its biochemical analysis was made possible by the development of techniques for the propagation of clonal populations of trypanosomes that undergo antigenic variation at a low rate and thus allow the production of large numbers of antigenically identical trypanosomes (Cross, 1975). The variant surface glycoprotein (VSG) is by far the most abundant component of the cell surface coat and it is composed of a single polypeptide species. A series of analyses of VSGs purified from a range of trypanosome clones all led to the conclusion that different VSGs are markedly different from each other in primary sequence. However, it was also clear that the different VSGs have certain properties in common. They are homodimers (Auffret and Turner, 1981), present at 5.5×10^6 dimers per

Table 2.1. VAT repertoires in *Trypanozoon.* A set of 34 different VATs, isolated as trypanosome clones from a number of different trypanosome stocks, were examined for presence in eight stocks. This was by a combination of cloning of trypanosomes expressing those VATs, detection by immunofluorescence during infections and reaction with lytic antibodies present in serum from rabbits chronically infected with the different stocks. Most of the stocks were cloned, as shown. Data are collated from Tables 7 and 8 in Van Meirvenne *et al.* (1977).

VAT	A *brucei* Uganda (cloned)	B *rhodesiense* Uganda (cloned)	C *rhodesiense* Rwanda	D *rhodesiense* Rwanda	F *gambiense* Ivory Coast (cloned)	H *brucei* Nigeria (cloned)	I *evansi* S. America (cloned)	J *equiperdum* ? (cloned)
A 1.1	+	–			–	–	–	–
A 1.2	+	–			–	–	+	–
A 1.3	+	–			–	–	–	–
A 1.4	+	–			–	–	–	–
A 1.5	+	–	+		+	+	–	–
A 1.6	+	–	+	+	–	–	–	–
A 1.7	+	–			–	–	–	–
A 1.8	+	+			+	+	+	+
A 1.9	+	–			–	–	–	–
A 1.10	+	–			–	–	–	–
A 1.11	+	+			–	–	–	–
A 1.12	+	–			–	–	–	–
A 1.13	+	–	+		–	–	–	–
B 1.1	–	+			–	–	–	–
B 1.2	–	+			–	–	–	–
B 1.3	–	+	+		–	+	+	+
B 1.4	–	+					–	
B 1.5	+	+				+	–	
B 1.6	+	+			–	–	+	+
B 1.7	+	+			–	–	–	–
B 1.8	+	+				+	+	
B 1.9	–	+					–	
B 1.10	+	+	+				–	
B 1.11	–	+					–	
B 1.12	–	+					–	



cell and represent 15–20% of the total cell protein and > 95% of the externally disposed cell surface protein. The VSG is attached to the surface of the trypanosome by a covalent linkage from the carboxyl group of the C-terminal amino acid to a glycosylphosphatidylinositol (GPI) anchor in the outer leaflet of the plasma membrane. The GPI anchor consists of a core, present in all VSGs, of ethanolamine-phosphate-6-mannose α1–2 mannose α1–6 mannose mannose α1–4 glucosamine α1–6 *myo*inositol-1-phosphate-*sn*-1,2-dimyristoylglycerol and, for most VSGs, is modified with galactose residues (see below). As a consequence of this means of attachment, the VSG is able to diffuse rapidly within the plane of the membrane (Bulow *et al.*, 1988) and almost certainly rotate around its axis perpendicular to the plane of the membrane, though this has not been demonstrated experimentally. The presence of the GPI anchor means that the membrane form of the VSG is not water-soluble. The purification of VSGs originally relied on the action of an endogenous phospholipase C that hydrolyses the GPI anchor, releasing a water-soluble form and dimyristoylglycerol (Fox *et al.*, 1986; Hereld *et al.*, 1986). The residual oligosaccharide from the GPI anchor of the VSG is moderately immunogenic and is the source of the only immunological cross-reactivity between many VSGs – the cross-reacting determinant.

Primary structure of the VSG monomer

Since the pioneering work described above, the complete primary sequences of about 60 individual VSGs from *T. brucei* ssp., *T. evansi*, *T. equiperdum*, *T. congolense* and *T. vivax* have been derived from the corresponding cDNA sequences (Carrington *et al.*, 1991; Cross, 1996; Ferguson, 1999). The sequences can be retrieved from the EMBL or Genbank databases. This number of VSGs is sufficiently large to enable some general conclusions to be made about VSG primary sequences. The VSGs of *T. brucei, T. evansi* and *T. equiperdum* are all very similar to one another but differ significantly from those of *T. congolense* and the single sequence available for *T. vivax.*

As found on the cell surface of *T. brucei*, *T. evansi* and *T. equiperdum*, the VSG polypeptide is between 400 and 500 amino acids long, with most having between 420 and 460 residues. The majority of the sequence forms a single N-terminal domain of 350–400 residues, one of the largest single protein domains known, and one or two smaller C-terminal domains of 40–80 residues each (Allen and Gurnett, 1983; Carrington *et al.*, 1991). Each domain contains an independently conserved pattern of cysteine residues (see Plate 1). To date, there are three alternative patterns of cysteines found in the N-terminal domain (A, B and C), and four patterns of cysteines in the C-terminal domain(s) (1, 2, 3 and 4). Types 2 and 4 are single domains, each containing four cysteine residues, whereas types 1 and 3 contain eight cysteines and indicate two separate domains, each containing four cysteines. Any VSG can thus be described by the distinct pattern of cysteine residues within its N- and C-terminal domains (A1, A2, A3, A4, B1, B2 etc.) and there appears to be no restriction on combinations; there is also a case now of two VSGs with similar N-terminal domains but different C-terminal domains. Other than the cysteine residues, there is little conservation of primary sequence within the N-terminal domains, though the physicochemical nature, such as hydrophobicity, is conserved in a residue at a particular location. The C-terminal domains have a greater degree of primary sequence identity, especially within each type. This conservation of sequence in the C-terminal domain does not compromise antigenic variation, as the epitopes recognized by antibodies on the surface of living trypanosomes all fall within the N-terminal domain (Miller *et al.*, 1984). Further evidence for the inaccessibilty of the C-terminal domain to external immunoglobulins comes from the finding that antibodies raised against the recombinant C-terminal domain of a VSG do not bind living cells (P. Voorheis, Dublin, 2002, personal communication). Amongst the VSG sequences available, there are two sets that were expressed sequentially during an infection and thus represent a series of VSGs produced as a result of antigenic variation (Miller and Turner, 1981). The primary sequences of these VSGs are remarkably different from each other, with a basal level of 15–20% identity, confirming that antigenic variation results from variation of the primary amino acid sequence in VSGs.

As denoted by their name, VSGs are glycoproteins. Their GPI anchor is modified by the addition of galactose residues, the number and location of which are determined by the type of C-terminal domain. Type 1 and type 2 domains have between three and six galactose molecules on the core GPI anchor, with type 2 having more galactose on average, whereas type 3 C-terminal domains have none. It has been proposed that this heterogeneity results from a space-filling function for the galactose residues and thus is determined solely by the primary structure of the VSG. There are also N-linked glycosylation sites, of which *T. brucei* and *T. equiperdum* VSGs have at least one, as do most of the VSGs from *T. evansi*, but it is worth noting that two *T. evansi* VSGs have no such sites. There is a single site within each of the C-terminal domain types 1 and 2, and the location of this site in the primary sequence is conserved within each domain type. The C-terminal domain type 3 does not contain an N-linked oligosaccharide, despite the C-terminal asparagine being in an N-linked glycosylation motif, but it is linked to the GPI oligosaccharide. The oligosaccharide in the C-terminal domain varies with the type of domain and is not homogeneous. In type 1 C-terminal domains, it is predominantly of the branched oligomannose-type and varies in size from mannose$_9$-*N*-acetyl glucosamine$_2$ to mannose$_5$-*N*-acetyl glucosamine$_2$. A similar situation is present in type 2 C-terminal domains, except that the size range is more restricted, being between mannose$_9$-*N*-acetyl glucosamine$_2$ and mannose$_7$-*N*-acetyl glucosamine$_2$ with a more complicated oligosaccharide with galactose and *N*-acetyl lactosamine residues decorating a branched mannose$_3$-*N*-acetyl glucosamine$_2$ core (Zamze *et al.*, 1990). There is further heterogeneity in the fraction of each type of oligosaccharide present at each site: in some

VSGs the oligomannose oligosaccharide is present on most VSG molecules, whereas in other VSGs the more complex galactose and *N*-acetyl lactosamine containing oligosaccharide predominates (Zamze *et al.*, 1990; Mehlert *et al.*, 2002). There are between zero and three N-linked sites within the N-terminal domain. Unlike the C-terminal domain, the location of the N-linked sites in the N-terminal domain is variable but not random. The nature of the oligosaccharide present at these sites is also more variable, ranging from mannose$_9$-*N*-acetyl glucosamine$_2$ to mannose$_3$-*N*-acetyl glucosamine$_2$. In addition, some are decorated with galactose and/or *N*-acetyl lactosamine (Zamze *et al.*, 1990; Zamze *et al.*, 1991; Mehlert *et al.*, 2002). The heterogeneity of glycosylation at both N-linked sites and the GPI anchor were only slightly affected when a cell line forced to express two VSGs was analysed. This result implies that the most important consideration in determining the glycosylation pattern of a VSG is its tertiary structure.

The function, synthesis and properties of the VSGs from *T. congolense* appear to be similar to those of other VSGs (Gerold *et al.*, 1996). The major point of difference between the VSGs described above and the ten available complete VSG sequences from *T. congolense* lies within the C-terminal domain and GPI anchor. The cysteines characteristic of the different C-terminal domain types present in other VSGs are not present, and it is probable that the C-terminal domain of *T. congolense* VSGs is smaller than the equivalent domain in *T. brucei* VSGs (see Plate 1). The latter possibility is supported by the overall length of the mature *T. congolense* VSGs, which ranges from 350 to 410 residues – significantly less than the *T. brucei* VSGs. The GPI anchor on *T. congolense* VSGs has the same core structure as that of other VSGs but is modified with *N*-acetyl glucosamine as well as the galactose found in other VSGs (see Plate 1). The N-terminal domains of all of the *T. congolense* VSGs characterized are related to those of *T. brucei* VSGs, but all ten have an N-terminal domain of type B. It is not clear yet whether this more restricted variability represents a more limited repertoire of VSGs, or whether it is just chance that only this type has been found to date. The single VSG sequence from *T. vivax* is not complete but has primary sequence identity to the N-terminal domain of a putative *VSG* gene in the *T. brucei* genome. The *T. vivax* VSG does not contain a cysteine-rich C-terminal domain.

Tertiary structure of the VSG

The VSG, in addition to undergoing antigenic variation, has to form an effective barrier to prevent access of immunoglobulins and complement components to other cell surface proteins. For example, on living cells the VSG prevents any substantial binding of immunoglobulins against *T. brucei* invariant surface glycoproteins (ISGs), presumably by preventing their access (Ziegelbauer and Overath, 1993). This raises the intriguing question of how a large number of VSGs, each with the different primary sequences required for antigenic variation, are all also able to form a protective barrier. The question was answered when the structure of two different VSGs was solved by X-ray crystallography (Freymann *et al.*, 1990; Blum *et al.*, 1993). These were the N-terminal domains of two type A VSGs, with a basal level of ~20% amino acid sequence identity. It is believed that the C-terminal domain was removed by a contaminating protease during crystallization. Subsequent attempts to solve the structure of a complete VSG have not been successful (Garman, 1994). The VSG N-terminal domain is a homodimer and has a long axis of ~10 nm. The backbone of this long axis is the two α-helices in each monomer that form a coiled coil and give the domain its characteristic elongated shape. The two known VSG N-terminal domain structures are compared in Plate 2, from which it is immediately apparent that they have a remarkably similar tertiary structure, and they remain a paradigm for the conservation of tertiary structure despite divergence of primary sequence. It is believed that the long axis of the VSG is perpendicular to the cell surface and that the VSG forms a monolayer covering the entire surface. The 10 nm length of the VSG N-ter-

minal domain is in accordance with the 15 nm thickness of the cell surface coat measured in electron micrographs, the remaining ~5 nm presumably being occupied by the C-terminal domain and the GPI anchor.

Although only two VSG N-terminal domain structures have been solved, it is possible to produce models of other VSGs (Mehlert *et al.*, 2002) (see Plate 2). The conclusion from the modelling is that all types of VSG N-terminal domain probably have a very similar tertiary structure. Thus, the N-terminal domain of the VSG is under two opposing selection pressures: (i) extreme sequence variation in the primary sequence to maximize antigenic variation; and (ii) conservation of tertiary structure to maintain a barrier to protect other cell surface components from effectors of the host immune system. The conserved tertiary structure means that, although there are residues within the N-terminal domain that are internal and not accessible to host immunoglobulins, the sequence variation at these positions is as great as elsewhere. The selection pressure for variation at these positions is not understood, but is consistent with a strategy evolved to minimize continual activation of T-helper cells by processed peptides derived from VSGs that might otherwise occur during a persistent infection.

Structure of the VSG monolayer

In addition to a central role in antigenic variation, VSGs must all have the ability to pack to form the dense barrier outlined above. This is necessary for the survival of the trypanosome population in the host as, during the course of an infection, the host produces antibodies not only against VSGs but also against many other trypanosome proteins, which presumably stimulate immunity after being released following anti-VSG-mediated lysis of trypanosomes. The stunning conservation of secondary and tertiary structure of the VSG N-terminal domain almost certainly results from a requirement to form a physical barrier between invariant cell surface components and host immunoglobulins. It is also possible that the VSG monolayer forms a barrier between the complement components that initiate the alternative pathway and the cell surface component, but to date no activators of the alternative pathway have been identified.

It is assumed that the VSG dimers are closely packed on the cell surface to form the protective barrier. There are estimates, but no direct measurements, of the mean distance between dimers. The most detailed estimate of the mean surface area per VSG dimer (30 nm^2) comes from an estimate of the packing density of the VSG, itself derived from a measurement of the mean number of VSG molecules per trypanosome (1.1×10^7 monomers) and an estimate of the surface area (165 μm^2) (Jackson *et al.*, 1985). The proximity of the VSG dimers to each other in the surface coat, at least in the N-terminal domain, is limited by the widest part, which is at the base, nearest the cell surface (see Plate 2). These 'hips' at the base of the N-terminal domain are significantly wider than the rest of the molecule and have an area of ~22 nm^2, a measurement that does not take account of waters of hydration. A model of the monolayer based on these measurements has gaps between the VSG dimers, both in a vertical section and when viewed from outside the cell. The gap is particularly apparent in the middle of the VSG layer and is reduced but still pronounced at the top of the VSG. Importantly, the gap appears to be too small to allow complete penetration of immunoglobulins through the monolayer, but large enough to permit penetration as far as the 'hips' (Hsia *et al.*, 1996). The model presented here takes no account of the N-linked oligosaccharides occupying space within these gaps that would further hinder immunoglobulin penetration (Mehlert *et al.*, 2002). Some evidence for a space-filling role for the oligosaccharides comes from the observation that, although only part of the structure of the N-linked oligosaccharide on MITat1.2 was resolved in the crystal structure, this occupied a space filled by an α-helix in the second structure. The model is very tentative in the absence of firm information on the structure of the C-terminal domain but does appear to explain why immunoglobulins cannot penetrate the VSG monolayer. It is hoped in the future that the model can be

developed to include the VSG C-terminal domain and non-VSG cell surface proteins and become predictive of the penetrance of any potential therapeutic.

Genetic Basis of Antigenic Variation

When it was realized that different VSGs had entirely distinct N-terminal sequences, the possibility emerged that each was encoded by a distinct gene, and the field of molecular parasitology took off in earnest with the cloning of a number of cDNAs complementary to VSG mRNAs (Williams *et al.*, 1979; Hoeijmakers *et al.*, 1980). These materials led rapidly to the uncovering of a very complex system for the differential expression of VSGs. To this day, the underlying mechanisms remain elusive, partly due to some of the experimental limitations that accompany the study of trypanosomes.

Nevertheless many, and perhaps most, of the basic features have been described (Cross, 1996; Barry and McCulloch, 2001; Vanhamme *et al.*, 2001a; Borst, 2002). Each VSG is encoded by a distinct gene, or in pieces of different genes, which is kept silent until activated. Many of the silent genes are interstitial, occupying loci within chromosomes, while others are subtelomeric, lying just upstream of the set of simple DNA repeats that form the telomere, the end of the chromosome. The genomes of *T. brucei* and of *T. congolense* have become augmented by a very large set of mini-chromosomes which, at least in the former species, contain almost no genes other than a set of up to 200 subtelomeric *VSG* genes. Potentially, gene counting could reveal the extent of the repertoire available for antigenic variation. However, whereas the repertoire of interstitial genes was estimated initially at about 1000 arranged in tandem arrays, the reality appears more complex. As the sequencing project gradually unveils the contents of the genome, up to 90 *VSG*s have become apparent (M. Carrington, P. Burton, L. Marcello and J.D. Barry, unpublished). Some are in arrays; it is too early to conclude about the rest. Surprisingly, at least three-quarters of the genes are incomplete and are incapable of yielding an intact surface coat. While acknowledging that hundreds of intact *VSG*s may lie undiscovered as yet, the situation emerging is one or both of the following: that the effective repertoire is much smaller than anticipated, or that trypanosome antigenic variation uses, besides the conventional mechanism of switching between intact genes, a system found in contingency systems in some bacterial pathogens. There, silent information is encoded in incomplete genes, which are assembled into intact genes by a copying process (Restrepo and Barbour, 1994; Brayton *et al.*, 2002). There already is evidence that *T. brucei* undertakes the latter route for some *VSG* genes, as described below. One way or another, the trypanosome has an enormous bank of silent information at its disposal for undergoing extensive antigenic variation.

There is a substantial logistical problem in regulating a gene family of this size such that only one member is active at a time in each trypanosome. Much of the problem is solved by transcription being restricted to transcriptionally active sites known as bloodstream expression sites (BESs) (see Plate 3) (reviewed by Pays *et al.*, 2001). Silent genes must move there to become active. To do so, as shown in Plate 3, they are duplicated in a reaction that leaves the silent gene intact, while replacing the active gene in the BES with the new copy (reviewed by Barry and McCulloch, 2001). The silent gene is thus kept intact for use in subsequent infections. Because this gene conversion reaction operates via the machinery of homologous recombination, it requires sequence homology between the donor and acceptor sites, but this creates a dilemma. There must also be substantial difference in sequence between them, in the region of the gene that encodes the variable epitopes necessary for antigenic variation. The dilemma is solved by the homology being restricted to the flanks of the genes. There is a set of common repeats ('70 bp repeats') lying upstream of most *VSG* genes, and there are common sequences lying within the other end of the coding region or immediately downstream. The all-important region encoding the variable domain of the protein is thus recom-

bined into the BES within a cassette, the flanks of which provide the substrates for homologous recombination. Some incomplete, silent genes lack the downstream homology and they recombine into the BES using homology further within the coding sequence. Presumably this results in a tendency for switching between genes related in their coding sequences. One outcome could be that there is more of a pattern to switching than previously suspected, and this could be fairly prominent if it does transpire that most *VSG*s are incomplete in this way. For most of the subtelomeric silent genes, including those on mini-chromosomes, gene conversion is also the route to activation. In these cases, however, duplication spans from the 70 bp repeats, through and beyond the *VSG*, possibly as far as the end of the chromosome. Not only is this indicative that the high rate of recombination between the subtelomeres of different chromosomes may account for the preferential use of telomeric genes as donors in antigenic variation, but it also provides evidence that interstitial and telomeric silent genes are duplicated by distinct recombinational mechanisms.

Convenient as these recombinational mechanisms may be for the exclusive and individual activation of *VSG*s, the story is complicated by the fact that there are many BESs in the genome. The system has to maintain only one BES active at a time, to prevent wasteful, simultaneous expression of more than one *VSG*. Exclusive expression is achieved through the use of a unique structure, the expression site body (ESB), for which BESs apparently compete (Navarro and Gull, 2001). The ESB is thought to contain a transcription factory that ensures correct initiation and progression of transcription, and that has also been suggested to be associated with proper export of the mRNA from the nucleus (Vanhamme *et al.*, 2001b). Gaining access to the ESB, and hence the privilege of being transcribed, probably involves communication between the two BESs involved. The inactivated BES then appears to revert to a state of basal expression from the promoter that terminates a short way downstream, well before the resident *VSG* gene. Consequently, no *VSG* is transcribed from these silent sites, but there can be expression of the genes adjacent to the promoter. Thus, although antigenic variation in the bloodstream is driven primarily by homologous recombination, it also involves transcriptional silencing and derepression. Interestingly, this type of regulation operates in the metacyclic population in the tsetse. The MVATs express metacyclic VSGs (MVSGs), which are located at subtelomeres of large chromosomes (reviewed by Donelson *et al.*, 1998; Barry and McCulloch, 2001). Unusually for the trypanosome, these genes have their own promoters. The promoters are completely silent throughout the life cycle until, as the metacyclic stage differentiates, individual trypanosomes activate one of them, at random. Because this is a fixed set of genes at the subtelomeres, and because of their random activation, the metacyclic population emerges from the fly as a mixture of MVATs, which is thought to enhance transmission in the field. The promoters used in the fly differ from those used in the bloodstream, which possibly tallies with the distinct levels of silencing applied to both when inactive, although the *MVSG* promoters continue to be used for several days in mammals following transmission. There are a number of theories about the regulation of *VSG* genes that are addressed regularly in reviews.

The existence of many BESs, when only one is needed at a time, and of many genes within each, is enigmatic. Between the stretch of 70 bp repeats and the promoter lie typically about eight genes known as expression site-associated genes (*ESAG*s) (see Plate 3). They encode a number of proteins, including several destined for the cell surface and one that has a role in the nucleolus. For example, *ESAG4* forms a diverse family, several hundred strong, encoding what appear to be cell surface receptors. There could be a number of reasons for inclusion of these genes in BESs, ranging from coincidental to a system for co-regulation of genes required in the mammalian phase of the life cycle. Like the transcription units encoding the procyclin surface coat that is present on all tsetse stages except the metacyclic form, the BES is unusual, in that it is fully transcribed

in only specific life cycle stages. It was hoped early on that the *ESAG*s might thus be a group of genes required for specific functions related to trypanosome life in the mammal, but for most there is little evidence of this. Except for *ESAG6* and *ESAG7*, which encode the two subunits of the essential transferrin receptor of the trypanosome, all the *ESAG*s have family members elsewhere in the genome that are expressed in both mammal and vector hosts (Vanhamme *et al.*, 2001a). Unless the family members in BESs are specific isoforms required in the mammalian phase, the co-regulation of most BES genes seems to be coincidental. An attractive hypothesis is that diversity between BESs expands the host range of the trypanosome (Gerrits *et al.*, 2002). The ESAG6 and ESAG7 proteins contain short, hypervariable regions, which have been proposed to correlate with structural differences between transferrin from different host species, so that different BESs are best suited to different hosts. As the high concentration of transferrin in plasma means that the different receptor isoforms would bind, equally well, transferrin from any source, regardless of structural differences, it has been proposed further that there is competition between antibodies and transferrin for the receptor in any host (Gerrits *et al.*, 2002). It would be interesting if this were shown to be the case. The concept of multiple BESs extending the host range can be applied also to an unusual gene, known as the serum resistance-associated (*SRA*) gene, which confers resistance to the lytic effect of non-immune human serum and is the main feature distinguishing *T. b. rhodesiense* from *T. b. brucei*. It is present in one BES and becomes activated when that BES is switched on (Xong *et al.*, 1998). This is a clear case of the use of BESs to alter host range. Another theory for the multiplicity of BESs is that they are needed to allow the construction of complete *VSG* genes from incomplete ones before becoming activated.

The hierarchical switching of VATs may have some basis in gene activation. At the beginning of infection, subtelomeric genes are activated and, much later, incomplete genes are involved. Not a lot is known about what happens in between but there is some indication that the interstitial genes follow on from the subtelomeric group. It is expected that these gene groups overlap in expression. The reason for the gene groups being expressed sequentially is not that there is a highly regulated gene-switching programme. Instead, it is likely that each gene has a distinct probability for activation, so that when an individual switch is occurring, the highest likelihood is that a subtelomeric gene will be involved. At the other end of the scale, incomplete genes may require several recombination events to yield a complete gene, meaning that they have a very low probablility for activation. As infection proceeds, therefore, subtelomeric genes will be activated continually, but after antibodies are present the switch product is killed, leaving the way open for proliferation of VATs not activated so frequently. As mentioned above, the discovery of many incomplete genes suggests that similarities in coding sequences may impose a degree of order to the process.

Conclusions

The antigenic variation system in trypanosomes operates at two levels. At the level of the population within the host, there is much imprecision, resulting in a highly varied course of antigenic variation and of infection. This appears to enable an overwhelming evasion of immunity and high chances for transmission to tsetse. At the cellular and molecular level, there is a highly sophisticated set of systems that ensure precision in the expression of only one VSG at a time, and that yield a highly diverse, yet structurally sound, protective surface coat.

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3 The African Trypanosome Genome

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Introduction

The African trypanosomes have two genomes, one within the nucleus and the other within the single mitochondrion (the kinetoplast), with the latter accounting for some 10–20% of the total DNA content. Renaturation experiments have indicated that the nuclear genome is of relatively low complexity with around 68% single copy sequences and the remainder comprising middle and highly repetitive sequences. A number of independent pieces of evidence show that the nuclear genome is essentially diploid; these include interpretation of Southern blot data using single copy gene probes, isoenzyme profiles, micro- and minisatellite genotypes, single nucleus DNA content in relation to kinetic complexity, and the molecular karyotype (see below). The key point in this evidence is the observation of two alleles for many of the genes or markers examined and segregation of these in the progeny of laboratory crosses (Tait *et al.*, 2002). There are, however, parts of the genome that are not diploid (e.g. the variant surface glycoprotein genes and their expression sites, and mini-chromosomes). Estimates of the size of the haploid nuclear genome content from analysis of DNA content and kinetic complexity provide values of approximately 3.5×10^7 bp (35 Mb). This is about three times the size of the yeast genome and roughly the same size as the genomes of *Plasmodium falciparum*, *Leishmania major* and *Trypanosoma cruzi*, but an order of magnitude smaller than higher eukaryotic genomes.

Despite the great economic importance of *Trypanosoma* (*Nannomonas*) *congolense* and *T.* (*Duttonella*) *vivax,* two species of pathogenic trypanosomes that cause serious disease in livestock, knowledge of the genomes of African trypanosomes is almost entirely based on study of *T.* (*Trypanozoon*) *brucei*. At the time of writing the shotgun sequencing of the genome of the South American trypanosome, *Trypanosoma cruzi,* is underway, although the molecular karyotype is only partially elucidated and the chromosome structure remains unresolved. While there must be significant genetic differences between the species, underlying the variation in the life cycle, host range and pathogenesis, it is likely that the overall architecture, gene content and gene organization of the trypanosome housekeeping chromosomes will be substantially similar (see later).

In the early period of molecular research with African trypanosomes the available techniques allowed the isolation of genes, their manual sequencing and analysis by Southern and Northern blotting, as well as heterologous expression of the gene product

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in bacteria or yeast. The main focus of research was on analysing the molecular basis of antigenic variation but this subsequently broadened into analysis of the kinetoplast, RNA editing, metabolic pathways and the cell cycle. This type of research provided much novel data on gene structure and function but only accessed a very small portion of the total genome and, in terms of the search for new genes, was laborious and time consuming. In 1997, the World Health Organization (WHO) set up a genomics initiative to stimulate and facilitate efforts aimed at sequencing the nuclear genomes of *T. brucei* and other pathogens that affect peoples of the developing world. Despite initial reservations of a significant sector of the trypanosome research community, this project was fully funded by National Institutes of Health (NIH) and the Wellcome Trust in 1999 as a joint effort by the Sanger Institute and The Institute for Genomic Research (TIGR). The choice of parasite stock TREU927/4 GUTat 10.1 (GPAL.KE/70/EATRO 1534) (van Deursen *et al.*, 2001) for this project has been somewhat controversial as several other stocks (notably 427) have been worked on more extensively in many laboratories. Nevertheless, the chosen stock, isolated from a tsetse fly in Kenya in 1962, has a complete history, is biologically intact and has undergone relatively few passages since its isolation. It is fly transmissible, has high switching rates during antigenic variation and has been used in genetic crosses in the laboratory to produce hybrid progeny (Tait *et al.*, 2002).

The aim of the genome project is to describe all the genes in one cloned stock of *T. brucei* by determining their structures and DNA sequence, and their organization along the chromosomes. Additionally, genomic analysis provides details of chromosome structure, the nature of the telomeres (the chromosome ends), the features of the sequences between genes (intergenic regions) and the nature of a range of DNA elements within the genome. This information underpins a wide range of studies, from understanding basic biological phenomena to more applied goals such as new diagnostics, epidemiological tools and novel drug targets. Essentially, genomics is an enabling technology that will facilitate much of the future research on trypanosomes.

The target date for completion of the sequence of the nuclear genome of *T. brucei* is 2004. This chapter describes the current knowledge of chromosome structure and content, based on the sequence of chromosomes I and II. In the near future there will be access to a wealth of information about the genome and its gene composition. Then the research community faces the significant challenge of moving from genomics into functional analysis, especially of genes with no homology to genes of known function in other organisms. This latter group is likely to include the majority of the kinetoplastid- and trypanosome-specific genes and, indeed, some of the most interesting genes. Their study should lead researchers to new insights into the biology of this evolutionary ancient pathogen in the coming decades as well as exploiting the information for practical applications.

The Karyotype

Identification of the nuclear chromosomes by classical cytogenetic approaches has proved impossible because the chromosomes of *T. brucei* do not appear to condense and so cannot be readily visualized. However, pulsed field gel electrophoresis (PFGE), a novel method of electrophoresis that allowed the separation of very large DNA molecules on agarose gels, was developed in the early 1990s. Combined with the isolation of intact chromosomes, maintained in an unbroken state by embedding the organisms in agarose prior to lysing the cells, this technique has been used to determine the numbers and sizes of chromosomes in numerous organisms, to generate molecular karyotypes. Based on these separations, three classes of chromosome have been defined in trypanosomes: the large megabase chromosomes (≥ 1 Mb), the intermediate chromosomes (> 100 kb) and the abundant mini-chromosomes (30–100 kb) (Fig. 3.1a). The latter two classes are considered to be aneuploid.

Mini- and intermediate chromosomes

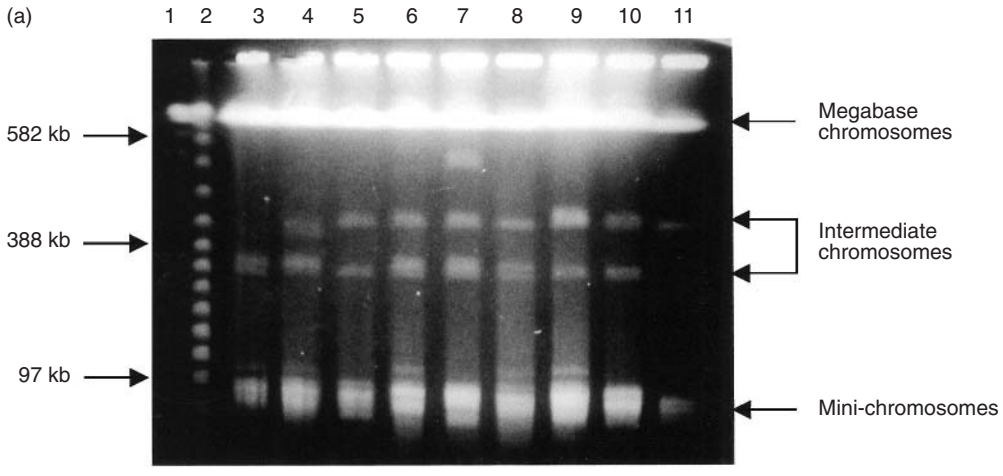
It is estimated that there are approximately 100 linear mini-chromosomes ranging in size from 50 to 100 kb, accounting for some 20% of the nuclear DNA content. Based on the sequence analysis of a subset of these chromosomes, they are primarily composed of a 177 bp repeat sequence (90% of the mini-chromosome), telomere repeats and one or two variant surface glycoprotein (*VSG*) genes (Weiden *et al.*, 1991). They do not appear to have any other coding regions or recognizable promoters and it has been suggested that they act as a reservoir of unexpressed *VSG*s that may be transposed into expression sites in the larger chromosomes (see later). The intermediate chromosomes can be differentiated from the mini-chromosomes by their size (100–700 kb) and the absence of the 177 bp repeat. Depending on the stock, there are between one and five of these chromosomes, representing only 0.2–1% of the genome. Based on hybridization with cDNAs or markers for the *VSG* expression site (ES) (see later) and sequencing (Berriman *et al.*, 2002), these chromosomes have been shown to contain ESs, telomeric sequences and members of gene families that are common to a number of other chromosomes (see later). In the absence of full sequence information it is unclear whether they are specialized for surface antigen gene expression or contain other coding sequences also.

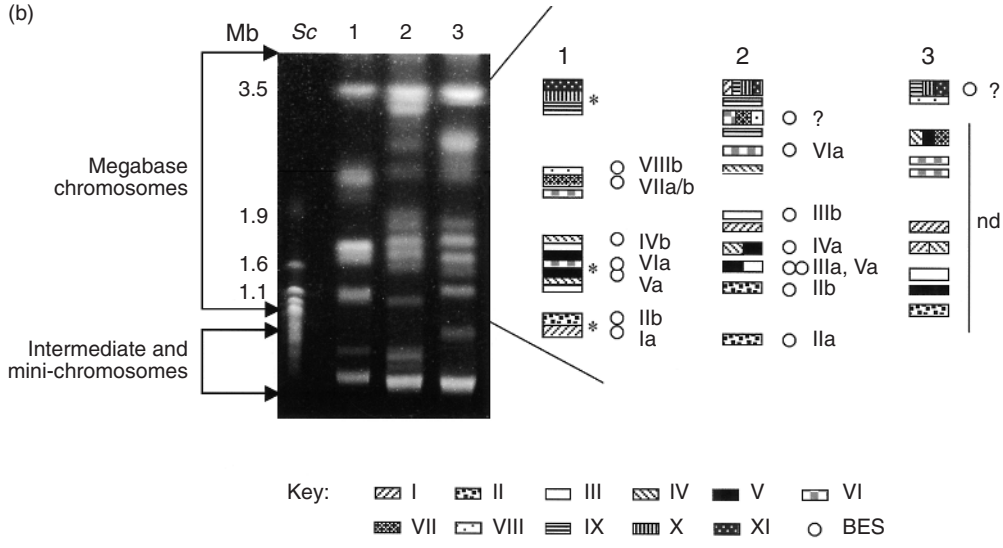
Housekeeping chromosomes

Approximately 80% of the nuclear genome is comprised of the megabase chromosomes, also called the housekeeping chromosomes because they carry most, perhaps all, of the genes involved with the basic functions of the trypanosome (housekeeping genes). These chromosomes have been characterized by PFGE separation followed by Southern blotting and hybridization of a large number of different cDNA probes to multiple stocks, including 427 and TREU927 (see section on expressed sequence tags, below). Based on this analysis, 11 pairs of diploid chromosomes have been identified that range in size from 1 to over 6 Mb (Melville *et al.*, 1998, 2000). The chromosomes have been assigned numbers I–XI (Fig. 3.1b) and cDNAs identified that define each of them. The gene content of each chromosome seems to be conserved between stocks, though there is one exception to this reported in the literature for chromosome I. A small proportion of these cDNAs hybridize to several or most chromosome bands and these presumably represent multi-copy genes or members of closely related gene families. In addition to the characterization using housekeeping cDNAs, analysis with probes for the bloodstream *VSG* expression sites (BES) revealed that some chromosomes probably have no BES and that a BES may be found on one homologue but not on the other, while some chromosome pairs have BES on both homologues. Moreover, distribution of these ESs does not seem to be conserved between stocks (Fig. 3.1b), suggesting that occasional recombination may occur between homologous sequences on non-homologous chromosomes that results in the transfer of ES between chromosomes.

Ploidy

Most of the data from the analysis of the molecular karyotype is consistent with a diploid nuclear genome. Exceptions to this occur when considering the *VSG* genes, their expression sites and the mini- and intermediate chromosomes. The genome contains numerous *VSG*s (an estimated 1000 genes in stock 427, though this was an extrapolation from a limited survey (van der Ploeg *et al.*, 1982)). These unexpressed basic copies are located in mini-chromosomes and embedded in the main diploid part of the megabase chromosomes, with a small number in inactive expression sites at the end of intermediate and megabase chromosomes. Southern blot analysis with single *VSG* probes defines them as haploid in all these contexts (Melville *et al.*, 2000). This is thought to be due to the haploid nature of the teleomeric region containing ES on the larger chromosomes, over a


(a)
1 2 3 4 5 6 7 8 9 10 11
582 kb
388 kb
97 kb
Megabase chromosomes
Intermediate chromosomes
Mini-chromosomes


(b)
Mb
Sc
1
2
3
3.5
1.9
1.6
1.1
Megabase chromosomes
Intermediate and mini-chromosomes
VIIIb
VIIa/b
IVb
VIa
Va
IIb
Ia
?
VIa
IIIb
IVa
IIIa, Va
IIb
IIa
?
nd
Key:
I II III IV V VI
VII VIII IX X XI BES

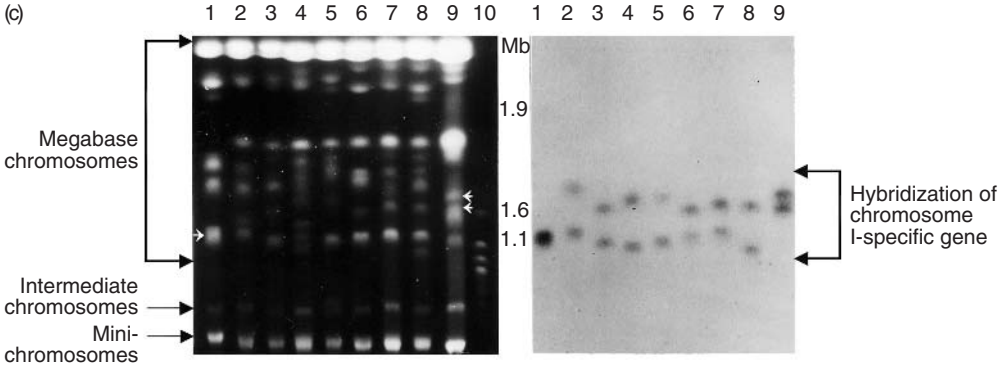

(c)
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9
Mb
1.9
1.6
1.1
Megabase chromosomes
Intermediate chromosomes
Mini-chromosomes
Hybridization of chromosome I-specific gene

Fig. 3.1. (*opposite*) **(a)** The three classes of trypanosome chromosome may be separated according to size by pulsed field gel electrophoresis (PFGE). Lane 1, *Saccharomyces cerevisiae* chromosomes (most of which are too large to migrate under these conditions); Lane 2, Lambda DNA ladder (multiples of 48.5 kb, the smallest band is too faint to detect); Lane 3, *T. brucei* stock TREU927/4; Lanes 4–10, genetic hybrids from a cross between TREU927/4 and STIB247L (Tait *et al.*, 2002); Lane 11, *T. brucei stock* STIB247L. **(b)** Molecular karyotypes of the megabase chromosomes of *T. brucei* stocks are very variable, although each stock contains 11 homologous diploid pairs. **Both panels:** Lane 1, *T. brucei* stock TREU927/4 (East Africa); Lane 2, *T. brucei* stock 427 (commonly used laboratory strain); Lane 3, *T. brucei* stock STIB386AA (West Africa). **Left panel:** The position and size of four *S. cerevisiae* chromosomes (*Sc*) are indicated (Mb = megabases). All chromosomes over 3.5 Mb in size are compressed at the top of the gel. This gel was prepared by Ms Vanessa Leech. **Right panel:** Diagrammatic representation of the megabase chromosomes separated in Panel (a), showing the location of each of the diploid homologues. The diagram is not exactly the same scale as the gel, due to the detail shown. Where a single band of one pattern is observed in a stock, the diploid homologous chromosomes are the same size; where two are observed, the homologues are of different size. Asterisk (*) indicates that the relative positions of chromosomes compressed into one band on the gel in Panel (a) have been determined in other gels. The chromosomes that carry sequences associated with bloodstream-form *VSG* expression sites (BES) are indicated by open circles and the chromosome number: a, smaller homologue; b, larger homologue; nd, location of BES on these chromosome has not been determined; ?, one or more BES are found in the compression of chromosomes (Melville *et al.*, 1998, 2000). **(c)** Size changes are observed in homologous chromosomes after genetic exchange. **Left panel:** Lane 1, *T. brucei* stock TREU927/4; Lanes 2–8, genetic hybrids from a cross between TREU927/4 and STIB247L (Tait *et al.*, 2002); Lane 9, *T. brucei stock* STIB247L; Lane 10, *S. cerevisiae* chromosomes. The positions and size of three *S. cerevisiae* chromosomes are indicated. The PFGE parameters used have separated chromosomes of 1–2 Mb only and several chromosome bands that were comigrating in (b) have separated in this gel. **Right panel:** autoradiograph resulting from hybridization of a radioactive chromosome I-specific DNA probe (a cloned tubulin gene), revealing the chromosomal size variation commonly observed between parent and hybrid trypanosome stocks.

region spanning up to 100 kb, as well as of the mini-chromosomes. In the case of the basic copy genes within the megabase chromosomes, this is likely to be due to the high level of sequence divergence between individual alleles so that the genes appear haploid, although the relative location and organization of the *VSG* arrays in homologous pairs of chromosomes is yet to be determined.

The ploidy of the intermediate and mini-chromosomes remains uncertain, as it has not been possible to identify chromosome-specific markers that would allow the identification of specific pairs. The available evidence suggests that both classes of small chromosome segregate faithfully at mitosis even though they appear to be essentially aneuploid (Ersfeld and Gull, 1997). Their mode of segregation during genetic exchange is less clear.

Chromosome size polymorphism

A characteristic feature of the molecular karyotype is the high level of size polymorphism between homologous chromosomes (Melville *et al.*, 1998, 2000) (Fig. 3.1). This may be observed within a single parasite; for example, the estimated sizes of the chromosome I homologues in stock 427 differ by approximately 2 Mb. Size polymorphism is also observed between different stocks of the parasite and this possibly accounts for the differences in estimated DNA content that has been observed between stocks. The smallest chromosome I homologue (0.9 Mb) is observed in a laboratory hybrid while the largest is 3.6 Mb (in stock 427, amongst others). In contrast, chromosome II has never been observed to vary by more than about 150 kb (15%) between stocks. It appears that some chromosomes are subject to greater size variation than others.

The chromosomal regions involved in these size differences have been determined for chromosome I by long-range restriction digestion (Melville *et al.*, 1999). A significant proportion of the size difference can be accounted for by variation in the multi-copy sequences adjacent to one end of the chro-

mosome (see later). However, there are also differences in the sizes of fragments in the internal regions of the chromosome. This is not due to restriction site polymorphism and the basis for this size variation remains largely unexplored. As gene content appears to be conserved, it seems likely that the differences in size are due to amplification of specific sequences or of chromosomal segments. This would be a most interesting observation if it could be shown that such amplification affected the survival of the parasite, i.e. was adaptive, by altering levels of gene expression for example. To date, it remains merely an intriguing observation.

Another remarkable feature of the karyotype, given the large size differences between the homologues, is the faithful segregation of chromosomes at mitosis, when pairing between very disparate homologues must occur. Further evidence that the two putative homologues of each chromosome identified by Southern blotting are genuine homologues in a diploid genome, despite the considerable size variation, has come from analysis of the inheritance of karyotype in crosses between different stocks of *T. brucei* (Hope *et al.*, 1999). It has been shown that each F_1 progeny clone inherits one homologue of chromosome I from each parent, indicating that the bands observed on the agarose gels are indeed homologues. Analysis of the progeny also shows that the majority of homologues undergo size changes during genetic exchange (Fig. 3.1c), while analysis of the karyotype after multiple rounds of vegetative division suggests that the sizes of the chromosomes are mostly stable at mitosis (notwithstanding small-scale size changes as a result of *VSG* gene transposition). It seems, then, that meiosis is a key mediator of chromosomal size polymorphism. Size changes presumably arise from crossing over between homologues and, where arrays of perfectly conserved tandem repeats occur (see later), may also involve gene conversion. Recently, crossing over has been formally demonstrated and the small size of the recombination unit (15–30 kb/centimorgan) suggests that at least one crossover will occur per chromosome at meiosis (Tait *et al.*, 2002).

Sequencing of the Nuclear Genome

Searching for genes that are responsible for different aspects of an organism's biology or for different phenotypes is a laborious task using conventional approaches. Even in a relatively simple organism such as the trypanosome, such efforts can take many years – sometimes without success. It is increasingly recognized that the high-throughput determination of the sequence of all genes in a genome, and the provision of such data in databases with powerful search and analysis tools, allows researchers to progress much more rapidly to functional analysis. Such global information may also lead to novel insights on which to base further hypotheses. The gold standard is the determination of the entire sequence of each chromosome from end to end. However, many researchers are primarily interested in the coding sequence of the genome, i.e. the gene content, and it is possible and useful to provide such data without full knowledge of their genomic locations or of genome organization. A phase of gene discovery took place at an early stage in the *T. brucei* genome project, long before the complete sequence of any chromosome was available, and proved to be a turning point in speeding up the identification of specific genes.

Gene discovery from expressed sequence tags

Complementary DNA clones, or cDNAs, are double-stranded DNA copies of the single-stranded messenger RNA (mRNA) molecules transcribed from the genome. The sequencing of randomly selected cDNAs has proved to be an efficient approach to novel gene discovery in many organisms. These sequences are designated expressed sequence tags (ESTs). In EST analysis, it is necessary to sequence only the 5′ end of each cDNA inserted directionally into a cloning vector, to provide a tag, since this is the segment nearest to the start of the protein-coding region.

From the size of its nuclear genome, *T. brucei* is expected to produce approximately 8000 unique nuclear transcripts. Even the

relatively small EST discovery project summarized here, carried out for the most part at the International livestock Research Institute (ILRI) in Kenya by a PhD student (Djikeng *et al.*, 1998), yielded tags for over 3000 of them. As described above, the *T. brucei* ESTs were used as specific tags for each of the 11 large chromosomes, and in chromosome mapping (Melville *et al.*, 1998, 1999). Since ESTs provide evidence for transcription of a chromosomal locus, they may also aid annotation of the genome sequence.

Abundance of different transcripts amongst the ESTs

There are over 5000 ESTs from *T. brucei* deposited in GenBank, each with a unique accession number (El Sayed *et al.*, 1995; Djikeng *et al.*, 1998; E. Ullu and C. Clayton, unpublished). An iterative clustering process yields 2686 singletons, i.e. genes that have been tagged only once. The remaining 2467 fall into 427 clusters containing from two to 61 ESTs of identical or similar sequence. The sum of the number of singletons and the number of unique clusters reveals that 3113 unique sequences have been identified.

The number of times a gene is tagged by this random process is correlated to its level of expression, i.e. its relative representation in the mRNA from which the cDNA is prepared. There is always over-representation of abundant transcripts among random ESTs derived from an unbiased cDNA library constructed without normalization. The rate of gene discovery can be improved by eliminating cDNA clones already sequenced, thus enriching for new ones. This is achieved by hybridizing with radio-labelled pools of inserts from sequenced ESTs, by subtractive enrichment or by normalization of the cDNA library from which the ESTs are to be generated. The suggested criteria for exclusion of an EST are that: (i) the transcript appears among the ESTs at a frequency of > 10%; (ii) the EST belongs to a functional group, the proportion of which is > 10%; and/or (iii) a full-length or near full-length cDNA clone has been identified that represents that particular group of transcripts.

Approximately 52% of the *T. brucei* ESTs have no significant similarity to anything in the sequence databases; the remaining are distributed among seven functional categories as summarized in Fig. 3.2. At the time of writing, of all the trypanosome ESTs

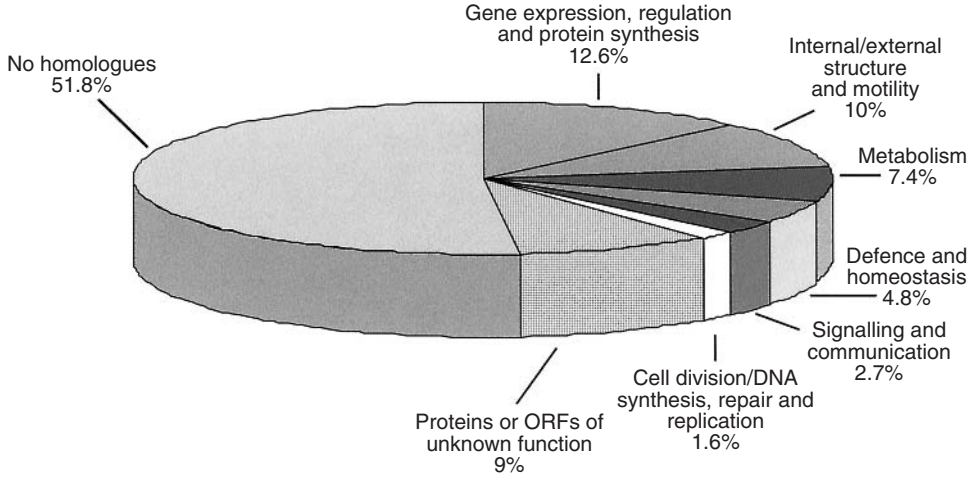


Fig. 3.2. Proportion of ESTs assigned to each functional category, based upon the most significant sequence similarity detected in the public domain databases using BLAST searches. As more genomes are sequenced, the number of coding sequences in the *T. brucei* genome sequence for which no homologues have been identified is declining.

available in the public domain databases, approximately 48% are clearly homologous to known proteins whose functions may or may not have been determined in other organisms, including humans, rodents, yeasts and plants. Based upon protein homology and/or structural motifs, some of the *T. brucei* ESTs encode homologues of proteins involved in intra- or extracellular signal reception and transduction, cell division, gene regulation, DNA repair and replication, general metabolism, and internal or surface structures. So far, the proportion of *T. brucei* ESTs homologous to genes already described in trypanosomatids is approximately 20%. ESTs of genes encoding tubulin, cysteine protease precursor, histone H2B and 40S ribosomal protein S17 are the most abundant. Fewer than 10% of the ESTs are derived from *VSG*s, which is surprising given that the VSG protein comprises 10% of trypanosome cellular protein.

ESTs from developmentally transcribed genes

Early during infection of a mammal, a majority of trypanosomes in the blood are long-slender dividing forms. Later short-stumpy forms appear and predominate at the peak of parasitaemia. Once the parasites are taken up by the tsetse fly in a blood meal, they differentiate yet again. The adaptive capacity of the trypanosome is presumably effected through variation in the types and amounts of proteins produced in the cell. This may be controlled at the level of mRNA production or by post-transcriptional regulation processes, such as mRNA degradation or rate of translation. ESTs provide a catalogue of gene transcripts produced at a particular developmental stage. Most of the ESTs in the databases were derived from mRNA representative of one peak of parasitaemia, harvested at the logarithmic phase of growth. A smaller number of ESTs derive from procyclic trypanosome mRNA.

The transcriptional status of the *T. brucei* ESTs, identified as either putative developmentally regulated or as a homologue of genes known to be developmentally transcribed in other organisms, is normally confirmed by experimentation. Inserts from each EST can be manually slot-blotted on to membranes or (in larger numbers) robotically arrayed on glass slides to produce microarrays (see penultimate section), then hybridized with radio-labelled total cDNA prepared from different developmental forms of the parasite. Typically, some of the ESTs will hybridize more efficiently with mRNA from one and not the other developmental stage, indicating that the corresponding transcripts may be more abundant, or present only, in the trypanosomes at these specific life-cycle stages. Where necessary, if RNA is limiting, amplification using reverse transcriptase (RT-PCR) can be used to investigate the abundance of a transcript at any developmental stage. Identification of developmentally regulated ESTs will make it possible to add additional biological information to the annotation of the *T. brucei* chromosome sequences as these become available.

Gene discovery from genome survey sequences

An alternative method of gene discovery is the random sequencing of short fragments of genomic DNA to create genome survey sequences (GSSs). This is very inefficient in many animal and plant genomes, which contain larger amounts of intergenic and repetitive DNA. However, the trypanosome genome is small and compact with short intergenic sequences, and this approach is feasible (El Sayed and Donelson, 1997). The advantage of ESTs is that they provide evidence of transcription of a DNA sequence, but their major disadvantage is the under-representation of some transcripts. This problem is avoided by sequencing genomic DNA, which essentially represents a *normalized* library, but gene discovery is based on automated sequence analysis and provides no corroborative evidence of transcription. GSSs, like ESTs, are short sequence tags produced by the random selection of clones from a clone library. In this case, each end of the cloned genomic DNA fragment is sequenced using primers for the plasmid vector.

At an early stage in the *T. brucei* genome project, it was decided that a gene discovery

project based on GSSs had great potential to promote rapid research progress in the short term, prior to providing the complete genome sequence. In response to requests from the community, TIGR produced two plasmid libraries containing inserts of sheared genomic DNA (inserts ranging from 1–2 kb and 2–4 kb, respectively). The two participating sequencing centres produced approximately 97,000 end-sequences from these genomic fragments. Based on theoretical calculations, this level of coverage provides a greater than 90% probability that every gene in the genome will be tagged.

As part of the same programme, TIGR also produced sequence tags from over 11,000 ends of very large genomic DNA inserts in P1 and BAC libraries (bacteriophage P1 vector p*Sac*BII, average insert size of 65 kb; bacterial artificial chromosome vector pBACe3.6, average insert size of 145 kb). These serve not only as part of the gene discovery programme, but also to aid mapping and sequencing of the entire genome (see later).

An important aspect of sequence tags of any sort is that they are produced randomly and rapidly; therefore sequence accuracy is lower than that of the finished sequence described later. These approaches also suffer from the disadvantage that the complete sequence of the *T. brucei* gene may not be present, such that additional work is required to obtain the full sequence of the gene and its genomic location. However, they provide a rapid means of identifying genes with homology to those already described in other organisms and these sequences can be generated relatively rapidly and cheaply.

Genome Organization and Gene Content

Following the development of pulsed field gel electrophoresis, much of the initial work on genome and chromosome organization in the 1980s arose out of an overriding interest in the recombination mechanisms involved in antigenic variation. As described above, all size classes of chromosomes were found to carry *VSG* genes, but the extra copy that appeared during duplicative transposition was always present on a larger chromosome (intermediate or megabase size) and it was proposed that the mini-chromosomes represent a reservoir of unexpressed *VSG*s (see Chapter 2). This remains the current thinking, although only very few mini-chromosomes have been partially mapped and sequenced (Weiden *et al.*, 1991) and it remains possible that some chromosomes in this size range are of different structure and sequence content.

VSG expression sites

The fragmentary knowledge gained on the nature of the 'expression sites' (ES) during this time – that there are multiple ESs in the genome, always on larger chromosomes, all transcriptionally quiescent except for one 'active site' – coalesced into a coherent picture with the publication of the first DNA sequence of an ES active in the bloodstream form of the parasite (BES) (Pays *et al.*, 1989). This revealed that the promoter lay upstream of a tandem series of other genes, termed expression site-associated genes (*ESAG*s). Some were known and some were novel, and all appear to be surface-associated proteins. BESs are separated from the remainder of the chromosome by a tandem array of imperfect 50 bp repeats. Several potential BESs have now been sequenced, including the active site from the genome project reference stock (LaCount *et al.*, 2001). The general structure is common to all, though some regions may be duplicated.

However, the metacyclic trypomastigotes found in the salivary glands of the tsetse vector and pre-adapted to infection of the mammal are also coated by the VSG. The metacyclic *VSG* expression sites are distinct from BES, also subtelomeric but lacking the 50 bp repeats and the BES promoter and with fragmentary *ESAG*s (Bringaud *et al.*, 2001). Thus the telomeric ends of most, perhaps all, *T. brucei* chromosomes are dedicated to the system of antigenic variation (for a more detailed description, see Chapter 2).

Megabase chromosome structure

In order to determine the structure of an entire megabase chromosome, it was necessary to fragment the chromosome into manageable pieces. This was achieved in two ways: (i) by creating clone libraries containing large fragments of genomic DNA in cosmid, bacteriophage P1 and bacterial artificial chromosome (BAC) vectors; and (ii) by long-range restriction digestion of genomic DNA or of chromosomal DNA excised from a pulsed field gel (Melville *et al.*, 1999). While recognition sites for some of the commonly used 'rare-cutting' enzymes occur too frequently in *T. brucei* DNA due to the relatively high GC content (e.g. *Not*I), the enzyme *Pme*I produces fragments ranging from 10 kb to over 300 kb. This produces a sufficiently small number of large fragments to enable entire chromosomes to be mapped.

As described above, the EST project provided chromosome-specific markers for chromosome mapping. Chromosome I was the first target of whole chromosome mapping because it is the smallest chromosome. It was also of interest as one of the most size-polymorphic chromosomes yet described. The chromosome I-specific cDNAs were hybridized to the genomic libraries and to *Pme*I-digested DNA. Markers specific to *VSG* BES were also hybridized to digested chromosome I DNA to define the size of any expression sites. It became clear that this method could rapidly produce a map of gene-rich sections of the chromosome which, even with a generous estimate of BES size, could not account for the full length of the chromosome. Hybridization of known repetitive elements, the putative retrotransposons RIME (ribosomal inserted mobile element) (Hasan *et al.*, 1984) and *ingi* (*many* in Swahili) (Kimmel *et al.*, 1987), revealed that these were clustered in genomic clones with sparse EST assignments, and also in the unmapped chromosome fragments (see Fig. 3.3). Furthermore, some ESTs previously observed to hybridize to multiple chromosomes hybridized to these same clones and chromosome fragments.

The structure and organization of these segments of chromosome I was solved when the genomic location of a novel family of genes was analysed (Bringaud *et al.*, 2002a). Many members of this family (both genomic and cDNA clones) had RIME and *ingi* elements inserted into the gene sequence at exactly the same position. This was clearly the missing link in the structure of megabase chromosomes. The so-called *RHS* (*r*etrotransposon *h*otspot) genes have two conserved regions and are subdivided into six subfamilies based on level of sequence identity in the less conserved parts of the genes. Variable numbers of *RHS* genes and pseudogenes are present on many megabase and intermediate chromosomes, and just a few in the mini-chromosome size range (50–100 kb). It is possible, therefore, that a subset of the mini-chromosomes is of different structure to that described above. They may derive from the ends of larger chromosomes, as observed in mini-chromosomes in *Leishmania*. No genes with significant similarity to *RHS* have been detected in *Leishmania major*, which also appears to lack large transposon families, but homologous sequences are found amongst the *T. cruzi* GSSs and observed to be associated with putative transposon sequences (Bringaud *et al.*, 2002b). Unlike *T. brucei*, *L. major* chromosomes seem to have long tracts of simple sequence repeats in subtelomeric regions while the *T. cruzi* genome project may soon reveal subtelomeric regions with similarity to those in *T. brucei* chromosomes.

The complete sequences of the megabase chromosomes I and II

At a meeting at the NIH, Bethesda, USA, in 1999 it was agreed that half of the megabase chromosomal DNA would be sequenced at each of two centres: chromosomes II to VIII at TIGR in the USA and chromosomes I, IX, X and XI at the Sanger Institute in the UK. While the aim at each centre is to provide complete chromosome sequences, the methods employed are different. At TIGR, a large number of BAC clones were end-sequenced as part of the GSS project (see above). From

these, chromosome-specific BACs are selected using cDNA markers specific to the target chromosome (see karyotype section, above), subcloned into plasmids and sequenced to completion. Then the database of end-sequences is searched to retrieve BAC clones that overlap by only 5–10 kb and these are sequenced to completion. The BACs may derive from either of the diploid homologues, and this is called 'chromosome walking'. At the Sanger Institute, individual chromosome homologues are separated by PFGE and excised from the gel. This chromosomal DNA is sheared and cloned into a plasmid vector, and the clones are randomly end-sequenced to a (theoretical) 8–10 × coverage of the chromosome. This is called 'whole chromosome shotgun' (wcs) and is supplemented by low-level shotgun sequencing of mapped BAC clones. Both methods have their advantages and disadvantages: it is difficult to obtain sequence of the chromosome ends by 'walking', while large repetitive regions are difficult to complete by wcs in regions that lack anchored genomic clones. The main difference for the research community is that 'walking' provides sections of chromosome that are finished and annotated one by one, while wcs provides almost all the chromosome sequence in the first month of the project but the presentation of the chromosome annotation is delayed until the end of the project.

The completion of the sequence of chromosomes I and II provides a gene catalogue for these chromosomes (El-Sayed *et al.*, 2003; Hall *et al.*, 2003). However, although all coding sequences are necessarily present in these data, sequence analysis may fail to identify some novel genes. In addition, some annotated putative coding sequences may in fact not be expressed. Bioinformatic identification of sequence features is very powerful but, in the end, experimental verification is required for all annotated features.

The chromosome sequences also provide the definitive picture of megabase chromosome structure. Both chromosomes have a gene-rich core, and a region containing *RHS* and other gene families and transposons; they both have *VSG*s adjacent to telomeres, and may carry a BES (in TREU927, chromosomes Ia and IIb have BES, while Ib and IIa do not) with upstream 50 bp repeats. Most genes are contained within directional gene clusters, i.e. there are long arrays of genes located on the same coding strand, each array separated by a strand-switch region. This arrangement likely reflects the previously described polycistronic transcription of trypanosome genes (Tschudi and Ullu, 1988), whereby long mRNA transcripts of multiple genes are processed to individual mRNAs by *cis*-splicing of a 5′ spliced leader and addition of a poly-A tail (Matthews *et al.*, 1994).

Figure 3.3 summarizes in diagrammatic form the current knowledge on megabase chromosome structure derived from all the sources cited here.

DNA sequences that contribute to the size polymorphism of chromosome I

The astonishing level of size polymorphism observed in *T. brucei* chromosomes was described in the karyotype section (above), where it was shown that, with a chromosome map in hand, it was possible to determine which sections of the chromosome contribute to the size differences in chromosome I homologues. Surprisingly, the presence of a BES is not correlated with increased size (see Fig. 3.1b). The presence of divergent members of gene families with multiple retrotransposons may promote recombination and hence high levels of diversity between strains, and indeed the *RHS* region is very variable in size, as discussed earlier. However, the completed sequences of chromosomes I and II reveal that these are found on both chromosomes, yet chromosome II shows little variation between strains. Chromosome I also contains several tandem repeats, including the α/β tubulin genes, histone *H3* genes, and several others of unknown function – all of which vary in number between strains. Yet the sum of all these does not account for the variation observed and the picture is not yet complete. Once the TREU927 genome

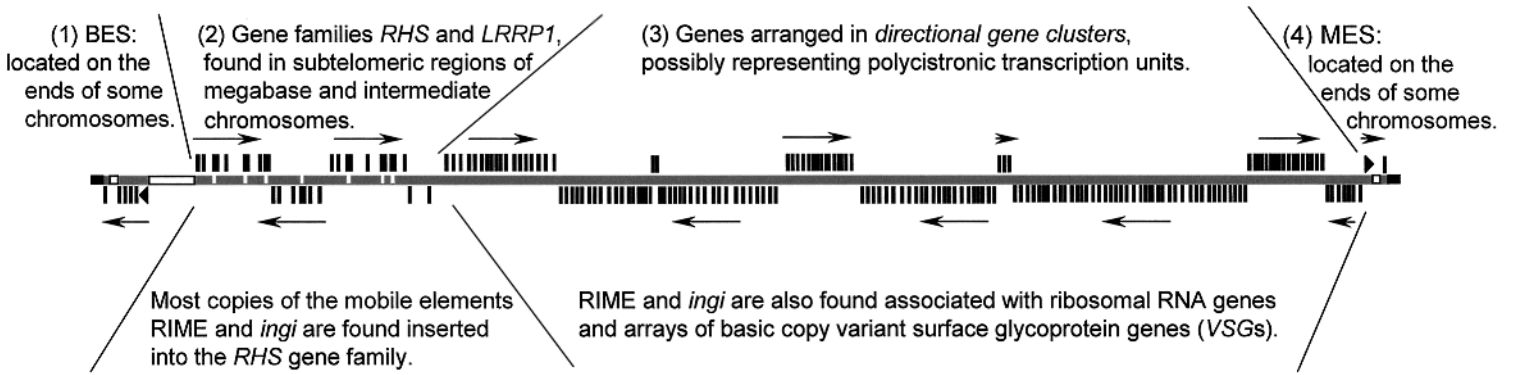


Fig. 3.3. Salient features of megabase chromosome structure in *T. brucei*, based on the data of Melville *et al.* (1999), Bringaud *et al.* (2002), Berriman *et al.* (2002), the sequences of chromosome I and II (El-Sayed *et al.*, 2003; Hall *et al.*, 2003) and references cited in Chapter 2. BES, bloodstream-form expression site; MES, metacyclic expression site. Clusters of genes are represented as black boxes above and below the line and the direction of transcription (5′ to 3′) of each cluster is shown by an arrow. The number of black boxes is not representative of the number of genes at this scale. The location of *VSG* promoters are shown (▶), but RNA polymerase II promoter sequences have not been identified between the gene clusters. White filled boxes on the central line indicate known repeated DNA elements (50 bp and 70–76 bp repeats, see text) and the mobile elements RIME and *ingi* inserted into *RHS* genes (see text). Black boxes represent the tandemly repeated telomeric sequences. Elements within the diagram are not to scale.

sequence is available, it will be important to study variation in the genomes of a range of strains, not least to search for the basis of phenotypic variation in important traits such as clinical pathology or transmission.

Finishing the *T. brucei* genome sequence

Funding has been provided to finish the sequence of the megabase chromosomes of *T. brucei* stock TREU927. Finishing in this context means, in an ideal world, assembling a fully contiguous sequence and annotating to the highest accuracy possible with current tools. Finishing is a long and complex process, in contrast to the provision of an unassembled shotgun sequence which occurs very rapidly near the start of the project. Inevitably, most 'finished' genomes still have gaps in their sequences: some regions of repeated sequence may not be fully resolved if it is decided that the biological importance of such sequences is not in due proportion to the effort involved. Also, the 'finished' sequence may not provide all the variations present in a diploid genome. It takes time, skill and persistence to produce an accurate and essentially finished genome and, in the future, few may reach this state. A genome also requires long-term curation, updating of annotation and, in places, correction, if it is to serve as an accurate resource for researchers.

Future sequencing

It is likely that further related genomes will be sequenced in the future, although not to completion. Once a finished genome is available as a backbone, the shotgun sequence of the genomic DNA (essentially a GSS survey to a minimum 5 × haploid coverage of the genome) of related organisms may be pinned to this backbone for comparative analysis. For example, sequence data from the related livestock-infective pathogenic African trypanosomes and the genetically homogeneous human-infective subspecies *T. b. gambiense* will provide considerable added value to the *T. brucei* sequence, allowing researchers to search for significant genomic variation underpinning differences in life cycle, biology and pathogenesis. Given the potential of genome sequence to facilitate rapid molecular analysis, such data are likely to be in increasing demand for a range of pathogenic organisms in the years to come.

The Kinetoplast Genome

The mitochondrial genome of the kinetoplastids occurs as a unique concatenated structure called the kinetoplast and contains two classes of circular genome: the maxicircles and the minicircles, which exist as a highly concatenated structure (Fig. 3.4). There are approximately 50 maxicircles per cell and, in *T. brucei*, this genome is 25–27 kb in size. The available evidence suggests that the 50 copies are identical although there is polymorphism between different parasite stocks. There are in the region of 5000–10,000 minicircles per cell of approximately 1 kb. These are highly heterogeneous with up to 200 different sequence classes per cell (Shapiro and Englund, 1995). The complete sequence of the maxicircle has been determined as well as the sequences of several minicircles. These data provide insight into the nature of the encoded genes and their organization. Structurally, within the cell, the kinetoplast occurs as a flattened disc of intercalated DNA circles.

The maxicircle encodes the 9S and 12S ribosomal RNA genes required for mitochondrial ribosomes and protein synthesis. There are a further 17 open reading frames (ORFs) of which 11 encode genes of known function (subunits of NADH dehydrogenase, cytochrome oxidase complexes I, II and III, ATPase 6 and cytochrome *b*). This is a relatively conventional mitochondrial gene complement, though the absence of any genes for mitochondrial transfer RNAs is exceptional. The genes are closely clustered with little or no intervening sequence but there is an 8 kb region that contains a number of repetitive sequences and no ORFs. This is called the variable region, because its sequence and length vary between stocks (Hensgens *et al.*, 1984).

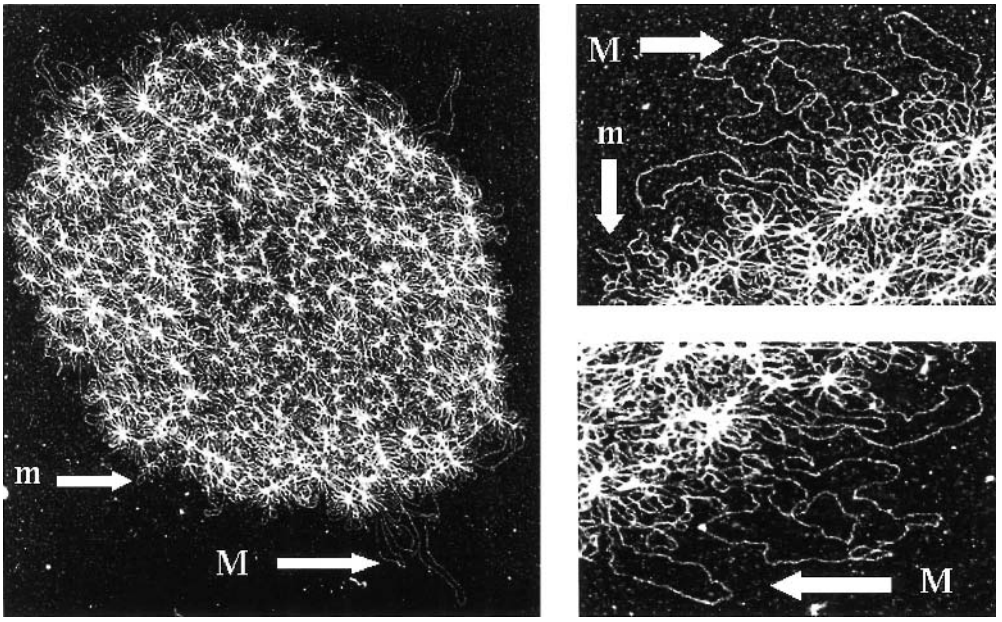


Fig. 3.4. Kinetoplast DNA, consisting of concatenated maxi- and minicircles (see text), viewed under the electron microscope. The left panel shows an entire genome from one kinetoplast; the two smaller panels show details of the circular DNA molecules at the edge of the concatenated mass. M, maxicircle; m, minicircle. Photographs reproduced by kind permission of Dr Douglas Barker.

The sequence of the CO II gene was found to have a frame shift suggesting that the gene was non-functional; however, sequence analysis of the transcript led to the discovery of mitochondrial editing (Benne, 1994). By sequence analysis of the transcripts of each gene, it was found that they differed from the genomic sequence largely in relation to the addition or deletion of uridine residues and it was only as a result of this editing that a full ORF was generated. A further feature was a remarkable variation in the level of editing between different genes; some gene transcripts are not edited (five genes) while others have up to 50% of their final sequence provided by editing. The process has been studied in detail and the components of an active editing complex fully defined (Stuart *et al.*, 1997).

The sequence analysis of a number of minicircles has shown that these do not encode any proteins, but encode guide RNAs (55–70 bp) that play a central role in editing. Each guide RNA sequence has a portion complementary to an edited RNA; also a 5–12 bp sequence complementary to the unedited RNA and immediately 3′ to the edited region that acts to direct the editing process. From the available data, it is clear that the full editing process involves multiple editing events involving many different guide RNAs in the generation of the mature transcript. A number of models of the process have been proposed but discussion of these falls outside the scope of this chapter. Analysis of the structure and organization of several *T. brucei* minicircles has shown that they each contain three to four guide RNAs flanked by 18 bp inverted repeats as well as a conserved origin of replication.

The concatenated disc-like structure of the kDNA raises a range of questions as to how it is replicated and distributed to daughter cells at mitosis. This has been studied in a number of kinetoplastids and a general model developed, with current research directed at characterizing the molecular machinery involved. In contrast to the replication of mitochondrial DNA in

many eukaryotes, kDNA replication occurs during a distinct S-phase and is coordinated with nuclear replication. Each individual mini- and maxicircle is replicated and then distributed to each daughter cell as part of the network. The current model involves release of minicircles from the network and association with a replication complex at either end of the plate-like network. After replication, the newly synthesized molecule is attached to the network and the network grows until it splits into two daughter complexes. This is a complex process and more detailed descriptions can be found elsewhere. The replication of the maxicircles, in contrast, occurs within the network without release to the periphery and this process remains to be fully elucidated (Shapiro and Englund, 1995).

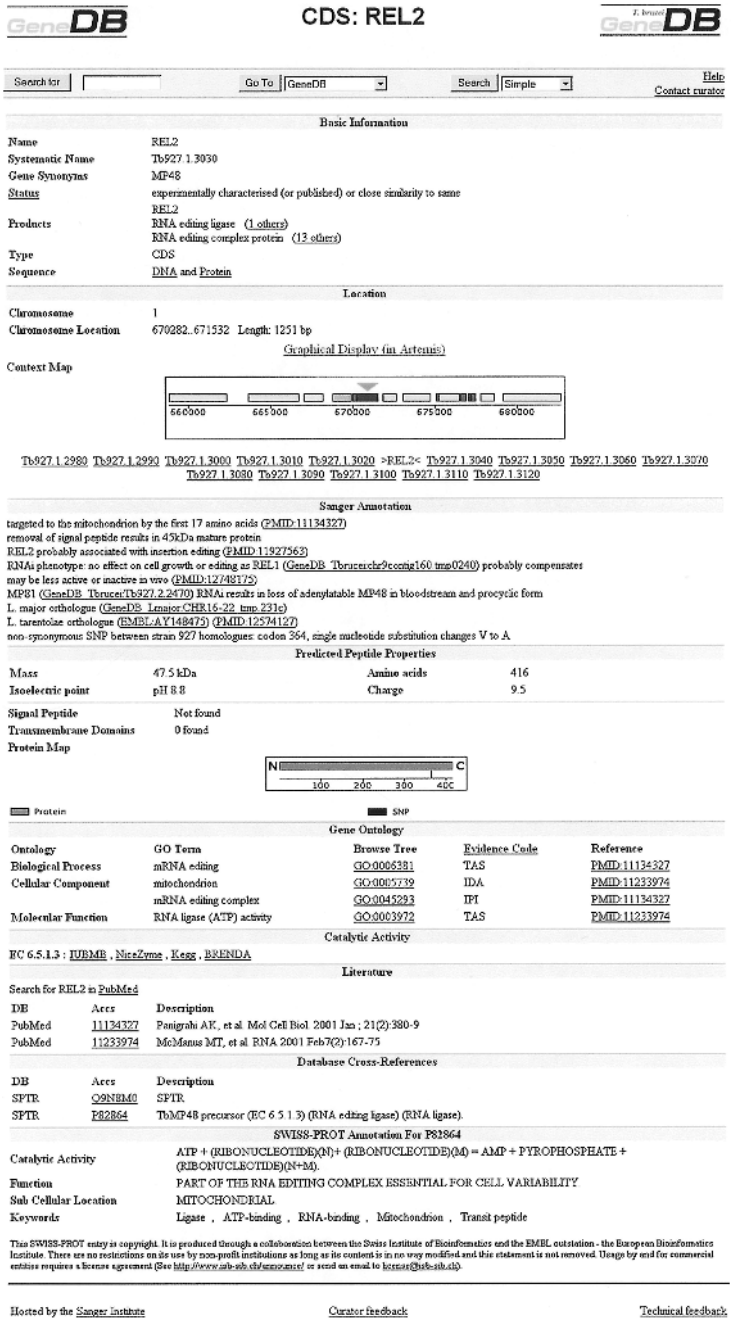
The Post-genomic Era

The complete sequence of the megabase chromosomes of one stock of *T. brucei* will provide a tremendous resource for future molecular analysis of the biology of African trypanosomes. However, it also poses considerable challenges. Bioinformatic analyses provide many clues as to the function of novel genes and pathways (Fig. 3.5) but, despite the deluge of new data, there is still the task of assigning or confirming the biological function of numerous coding sequences and interpreting complex genomic patterns.

It has been shown that considerable synteny is observed between related species, i.e. that large chromosomal segments contain homologous genes in the same order, notwithstanding occasional gene deletions or additions, large segmental inversions and chromosome breakage or fusion (Bringaud *et al.*, 1998; *T. brucei* Genome Project, unpublished). Therefore, further valuable insights should arise from comparative analysis of the sequences of related organisms, e.g. *T. brucei* with other kinetoplastid parasites such as *T. cruzi* and *L. major*. This will highlight the sequence features (genes, metabolic pathways etc.) that are common across genera, restricted to genera and, importantly, those that are specific to a single species. This type of data helps researchers to build testable hypotheses of biological function as well as to use this information for more applied goals, such as the identification of novel drug targets.

The genome sequence provides rapid access to gene sequences for functional analysis, and a range of molecular genetic tools are available for analysis of *T. brucei* genes. Transformation of *T. brucei* parasites is achievable and expression of transformed DNA within an inducible expression system is a very useful technique (Wirtz and Clayton, 1995; van Deursen *et al.*, 2001). Due to the high rate of homologous recombination, the removal of gene copies (gene knockout) is relatively efficient (Shen *et al.*, 2001). RNA interference, which circumvents the inherent difficulties of diploidy, is proving a powerful tool for ablation of gene expression in *T. brucei* (Ngo *et al.*, 1998). The challenge for the future will be to increase the rate of genetic analysis by medium- to high-throughput application of genetic techniques as appropriate.

The availability of the entire sequence also facilitates *global* analysis of gene expression, i.e. variation in the levels of *all* mRNAs or proteins in a parasite population at a given time. This is a major step forward in genetic analysis and an area of rapid technological innovation at present. Microarrays are created for analysis of mRNA levels by robotic spotting of DNA fragments on glass slides: the technological advances lie in the robotics, the biochemical materials used, and the hardware and software used for analysis, allowing researchers to analyse the entire genome in one experiment. Proteomic analysis (the identification of proteins and analysis of protein levels in the cell) will provide a wealth of peptide sequence information that can then be used to identify genes. These techniques are advancing rapidly but are outside the scope of this chapter. The relative applicability of these techniques to the global analysis of polycistronic transcription in the trypanosome genome remains largely unproven, but is likely to be tested in many laboratories in the coming decade.



GeneDB

CDS: REL2

T. brucei GeneDB

Search for | Go To: GeneDB | Search: Simple | Help | Contact curator

Basic Information

Name	REL2
Systematic Name	Tb927.1.3030
Gene Synonyms	MP48
Status	experimentally characterised (or published) or close similarity to same REL2
Products	RNA editing ligase (1 others) RNA editing complex protein (13 others)
Type	CDS
Sequence	DNA and Protein

Location

Chromosome	1
Chromosome Location	670282..671532 Length: 1251 bp

Graphical Display (in Artemis)

Context Map

660000 665000 670000 675000 680000

Tb927.1.2980 Tb927.1.2990 Tb927.1.3000 Tb927.1.3010 Tb927.1.3020 >REL2< Tb927.1.3040 Tb927.1.3050 Tb927.1.3060 Tb927.1.3070 Tb927.1.3080 Tb927.1.3090 Tb927.1.3100 Tb927.1.3110 Tb927.1.3120

Sanger Annotation

targeted to the mitochondrion by the first 17 amino acids (PMID:11134327)
removal of signal peptide results in 45kDa mature protein
REL2 probably associated with insertion editing (PMID:11927563)
RNAi phenotype: no effect on cell growth or editing as REL1 (GeneDB_Tbrucei:chr9contig160:tmp0240) probably compensates
may be less active or inactive in vivo (PMID:12748175)
MP81 (GeneDB_Tbrucei:Tb927.2.2470) RNAi results in loss of adenylatable MP48 in bloodstream and procyclic form
L. major orthologue (GeneDB_Lmajor:CHR16-22_tmp 231c)
L. tarentolae orthologue (EMBL:AY148475) (PMID:12574127)
non-synonymous SNP between strain 927 homologues: codon 364, single nucleotide substitution changes V to A

Predicted Peptide Properties

Mass	47.5 kDa	Amino acids	416
Isoelectric point	pH 8.8	Charge	9.5

Signal Peptide	Not found
Transmembrane Domains	0 found

Protein Map

N C

100 200 300 400

Protein SNP

Gene Ontology

Ontology	GO Term	Browse Tree	Evidence Code	Reference
Biological Process	mRNA editing	GO:0006381	TAS	PMID:11134327
Cellular Component	mitochondrion	GO:0005739	IDA	PMID:11233974
	mRNA editing complex	GO:0045293	IPI	PMID:11134327
Molecular Function	RNA ligase (ATP) activity	GO:0003972	TAS	PMID:11233974

Catalytic Activity

EC 6.5.1.3 ; IUBMB , NiceZyme , Kegg , BRENDA

Literature

Search for REL2 in PubMed

DB	Accs	Description
PubMed	11134327	Panigrahi AK, et al. Mol Cell Biol. 2001 Jan ; 21(2):380-9
PubMed	11233974	McManus MT, et al. RNA. 2001 Feb;7(2):167-75

Database Cross-References

DB	Accs	Description
SPTR	Q9NBM0	SPTR
SPTR	P82864	TbMP48 precursor (EC 6.5.1.3) (RNA editing ligase) (RNA ligase).

SWISS-PROT Annotation For P82864

Catalytic Activity	ATP + (RIBONUCLEOTIDE)(N)+ (RIBONUCLEOTIDE)(M) = AMP + PYROPHOSPHATE + (RIBONUCLEOTIDE)(N+M).
Function	PART OF THE RNA EDITING COMPLEX ESSENTIAL FOR CELL VIABILITY
Sub Cellular Location	MITOCHONDRIAL
Keywords	Ligase , ATP-binding , RNA-binding , Mitochondrion , Transit peptide

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Fig. 3.5. A portion of a GeneDB report (http://www.genedb.org/genedb/tryp/index.jsp) (search for Tb927.1.3030) for the RNA editing ligase gene of *T. brucei*. This report shows the location of the gene on chromosome I, with clickable links to neighbouring genes via an *Artemis* viewer (http://www.sanger.ac.uk/Software/Artemis), plus results of bioinformatic analysis of the DNA and protein sequences and the current gene ontology associations. Links are provided to relevant publications and to reports on orthologous genes in related organisms such as *Leishmania major*. GeneDB is being developed at the Sanger Institute and the *T. brucei* data are curated in close collaboration with the sequencing team at TIGR.

Access to Data

For those requiring more detailed information on the gene and chromosome sequences discussed here, the following sources are available with many being updated over time.

Websites include:

- The Sanger Institute: http://www.sanger.ac.uk/
- The Institute for Genome Research: http://www.tigr.org/
- The EMBL sequence database: http://www.ebi.ac.uk/embl/
- The GenBank sequence database: http://www.ncbi.nlm.nih.gov/Genbank/
- The DDBJ: http://www.ddbj.nig.ac.jp/
- The EST database: http://www.ncbi.nlm.nih.gov/dbEST/
- The genome database: http://www.genedb.org/

All DNA sequences are found in the public databases EMBL, GenBank and the DNA Data Bank of Japan (DDBJ).

- Chromosome I sequence (as a single DNA molecule) accession number: AL929608.
- Chromosome II sequence (individual BACs) accession numbers: AC007865, AC007862, AC073246, AC007864, AC079606, AC012647, AC008031, AC007866, AC008368, and AC009463.

EST sequences are also contained in dbEST. Additional clustering and annotation data may be viewed at http://www.tigr.org/tdb/tgi/tbgi/

All annotated *T. brucei* sequences are present in the genome database, GeneDB. A BLAST server for homology searching of *all* available *T. brucei* and other kinetoplastid-derived sequences is also available at this site. In the future, the results of functional analysis will be deposited here also, allowing the database curators to annotate the DNA sequence with additional biological information.

Useful search and analysis tools are available at the websites of both sequencing centres. Two excellent examples are the Artemis sequence viewing and analysis tool (http://www.sanger.ac.uk/Software/Artemis), which displays all annotation associated with an EMBL file; and the BAC end mapping tool at TIGR, which allows selection of BACs covering a genomic region of interest (http://www.tigr.org/tdb/mdb/tbdb/bac_end_search.html).

Acknowledgements

We especially thank the sequencing teams at TIGR and the Sanger Institute, in particular Drs Najib El Sayed, Neil Hall, Matt Berriman, Elodie Ghedin, Christiane Hertz-Fowler and all involved in GeneDB for their provision of sequence data to the research community and discussion of annotated chromosome sequences prior to publication. In the text we acknowledge their joint work, as submitted to the public access databases, with the phrase '*T. brucei* Genome Project, unpublished'. In addition, we would like to acknowledge here the many contributions of the research community to the genome project and apologize that we are unable to reference fully the decades of work on *T. brucei* that underpinned the work discussed in this chapter.

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4 Communication in Trypanosomatids

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Introduction

Parasitic trypanosomatids are dependent upon their extracellular environment, a parameter that is subject to radical changes during their life cycle. A key to the evolutionary success of these organisms is the efficient way in which they have adapted to meet multiple environmental challenges. Sensing mechanisms, coupled to signalling pathways, in which processes occurring across the plasma membrane occupy a pivotal position, enable parasites to survive within changing environments. In spite of their obvious significance, interactions between trypanosomatids and their hosts/vectors and the molecular mechanisms that underlie them are poorly understood. This chapter will take an outside-to-inside approach, covering successively the interactions between the parasite surface and the extracellular environment, pathways of signal transduction, cell and life cycle differentiation and subsequent alterations in gene expression.

Interactions with Host Components Involving Macromolecules and Surface Receptors

Broadly speaking, these interactions fall into two groups: those required for growth which involve the uptake of essential macromolecular growth factors; and those involved in the regulation of growth and differentiation of the parasite. Some of the basic elements involved have been reviewed recently elsewhere (Borst and Fairlamb, 1998; Pays and Nolan, 1998).

Transferrin receptor

Transferrin is a necessary growth factor for trypanosomes and the receptor responsible for its uptake is so far the only one to be characterized at a molecular level (Steverding, 2002). The transferrin receptor of *Trypanosoma brucei* differs from that of higher eukaryotes. It is heterodimeric, with subunits encoded by a pair of genes (expression site-associated genes, or *ESAG*s, 7 and 6), linked to the variant surface glycoprotein (*VSG*) gene within the *VSG* expression site (ES). The receptor is attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor located at the C-terminus of the ESAG 6 subunit only (Fig. 4.1). The number of receptors per cell is estimated at around 3000. Structurally the protein resembles a VSG dimer and each subunit folds like the N-terminal domain of the VSG. Amino acids involved in ligand binding are known and only a few substitu-

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Fig. 4.1. Characterized membrane components of *T. brucei*. The major surface antigen (VSG for variant surface glycoprotein) is attached to the plasma membrane via a GPI anchor. The cleavage of this anchor by a GPI-PLC termed VSG lipase (see Fig. 4.2) releases components such as DAG and inositol phosphoglycan, which may play a role in cell signalling. The transferrin receptor is a heterodimer encoded by two genes linked to the VSG gene (*ESAG 7* and *ESAG 6*). This receptor has evolved from VSG N-terminal domains and binds to the membrane by the GPI anchor of *ESAG 6*. *T. brucei* contains several transmembrane adenylate cyclase isoforms that share the same receptor-like structure, with a variable extracellular N-terminal domain and a catalytic site in the C-terminal domain, as exemplified here for the isoform encoded by *ESAG 4*. The enzyme is probably activated through dimerization of the catalytic domain, induced by either interaction of specific ligands with the extracellular domain of each isoform, or general stress-induced release of inhibitory factors. TF, transferrin; AC, catalytic adenylate cyclase domain.

tions in this region are needed to alter receptor affinity drastically for a given host species of transferrin. The variable affinity of a given receptor for transferrin from different mammalian hosts may be one reason for the multiplicity of receptors encoded in different *VSG* ESs, which may number as many as 20–30, allowing the parasite to overcome transferrin diversity between different mammals. The transferrin receptors from different ESs do show distinct affinities for various transferrins which appears to influence the ability of trypanosomes to grow in the sera of these mammals. On average each receptor takes 11 min to complete one cycle and is recycled approximately 60 times before being degraded.

Receptors for lipoproteins

Lipoproteins are required growth factors for trypanosomes in mammals, probably as a source of cholesterol, and are required for

cell-cycle growth-phase G_1 progression. A series of studies showed that bloodstream forms of *T. brucei* meet their lipid requirements mainly by receptor-mediated endocytosis of low-density lipoprotein (LDL) via a specific receptor located in the flagellar pocket (Coppens and Courtoy, 2000). Processing of the endocytosed LDL apparently involves an acidic compartment and it was proposed that receptor recycling occurred due to acid-induced ligand–receptor dissociation. A proposed 145 kDa LDL-binding protein appears to have been conserved throughout the order Kinetoplastida, even in organisms that have never encountered a mammal. So far, however, a gene encoding this protein has not been identified and we share the view of other reviewers (Borst and Fairlamb, 1998) that the 145 kDa protein is probably a contaminating host protein, possibly even a component of the LDL complex itself.

Other studies have shown that bloodstream forms adapted to axenic culture grow equally well when provided with bovine, rabbit or rat LDL, or high-density lipoprotein (HDL), as their sole lipid source. Lipids were acquired with equal efficiency from these different sources and did so without accumulating radiolabelled protein components (Vandeweerd and Black, 1989). Endocytosed LDL and HDL may be degraded very rapidly in an early endosomal compartment to release the required lipids, with the degraded peptides being ejected from the cell.

A cysteine-rich acidic integral membrane protein (CRAM) may play a role in HDL uptake in procyclic forms of *T. brucei* (Liu *et al.*, 2000). This protein has homologies with the cysteine-rich repetitive motif characteristic of the human LDL receptor. Binding of HDL in *CRAM* null mutants was reduced by about 80%, but ligand uptake apparently decreased by only 20%, which at the very least suggests that CRAM is not solely responsible for HDL uptake. Estimates of 10^6 exposed binding sites for HDL in procyclic forms of *T. brucei* seems unrealistically high since it indicates that, in procyclic cells, the HDL receptor is almost as abundant as procyclin, the major surface protein in these forms ($\sim 5 \times 10^6$ copies per cell). Moreover, a physiological role for receptor-mediated endocytosis of mammalian serum components in parasites proliferating within an insect is not immediately obvious.

Receptors for serum trypanolytic factors

Humans and certain ground-dwelling primates are resistant to infections with *T. b. brucei* due to the presence of a serum factor that lyses these trypanosomes (Raper *et al.*, 2001). This factor, termed TLF, was originally shown to co-purify with the HDL fraction and later to be present in a very high-density subfraction (VHDL) comprising less than 1% of the total serum HDL. Lytic activity was proposed to be due to receptor-mediated endocytosis of TLF, delivery to acidic intracellular organelles, disruption of these organelles and subsequent autodigestion and lysis of the cell (Hajduk *et al.*, 1994). Around 350 high-affinity TLF-binding sites, distinct from non-lytic HDL receptors, localized to the flagellar pocket have been proposed. Trypanolytic VHDL contains a haptoglobin-related protein (hpr) and paraoxonase-arylesterase and, in conjunction with bound or internalized haemoglobin, it was proposed that disruption of the lysosomal membrane was due to lipid peroxidation. Serum-resistant parasites appear to bind TLF to the same extent as sensitive ones but fail to internalize the ligand receptor complex (Hager and Hajduk, 1997). In *T. b. rhodesiense*, resistance to human serum was associated with antigenic variation and was apparently linked to the transcription of a gene termed *SRA,* for serum resistance-associated gene (De Greef and Hamers, 1994). In resistant strains the active *VSG* ES contains *SRA* and is unusual in that several *ESAG*s are deleted (Xong *et al.*, 1998). More importantly, transfection of *SRA* into *T. b. brucei* was sufficient to confer resistance to human serum, indicating that this gene was responsible for *T. b. rhodesiense* adaptation to humans. Precisely how this gene, which codes for a mini VSG-like protein, confers this phenotype remains to be established.

Whether a single species of VHDL is responsible for trypanolytic activity is questionable given that two distinct lytic factors in human serum were demonstrated, with one fraction larger than 1000 kDa principally responsible for the lytic activity of unfractionated serum. The highly purified VHDL trypanolytic fraction was shown to contain apoA-I, hpr and trace amounts of paraoxonase, apoA-II and haptoglobin but, significantly, no haemoglobin. The mechanism of lysis by this complex and its relationship to VHDL-mediated lysis has yet to be established.

Receptors involved in sensing host growth factors and cytokines

The idea that molecules secreted by the host or parasite might modulate parasite growth and development is attractive but controversial. A homologue of the mammalian epidermal growth factor (EGF) receptor on the surface of bloodstream and insect forms of *T. brucei* was inferred (Hide *et al.*, 1989) but the evidence was largely indirect; the putative receptor was not identified and its presence remains unverified.

The discovery that upon invasion of the mammalian host *T. brucei* apparently releases a 44 kDa T-lymphocyte triggering factor (TLTF) (Vaidya *et al.*, 1997), which binds to $CD8^+$ T cells and triggers interferon-γ (IFN-γ) production, provoked a great deal of excitement about the direct involvement of cytokines in growth of the parasites. Unfortunately other groups have failed to validate the *in vitro* effects of IFN-γ on cell growth. Indeed an inverse relationship between IFN-γ production and parasite growth has been reported (Hertz *et al.*, 1998), which could imply that IFN-γ is essential for resistance and exerts its protective effect via the macrophage. Products of IFN-γ-activated macrophages, including NO and tumour necrosis factor-alpha (TNF-α), are cytotoxic for trypanosomes. Interestingly, TNF-α-mediated cell lysis *in vitro* shares features with lysis induced by the trypanosome lytic factor (TLF) of human serum. For instance, it involves specific endocytosis of the cytokine, delivery to an intracellular organelle, probably acidic, followed by disruption of the organelle membrane and autodigestion of the cell (Magez *et al.*, 1997). TNF-α appears to be lytic only for cells harvested late in the parasitaemia, which suggests a possible involvement in maintaining a chronic infection. Available evidence suggests that uptake of TNF-α might be via a lectin-like interaction with a protein located in the flagellar pocket (Magez *et al.*, 1997).

Clustering of receptors in the flagellar pocket

Endocytosis and exocytosis in trypanosomatids occur exclusively at the flagellar pocket, an invagination of the surface membrane from where the flagellum emerges from the cell. This is probably because the subpellicular microtubule network, which forms a corset considered to be inimical to membrane fission and fusion, is interrupted in this region. Although it represents only about 0.5% of the cell surface in bloodstream forms of *T. brucei*, the flagellar pocket membrane turns over at very high rates. Receptors for macromolecules are sequestered there to shield them from the cellular arm of the host immune response. An obvious question is: how are receptors and other proteins constrained to this microenvironment?

One study suggested that protein-targeting domains might play a role in directing some proteins to the flagellar pocket (Hill *et al.*, 1999). An internal domain of 144 amino acids of TLTF directed a green fluorescent protein chimera to a membrane-enclosed structure that abuts the cytoplasmic side of the anterior flagellar pocket. Interestingly, this membrane-bound, electron-dense structure containing the fusion proteins was unlike any previously characterized vesicles in trypanosomes. The sequence of the TLTF targeting domain did not resemble previously characterized trafficking signals. Deletional mutagenesis of the CRAM transmembrane protein of *T. brucei* also suggested the presence of two trafficking motifs in the short cytoplasmic tail (Yang *et al.*, 2000). The

extreme C-terminal region (residues 8–19 from the C-terminus) was implicated in the export of CRAM from the endoplasmic reticulum (ER), while a region up to 40 residues from the C-terminus might contain a flagellar pocket retention signal. Neither TLTF nor CRAM contained functional motifs similar to those reported in other eukaryotes. Although fusion proteins containing the cytoplasmic domain of CRAM co-localized with endogenous CRAM in the flagellar pocket, there were also significant amounts of this protein in the ER. Overall, however, in both cases, the secondary possibility of aberrant trafficking of modified or misfolded proteins cannot be discounted.

These studies fail to address the question of how surface proteins that lack transmembrane and cytoplasmic domains are sequestered in the pocket. For example, the GPI-anchored transferrin receptor is retained in the flagellar pocket, while the VSG, which also GPI-anchored, is free to move over the entire surface of the cell. Recent data have provided evidence suggesting that *N*-glycans, containing linear poly-*N*-acetyllactosamine (pNAL), might act as sorting signals for endocytosis in bloodstream forms (Nolan *et al.*, 1999). These glycans are only associated with proteins of the flagellar pocket/endocytic pathway and so endocytosis and retention within the pocket might involve interaction with a lectin-like protein with high affinity for these glycans. This view was consistent with the inhibitory effect of free chitooligosaccharides on the uptake of three different ligands. Although this model can only be considered tentative in the absence of direct evidence, the use of a luminal or extracellular sorting signal has obvious advantages for exploiting the limited surface area of the flagellar pocket through a common uptake mechanism. The flagellar pocket in bloodstream forms appears to be filled with a glycoproteinic material that is also found in coated pits and vesicles. One possibility, consistent with the particularly heavy glycosylation of some pNAL glycoproteins (Nolan *et al.*, 1999), is that interactions involving these *N*-glycans are spatially extended within the pocket.

Proteins, Pathways and Molecules Involved in Communication

To date no complete signalling pathway from physiological effector through the transducing machinery to eventual target and effect has been delineated in any trypanosomatid. Moreover, there is increasing evidence, both biochemical and from genome sequencing projects, that these organisms might lack certain classes of signalling systems found in higher eukaryotes (Parsons and Ruben, 2000).

Adenylate cyclases in trypanosomatids: a family of receptors, but what are the ligands?

The *VSG* ES of *T. brucei* contains a gene, termed *ESAG 4*, which codes for a cell surface adenylate cyclase that is probably responsible for a bloodstream stage-specific, Ca^{2+}-dependent activity. This gene belongs to a very large family (Naula and Seebeck, 2000). These cyclase isoforms all share the same receptor-like structure, containing a large and relatively variable region, predicted to be extracellular followed by a single transmembrane helix and a conserved catalytic domain (Fig. 4.1). The overall structure of trypanosomal cyclases differs substantially from that of their mammalian counterparts, but their catalytic domain is very similar to the C1 and C2 domains of the mammalian adenylate cyclases. Trypanosomal cyclases are far more numerous than in higher eukaryotes (Naula and Seebeck, 2000). Similar cyclases in *Dictyostelium* are activated by the interaction of their extracellular domain with different specific ligands. As is the case for the transferrin receptors, the cyclases encoded in the various *VSG* ESs may recognize activators that are almost, but not quite, the same in different hosts and are designed to allow colonization of a wide variety of mammals. Isoforms not encoded in *VSG* ESs, may recognize stage-specific ligands and trigger distinct differentiation events during parasite development. A huge step forward would be the unequivocal localization of some of these proteins. For example, if located in the flagellar pocket then a macromolecular activa-

tor is possible, whereas if uniformly distributed over the surface then a low-molecular-weight activator is more probable (as the VSG surface coat would act as a significant impediment to large but not small activators). To date the only reported localization is along the flagellum, a region that may be considered intermediate between these two extremes (Paindavoine *et al.*, 1992).

In *T. cruzi* epimastigotes, adenylate cyclase appears to be stimulated by a peptide produced by proteolytic cleavage of α-globin in the hindgut of the *Triatoma* haematophagous insect vector (Fraidenraich *et al.*, 1993). Peptides released by proteolysis of fibronectin were found to increase the concentration of cyclic AMP (cAMP) in *T. cruzi* trypomastigotes. This peptide-mediated activation of adenylate cyclase in *T. cruzi* is consistent with its structural resemblance with membrane form guanylate cyclases of higher eukaryotes, which are typically activated by peptides. In *T. brucei*, experiments using random peptides failed to show any significant effects, while peptidic protein kinase C (PKC) inhibitors were potent activators. In contrast to higher eukaryotes, so far no direct evidence has been presented for the involvement of G proteins in the control of trypanosomal adenylate cyclase. Moreover, the trypanosomal cyclases lack the consensus sequence for interaction with heterotrimeric G proteins, probably because the receptor-like region and catalytic domain are constituents of the same polypeptide, which precludes the need for G-proteins, again as seen in structurally related guanylate cyclases in higher eukaryotes.

Adenylate cyclase: a relationship with the glycosylphosphoinositol-specific phospholipase C (GPI-PLC) and PKC

Although the physiological activators of these cyclases remain elusive, adenylate cyclase activity can be stimulated, by up to two orders of magnitude, by a variety of agents and treatments such as Ca^{2+}, local anaesthetics, inhibitors of PKC, osmotic shock and extracellular acidic or proteolytic stress (Rolin *et al.*, 1996). All of these treatments also activate GPI-PLC and result in cleavage of the GPI and release of the VSG. Although these two activities appear to form part of the same stress-dependent signalling pathway, experimental evidence demonstrated that activation of adenylate cyclase and GPI-PLC occur independently (Rolin *et al.*, 1996).

In *T. brucei*, PKC activities appear to be differentially expressed during the life cycle, with PKC-like enzymes activated by diacylglycerol (DAG) and Ca^{2+} being bloodstream stage-specific (Boshart and Mottram, 1997). A bloodstream stage-specific PKC might be involved in down-regulating both adenylate cyclase and the GPI-PLC (Rolin *et al.*, 1996). In addition, the inhibitory effect of sodium fluoride on adenylate cyclase activity in membranes and cells might be due to enhancement of phosphorylation by inhibition of protein phosphatases. Thus a finely balanced 'futile cycle' of phosphorylation and dephosphorylation of some unknown target might operate in bloodstream forms of *T. brucei* to regulate a common stress response pathway involving adenylate cyclase and GPI-PLC. If correct then effectively these parasites operate a 'hair-trigger' response, since the energetic input is required to repress rather than activate this stress response pathway.

Adenylate cyclase: dimerization and control of activity

Mammalian adenylate cyclases require dimerization of the catalytic domain for activity. Since the *T. brucei* adenylate cyclases contain a single catalytic site, dimerization of the enzyme is a probable prerequisite for its function too. Functional studies with adenylate cyclases from *Leishmania donovani* have suggested that the active form is a dimer and that interactions between N-terminal domains might modulate the catalytic activity (Sanchez *et al.*, 1995). *T. brucei* ESAG 4 cyclase also migrates as an apparent dimer under non-denaturing electrophoresis conditions (Pays *et al.*, 1997). Since the adenylate cyclase activity is minimal in cells growing under resting conditions, a model can be proposed according to

which an energy-dependent process prevents the proper dimerization of the catalytic domain, so that all cyclase isoforms are kept inactive unless either the binding of specific ligands to some isoforms, or a general membrane stress, releases this inhibition for some or all enzymes at a time (model in Fig. 4.1). There is evidence that oligomerization and the formation of higher-order complexes may also be involved in the regulation of GPI-PLC (see later).

Signal transduction pathways

No signal transduction pathway has been elucidated to date in trypanosomes. Thus, only fragmentary information about possible players can be provided. Several trypanosomal proteins display the hallmarks of trimeric G proteins but there is no direct evidence yet that these proteins are used in response to extracellular signals (reviewed in Pays *et al.*, 1997; Parsons and Ruben, 2000).

Protein kinases A and C appear to exist in trypanosomatids (reviewed in Boshart and Mottram, 1997) as do proteins homologous to intracellular receptors for activated PKC (RACK) in *Leishmania* and *T. brucei* (Matthews and Gull, 1998). The involvement of at least some of these kinases in differentiation is probable, and *T. brucei* RACK is strongly up-regulated during the cellular differentiation into stumpy forms (Matthews and Gull, 1998; S. Lips and E. Pays, unpublished data). PKC activity may also play a role in metacyclogenesis of *T. cruzi*.

Multiple serine- and tyrosine-protein kinase activities have also been detected in trypanosomatids and most of them show developmental regulation. Auto-phosphorylation of protein kinases also appears to be developmentally regulated. In *Leishmania*, stage-specific changes of protein phosphorylation were shown to be due to phosphatase activities (Dell and Engel, 1994).

Mitogen-activated protein (MAP) kinases constitute a superfamily of signal transduction kinases that are sequentially activated in response to extracellular stimuli. Multiple genes encoding proteins related to the MAP kinase pathway have been characterized in *Leishmania* and *T. brucei* (Hua and Wang, 1997; Wiese, 1998). One of these kinases might be activated by IFN-γ in *T. brucei* (Hua and Wang, 1997), although the relvance of this is uncertain while the true effect of IFN-γ on trypanosomes is not clear (see above). In *Leishmania mexicana*, a MAP kinase homologue is essential for parasite survival in the host (Wiese, 1998).

In *T. brucei* and *T. cruzi*, a membrane-bound tyrosine phosphatase activity has been characterized and a transmembrane glycosylated acidic ectoprotein phosphatase was also found in bloodstream forms of *T. brucei* (Bakalara *et al.*, 2000). In *Leishmania*, the activity of a specific ectokinase was associated with infective forms while another, purified from the plasma membrane, was able to phosphorylate members of the human complement system. Phosphatases related to serine/threonine protein phosphatases 1 and 2A have been implicated in cell cycle progression (Orr *et al.*, 2000) and in the processing of some mRNAs (see below). An inositol phosphate/DAG pathway was suggested for *T. cruzi* but the function and regulation of this pathway remain to be established. A gene potentially encoding a phosphatidylinositol 3-kinase has been identified in *T. brucei*. Interestingly, in contrast to higher eukaryotes, IP_3 does not promote release of calcium from internal stores in trypanosomes.

The glycosylphosphoinositol-specific phospholipase C

In *T. brucei*, the only characterized PLC, termed VSG lipase, is that able to cleave the GPI anchor of the VSG (reviewed by Carrington *et al.*, 1998). This lipase is unusual in that it behaves as an integral membrane protein even though the primary sequence does not contain any obvious hydrophobic stretches and the protein apparently localizes to the cytoplasmic face of small intracellular vesicles. This presents a topological problem concerning enzyme access to the GPI substrate of the VSG. The physiological role of the VSG lipase also

remains elusive. It is not obligatorily required during any stage of the life cycle (*GPI-PLC* null mutants are capable of cyclical transmission between host and vector) though the null mutants exhibited lower parasitaemia in some strains of mice, due to the induction of a TH2-type of cytokine response after the initial phase of infection (Namangala *et al.*, 2000). The null mutants were also compromised in their ability to undergo stress-induced differentiation from the bloodstream to procyclic forms *in vitro*.

Although the biological function of the VSG lipase in *T. brucei* remains equivocal, the enzyme must be tightly regulated since almost the entire VSG coat can be released within a few minutes under environmental stress, whereas under resting conditions only a few molecules are cleaved. Thioacylation of the protein appears to be involved (Paturiaux-Hanocq *et al.*, 2000). Stress-induced activation of this enzyme appears to be linked to a shift from hemi- to full thioacylation on a cluster of three cysteine residues (Paturiaux-Hanocq *et al.*, 2000). Thioacylation is not necessary for catalytic activity but rather appears to be required for access of the enzyme to the VSG substrate. Tetramerization of the enzyme also seems crucial for activation. A possible model accounting for the observations is shown in Fig. 4.2. Lipid modification was also reported for a phosphatidylinositol-PLC of *T. cruzi* that is activated during trypomastigote to amastigote differentiation, suggesting that the mechanism controlling activation of the *T. brucei* VSG lipase may apply to other trypanosomatid PLCs.

Acylation-dependent membrane targeting

In addition to its role in regulating the VSG lipase, acylation also appears to be important for recruitment of other kinetoplastid proteins to the plasma membrane. The N-terminal sequence of a flagellar calcium-binding protein of *T. cruzi* needs both palmitoylation and N-myristoylation for the calcium-dependent targeting to the flagellum membrane

Fig. 4.2. Speculative model for the activation of the GPI-PLC (VSG lipase) of *T. brucei*. Under resting conditions the enzyme appears to be in equilibrium between two forms: a non-acylated form and a form thioacylated on a cluster of three cysteine residues. Among other possibilities, this observation could be explained assuming a dimerization between these forms (Paturiaux-Hanocq *et al.*, 2000). Under stress conditions the entire enzyme population becomes acylated, possibly through transfer of fatty acids between forms. This dynamic thioacylation appears to be involved in the process bringing the enzyme in contact with its VSG substrate on the surface of the plasma membrane, and may be linked to enzyme tetramerization.

(Godsel and Engman, 1999). Similarly, double palmitoylation/myristoylation of the N-terminal sequence was found to be required for proper localization to the extracellular face of the plasma membrane (Denny *et al.*, 2000) of a family of *Leishmania* hydrophilic surface proteins, termed HASPs (hydrophilic acylated surface proteins).

GPI anchors as signalling molecules

Several studies have suggested that GPI anchors may trigger pro-inflammatory cytokines and NO synthesis by macrophages. GPIs from *Plasmodium* and *T. brucei* were active only in the micromolar concentration range, similar to that observed for the relatively inactive *T. cruzi* epimastigote glycoinositol-phospholipids (GIPLs) and epimastigote mucin GPI anchors. The physiological relevance of effects at such concentrations is not clear. However, a highly purified GPI fraction from *T. cruzi* trypomastigote mucin glycoproteins was found to be a potent pro-inflammatory agent at picomolar concentrations (Almeida *et al.*, 2000) rendering these GPIs, which have unique structural features compared with the other GPIs, at least as active as bacterial endotoxin and *Mycoplasma* lipopeptide, the most potent microbial pro-inflammatory agents known. Release of trypomastigote GPI-anchored mucins might therefore play a role in inflammation and tissue pathology observed during chronic Chagas' disease. Whereas the release of free GPI and/or GPI-anchored proteins may influence the host–trypanosome interactions, in the case of *L. mexicana* a study of a null mutant for the GPI:protein transamidase indicated that GPI-anchored surface proteins are not essential for the parasite entry into and survival within host cells (Hilley *et al.*, 2000).

Calcium and signalling

Ca^{2+} is crucial to many signalling pathways, and mechanisms that maintain the resting intracellular [Ca^{2+}] at submicromolar levels are key to this. Trypanosomatids have several different membranes capable of transporting Ca^{2+}: the mitochondrion, plasma membrane, acidocalcisomes and ER (Parsons and Ruben, 2000). Although the ER possesses a Ca^{2+} ATPase, the precise role of this pool remains to be established since, in contrast to higher eukaryotes, it remains refractory to inositol 1,4,5-triphosphate (IP_3). Acidocalcisomes are membrane-bound Ca^{2+} storage organelles in protozoan parasites (reviewed in Docampo and Moreno, 1999) with an average diameter of 200 nm, possessing a Ca^{2+}-H^+ translocating ATPase. They contain high concentrations of H^+ and Ca^{2+} that have been implicated in intracellular Ca^{2+} and pH homeostasis, energy transduction and possibly osmoregulation. Physiological molecules that might initiate a Ca^{2+} influx have yet to be identified, but several amphiphilic peptides and amines have been shown to cause such an influx in bloodstream and procyclic forms of *T. brucei*. Phospholipase A2 and concomitant release of arachidonic acid from membranes seems to mediate this process in trypanosomes whilst in other eukaryotes IP_3 is of central importance. Specific processes regulated by Ca^{2+} flux and the machinery involved are also less well characterized in trypanosomatids. To date a calmodulin dependency has been shown only for a limited number of targets, including phosphodiesterases, kinases and a plasma membrane Ca^{2+} ATPase. The cellular complement of calmodulin-binding proteins also varies during the life cycle. This observation may be related to the control of gene expression, as the translation elongation factor 1-α of *T. brucei* binds calmodulin. One interesting group of EF-hand Ca^{2+}-binding proteins has been shown to be associated with the flagellum. This location may indicate a potential role in motility or alternatively environmental sensing, since a Ca^{2+}-activated adenylate cyclase also appears to be located on the flagellum in bloodstream forms of *T. brucei* (Paindavoine *et al.*, 1992). A special role for Ca^{2+} has been proposed for *T. cruzi* where changes in the concentration of Ca^{2+} occur during parasite invasion of the host cell. The source of the mobilized Ca^{2+} has yet to be determined

but acidocalcisomes may act as a store for Ca^{2+} in intracellular parasites (Docampo and Moreno, 1999).

Another facet of Ca^{2+}-induced signalling is involved in cell invasion by *T. cruzi*. This process is initiated by a local recruitment of host lysosomes, which constitute the entry vehicles for the parasite (Burleigh and Andrews, 1998). A cytosolic trypanosome oligopeptidase, termed oligopeptidase B, is involved in the generation of a secreted factor able to trigger local increase of Ca^{2+}, which promotes exocytosis and fusion of lysosomes in the host cell. Recent data indicate that activation of adenylate cyclase, resulting in an elevation of intracellular cAMP, regulates the Ca^{2+}-driven recruitment of these lysosomes. Therefore, the primary target of the oligopeptidase B-dependent signalling factor could be the host adenylate cyclase.

Virulence factors and components affecting host–parasite interactions

Glycan molecules have been found to be important in several aspects of trypanosome–host interactions, especially during parasite–host adhesion events.

Lipophosphoglycans (LPG) are a major constituent of the promastigote surface of *Leishmania* and consist of repeated phosphorylated disaccharides attached to the membrane by a GPI anchor. One enzyme essential for LPG biosynthesis is LPG1, a β-galactofuranosyl transferase that is responsible for the addition of an unusual internal β-Gal_f residue to the LPG diphosphoheptasaccharide core (Ilg, 2000). Deletion of this gene blocked LPG biosynthesis selectively without affecting the biosynthesis of other glycans and, surprisingly, LPG-deficient *L. mexicana* mutants were as virulent as the wild-type strain in macrophage and mice infections (Ilg, 2000). This finding clearly challenges the generally accepted view that *Leishmania* promastigote LPG is a multifunctional virulence factor that is required for parasite development and survival in sandflies and mammals (reviewed in Beverley and Turco, 1998).

Sialic acids are negatively charged carbohydrates found at terminal locations of glycoproteins and glycolipids. Trypanosomes are unable to synthesize these molecules, but *T. cruzi* and the procyclic form of *T. brucei* express on their surface several trans-sialidases – enzymes capable of transferring sialic acid between host glycoconjugates and the parasite (Frasch, 2000). Sialic acids appear to be key molecules for cell invasion by *T. cruzi*. Invasion of the cell may depend on sequential binding and release reactions performed by reversible sialylation of both the parasite surface and host ligands. In addition, the parasite sialidases appear to activate the TGF-β signalling pathway, a prerequisite for parasite growth in mammalian cells. This process may result from the induction of apoptosis in cells of the immune system. Indeed, the activity of *T. cruzi* trans-sialidase can directly trigger apoptosis of these cells. Then, upon interaction with macrophages via the vitronectin receptor, apoptotic T lymphocytes induce the release of prostaglandin E_2 and TGF-β, immunosuppressive molecules which favour the growth of the parasite.

Tsetse flies appear to contain glucosyl and galactosyl lectins that are lytic to trypanosomes, presumably through interaction with the procyclic surface coat (Welburn and Maudlin, 1999). Development of the trypanosome infection could depend on the protective effect achieved by the release of lectin-inhibitory sugars from symbiotic organisms present in the midgut of some tsetse fly species. The binding of midgut lectins to terminal carbohydrate residues of procyclin may lead to cell death but binding to other parts of the molecule might stimulate proliferation. Similar lectin-based systems appear to operate in other vector–parasite systems.

Finally, in both trypanosomes and *Leishmania*, cysteine proteinases have been found to be involved in parasite virulence as well as modulation of the immune response (reviewed in Mottram *et al.*, 1998). As was the case for the GPI-PLC of *T. brucei* (see above), the mechanism by which these enzymes influence the immune system is unclear.

Control of the Cell Cycle and Differentiation

Trypanosomatid life cycles alternate between proliferative and quiescent stages. Only the latter appear to be competent for differentiation into the next developmental form and are thus infective (Matthews, 1999). The controls of the cell cycle and cell differentiation are interdependent, with differentiation only possible between defined windows of the cell cycle. Differentiation involves extensive reprogramming of gene expression, mainly through changes in post-transcriptional processes. In contrast to higher eukaryotes, gene expression in trypanosomes is not controlled at the level of transcription initiation but primarily during RNA processing.

An interplay between cell cycle and cell differentiation

In *T. brucei*, quiescent stumpy bloodstream forms differentiate rapidly and in a synchronous manner into insect stage procyclic forms (Matthews, 1999). While different trypanosome lines may exhibit some differential ability to respond to differentiation signals during the cell cycle, it should be noted that even highly monomorphic populations of dividing long-slender forms can also differentiate but the process is slower and less synchronous. It would appear that the efficiency of growth is inversely related to the efficiency of differentiation, as suggested by differentiation studies performed with GPI-PLC null mutants. The commitments to differentiate and re-enter the cell cycle seem to occur rapidly and simultaneously, but these processes are separable (Matthews, 1999). The situation is also complicated by the fact that stumpy bloodstream forms of *T. brucei* clearly possess prior adaptations to life in the insect vector. These biochemical changes relate to the elaboration of mitochondrial function and also the ability to tolerate significant extracellular acidic and proteolytic stress (Nolan *et al.*, 2000). Studies with tsetse flies show that most ingested parasites are eliminated in the midgut and only a few cells (possibly stumpy forms) survive to transform into procyclic forms.

A few genes, homologous to cell cycle control genes in other eukaryotes, have been described in trypanosomatids. For example, families of cyclins and cdc2-related protein kinases have been described in *Leishmania* and *T. brucei* (Mottram and Grant, 1996). So far only one of these kinases, crk3, has been shown to bind a cyclin, but its localization does not clearly indicate an involvement in cell division (Van Hellemond *et al.*, 2000). Members of Polo and Nima kinases, two subfamilies of protein kinases that play pivotal roles in cell division and proliferation, have also been identified in *T. brucei*. Finally, the use of inhibitors allowed the demonstration that protein phosphatases 1 and/or 2A are involved in coordinating mitosis, mitochondrial DNA division and cytokinesis in trypanosomes (Orr *et al.*, 2000). Therefore, it seems that at least some of the elements known to participate in the control of the eukaryotic cell cycle are present in these protozoans. Roles for chromatin structure have also been implicated in cell cycle control (Horn, 2001).

Cyclic AMP and differentiation

Cyclic AMP plays a major role in differentiation during the life and cell cycle of trypanosomatids. In *T. cruzi*, the differentiation of epimastigotes to metacyclic trypomastigotes is linked to activation of adenylate cyclase. A transient stimulation of adenylate cyclase also occurs at an early stage in the differentiation from the bloodstream to the procyclic form of *T. brucei* and cAMP levels increase in *T. brucei* prior the transformation of long-slender to short-stumpy forms. Replicating slender bloodstream forms appear to secrete a low-molecular-weight component, termed stumpy induction factor (SIF), which accumulates in the medium and triggers cell cycle arrest in the G_1/G_0 phase, followed by the induction of cellular differentiation into stumpy forms (Reuner *et al.*, 1997). This is linked to the synthesis of cAMP, and experimental increase of intracellular cAMP leads to the same effects as SIF. An obvious implication is that SIF might be a ligand for one of the receptor-

like cyclases and, given its size (< 500 Da), the cyclase responsible could be shielded by the VSG coat.

Triggers for life cycle differentiation

The best-studied example of cell differentiation in trypanosomatids is the *in vitro* transformation of *T. brucei* from the bloodstream to the procyclic form. This step requires a decrease in temperature (from 37 to 27°C) and the presence of *cis*-aconitate (Ziegelbauer *et al.*, 1990). *Cis*-aconitate appears to be the crucial factor, as lower temperature seems to be required only to allow the differentiated procyclic form to survive. A role for aconitase in the process is unlikely, since the gene for this enzyme is dispensable (Saas *et al.*, 2000), and the site of action of this metabolite remains to be determined. Indeed the role of *cis*-aconitate per se may also be questionable *in vivo*, since exposure of pleiomorphic bloodstream trypanosomes to mild acidic stress accelerated the switch to stumpy forms and also abrogated the requirement for the presence of Krebs cycle intermediates for subsequent differentiation to procyclic forms at 27°C. The fact that stumpy but not slender forms resist high extracellular concentrations of H^+ and the likelihood that parasites ingested by the fly are exposed to acidic conditions (Nolan *et al.*, 2000) might relate to this pH-dependent differentiation phenomenon. An additional finding was that GPI-PLC null mutants were refractory to this acid stress-induced differentiation but not to that induced by Krebs cycle intermediates. It may be that several pathways can deliver the same result but so far the machinery involved remains to be identified.

Gene expression and differentiation

T. brucei is an excellent model organism for the study of parasite differentiation at the gene level. Trypanosome genes are generally clustered in long polycistronic units whose primary transcription is constitutive, with the notable exception of those encoding the major stage-specific surface antigens, the VSG and procyclin. The latter units share the peculiarity of recruiting RNA polymerase I, which is normally devoted to transcription of ribosomal genes. There is evidence for developmental control at the level of RNA elongation in the VSG and procyclin units (Vanhamme and Pays, 1995). Post-transcriptional controls are of primary importance to ensure cellular differentiation and they occur mainly at the levels of RNA processing, RNA translation and protein stability (Fig. 4.3). How cellular signalling influences them is not clear. Cyclic AMP appears to be involved in the synthesis of procyclin in differentiating stumpy forms, whereas RNA elongation in the VSG and procyclin units is regulated by the stimuli of differentiation. The relative abundance of the mRNAs encoding the transferrin receptor was found to be dependent on the amount of iron in the culture medium, suggesting the involvement of iron in the specific processing of these transcripts. Similarly, glycerol was found to affect the stability of the procyclin mRNAs. One obvious possibility is that environmental signalling influences (de)phosphorylation of proteins involved in RNA processing and stability. For example, the activity of protein phosphatases 1 and/or 2A appears to be involved in the determination of the steady-state levels of β-tubulin mRNA in *T. rhodesiense*.

The earliest events detectable during differentiation from the bloodstream to the procyclic forms are the inverse regulations of primary transcription in the units for the major stage-specific antigens, VSG and procyclin. These transcriptional controls consist of a progressive blocking of RNA polymerase activity in the VSG unit and a simultaneous stimulation of RNA elongation in the procyclin units. As an immediate consequence, the VSG gene, which is the last of its transcription unit, is completely silenced, whereas at the same time the procyclin genes, which are the first of their units, are completely activated. These transcriptional controls are linked to downstream controls at the levels of RNA processing and stability, which dictate both a selective degradation of the VSG mRNA and a preferential production of the procyclin mRNAs. As a result, the

Fig. 4.3. Control of gene expression in *T. brucei*. Whereas transcription units recruiting RNA polymerase II (pol II) and containing housekeeping genes are fully active in both procyclic and bloodstream forms (PF and BF, respectively), the pol I units encoding the major surface components, procyclin (α and β) and VSG, are only fully transcribed at their respective developmental stages. The opposite transcriptional controls operating in these units may involve common elements of the RNA factory, possibly differentially modified by environmental signalling. In all cases, processing of the primary transcripts into individual mature mRNAs is heavily dependent on environmental signalling. ES7/6, *ESAG 7/6*; PAG, procyclin-associated gene.

production of procyclin transcripts rapidly amounts to more than 20-fold the levels measured in established procyclic forms, whereas the VSG transcripts, which represented 5–10% of the polyadenylated RNAs, become barely detectable. These dramatic changes are presumably designed to renew the surface coat rapidly and completely. The VSG coat is released concomitant with activation of a protease (Ziegelbauer *et al.*, 1993), while procyclin requirements are met by a strong hyper-production of mRNA (Pays *et al.*, 1993). These events are essentially completed within 4 h and clearly precede the cell commitment to entry into the S-phase (Pays *et al.*, 1993; Matthews and Gull, 1994), which lasts between 5 and 12 h and is accompanied by a strong accumulation of RNA (Pays *et al.*, 1993). General reprogramming of gene expression occurs during this period, as shown by the appearance of multiple procyclic-specific proteins after 4–6 h (Pays *et al.*, 1993; Matthews and Gull, 1994). The temporal distinction between the changes occurring at the level of the major surface antigens and that of other proteins may be related to the differences between the respective gene expression controls (Fig. 4.3). Mitosis and cell division occur after 10–12 h, at a moment where the genetic programme is extensively, if not completely, rearranged, as judged by the general and specific protein content of the cell (Pays *et al.*, 1993; Matthews and Gull, 1994).

Summary and Future Outlook

Communication in trypanosomatids is a broad and diverse field ranging over a variety of different parasites and touching on a wide number of interconnecting issues such as ligand uptake, host/vector–parasite interactions, cell signalling, cell and life cycle differentiation and gene expression. This is a subject that could do justice to an entire volume rather than a single chapter but it is fair to ask what can be said on the basis of this short review. Certainly the situation concerning uptake of macromolecular ligands from mammalian hosts has advanced over the past decade, as exemplified by the case of the transferrin receptor of *T. brucei*. Of course difficulties remain, e.g. for lipoproteins and the mechanism of endocytosis in general, but overall this area is in relatively good shape. The role of receptors involved in environmental signalling is less well established; a significant step forward would be the identification a physiological ligand that interacts with any of the many potential receptor-like adenylate cyclases. In this respect surely the characterization of the putative SIF is imperative. In a sense this frustration encapsulates the whole problem of signalling in trypanosomatids, namely that no complete pathway right through from physiological effector via a transducing machinery to an eventual target and effect has been delineated. Is it possible that there is a convergence of signalling responses in these parasites and that multiple stimuli share the same transduction pathway? Certainly this notion is consistent with the huge number of adenylate cyclase genes and also the fact that different treatments and agents are capable of producing the same cellular response. In addition, current evidence suggests the absence of certain classes of signalling pathways in these parasites. In metazoan animals many signalling pathways culminate in the activation of specific transcription factors, whereas in trypanosomatids they appear to be directed towards the machinery involved in RNA processing and turnover. What of agents involved in modulating host–parasite interactions? Despite initial enthusiasm, the view that these parasites modulate host cytokines and growth factors to regulate their own growth seems to have become weaker, at least in the case of INF-γ. Similarly, the role of certain parasite-derived molecules as virulence factors, e.g. LPGs, has been called into question by recent studies. Nevertheless, it is clear that these parasites can affect the host immune response, but how and why remain elusive. To end on a positive note, it is worth remembering that these parasites have had millions of years to adapt to and confuse their mammalian hosts and so a few decades of research seems a fair return to find out how. Maximal exploitation of ongoing but

soon to be completed genome-sequencing projects allied to focused functional analysis is likely to pave the way forward.

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5 Genetics and Molecular Epidemiology of Trypanosomes

Geoff Hide and Andrew Tait

Introduction

The population biology of trypanosomes has developed as a major subject of research since the early 1980s. The major thrust of this research has been directed at three main, interrelated topics: taxonomy, genetic exchange and molecular epidemiology. For disease control, it is crucially important to define and identify species, subspecies, strains and populations of trypanosomes to enable epidemiological investigation (reviewed recently in Hide, 1999; Tait, 2000). Molecular epidemiology, which can be defined as the application of molecular biological methods to the investigation of the epidemiology of disease, emerged as a general discipline in parasitology in the 1980s. This approach generates markers that can define and identify discrete taxonomic units (species, subspecies, strains, populations and variants) with the aim of tracking parasites through different hosts or vectors, determining associations with geographical information, analysing host-specific variants and investigating associations between disease and particular groups of parasites. The genetic structure of taxonomic units, however, can be influenced significantly by the effects of genetic exchange and recombination between organisms. To be able fully to interpret molecular epidemiological data, it is crucial to have an understanding of the mechanisms and effects of genetic exchange. The integrity of any taxonomic unit (from species down to population) is maintained by the absence of gene flow (i.e. absence of genetic exchange) between that population and any other. If genetic exchange occurs, the defining characters for that population are exchanged with other populations and distinctions between them become blurred. In such a situation it is difficult to apply any epidemiological tracking methods to identify those populations. Furthermore, gene flow between populations can introduce new characteristics to a population. For example, the introduction of clinically important traits, such as human infectivity introduced to a non-human trypanosome population, could occur by genetic exchange and equally the introduction of drug resistance into drug-sensitive populations.

The scope of this chapter is to review the molecular tools available for genetic and epidemiological analysis of trypanosomes, to consider the contribution of molecular tools to defining trypanosome taxonomic units, to discuss the role of genetic exchange in trypanosomes in relation to laboratory and field studies and to highlight the contribution of molecular epidemiology to our understanding of trypanosomiasis. Much of the available data has been obtained with

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Trypanosoma brucei and, other than isoenzyme studies in the 1980s and the development of species-specific DNA markers, there are few molecular epidemiological analyses of *T. congolense*, *T. vivax* and other species despite their importance to livestock rearing. As the major body of data has been obtained with *T. brucei*, the chapter will focus on this species complex but will draw on parallels with other trypanosome species where data are available.

Molecular Tools

Two basic sets of molecular markers have been developed: qualitative and quantitative (Hide and Tait, 1991). The former set relies on finding absolute differences between the populations or species under investigation, e.g. the presence or absence of a given gene sequence or protein. This approach is often more appropriate for distinguishing between trypanosomes from taxonomic levels at or above the species level and is of limited use for tracking the relationships between trypanosomes at an intraspecific level. The second approach relies on finding more subtle, quantitative differences which can be used at the intraspecific level for tracking individual strains or strain groups. The key requirements for both of these approaches are specific methods for the identification of parasite populations and the ability to utilize those methods in tracking these populations through their hosts and vectors. In trypanosome population biology, these types of studies were difficult prior to the late 1970s and early 1980s due to the paucity of genetic markers. Morphological similarities between species (or subspecies) and the necessity of using complex microscopic characters or electron microscopic techniques made species distinction controversial, population or subspecific identification almost impossible and large-scale analysis difficult. Therefore, prior to this time, taxonomic groupings tended to be constructed from characters that were chosen from a clinical viewpoint, such as host range, disease pathology and geographical range.

The advent of biochemical techniques such as the study of isoenzyme variation by multilocus enzyme electrophoresis (MLEE) (Gibson *et al.*, 1980; Tait, 1980) and subsequently the introduction of molecular biological approaches (Hide and Tait, 1991) opened up new possibilities. Initial studies were carried out on *T. brucei* and focused on developing markers that could be used to characterize populations of trypanosomes without recourse to traditional characters. Using MLEE, three basic approaches were taken to characterize *T. brucei* populations: (i) on the basis of allele frequencies (e.g. Tait, 1980; Tait *et al.*, 1984, 1985); (ii) on the basis of similarities of shared characters (e.g. Gibson *et al.*, 1980) (quantitative approaches); and (iii) on the absolute distinction based on presence/absence of a specific isoenzyme band in a given taxonomic group (e.g. Gibson *et al.*, 1980; Tait *et al.*, 1984) (qualitative approaches). These methods provided a clear distinction at some taxonomic levels (e.g. *T. brucei gambiense* and *T. b. brucei*) but not at others (e.g. *T. b. brucei* and *T. b. rhodesiense*).

These same two basic types of approaches (quantitative and qualitative) have been utilized more recently using DNA-based molecular markers (reviewed in Hide and Tait, 1991; Hide, 1997). One of the fundamental properties of DNA is the way in which it can be denatured and, under appropriate conditions, will renature in a sequence-specific manner. This property is the basis of a number of important molecular epidemiological techniques such as DNA hybridization and the PCR. The qualitative approaches have been dominated by these techniques: hybridization with specific DNA probes and specific amplification of target gene sequences.

The simplest hybridization method, which can be used for distinguishing trypanosomes, is the dot blot method. DNA samples are prepared from suitable material, such as blood, insect vector tissue or biopsy material, and immobilized on a DNA-binding membrane such as nitrocellulose or nylon. A specific fragment of DNA (probe) is then hybridized to the immobilized DNA. The choice of probes used for this technique is crucial to the success of the approach. The basic properties of a suitable probe should be a lack of cross-hybridization to either host or vector

DNA and specificity for the trypanosomatid species of interest. Probes covering repetitive DNA sequences are especially useful since they enhance the sensitivity of the hybridization reaction. Examples of suitable probes that have been used are the 177 bp repeat which hybridizes with a variety of *Trypanozoon* species and the *Antat1.1* gene which is absent from *T. b. gambiense* but present in *T. b. brucei* (reviewed in Hide and Tait, 1991). Probes for detecting different species of trypanosome and subgroups of *T. congolense* have been developed and these have been reviewed and listed elsewhere (Hide and Tait, 1991; Desquesnes and Davila, 2002). In some cases, it is not possible to find appropriate DNA sequences that have the correct specificity for hybridization. Such problems can be overcome by the synthesis of specific oligonucleotide probes based on known DNA sequences that differ in only a few base pairs between strains of parasite. Once the exact hybridization conditions of such oligonucleotide probes are known, considerable specificity can be achieved in dot blot assays (Hide, 1997).

One of the main advantages with the dot blot approach is that many samples can be processed simultaneously and with relatively high sensitivity (10^2–10^3 trypanosomes detected using a repetitive probe). The main disadvantage is the requirement for DNA to be extracted from each sample. A modification of this approach has been the 'touch blot'. In this technique, biological samples are directly touched on to DNA-binding membrane. This has been successfully used for analysing the trypanosomes found in tsetse flies, using the abdominal touch blotting method (reviewed in Hide and Tait, 1991) where as few as 10–100 trypanosomes per vector can be detected. A further hybridization method, which could be an important epidemiological tool, is that of *in situ* hybridization. With this technique, probes are hybridized directly to trypanosomatids fixed on a microscope slide and microautoradiography identifies those parasites containing the probe sequence. This approach has been used to identify mixed infections of *T. brucei* and *T. congolense* in tsetse flies (reviewed in Hide and Tait, 1991).

The application of hybridization-based methods has been limited in large-scale field-based studies and this is, in part, due to the requirement for sources of radioactive nucleotides and hybridization equipment that are not readily available in many developing country laboratories. Furthermore, the development of PCR-based approaches, which have many advantages in terms of specificity and sensitivity, has meant that these techniques have superseded hybridization-based approaches.

The PCR detection of parasites is based on the sequential amplification of a target sequence such that, with 30 cycles of amplification, some 2^{30} copies of the original target sequence are generated. PCR primers, designed from the 177 bp repeat or other sequences such as the mini-exon, have been used for species-specific detection (e.g. Masiga *et al.*, 1992). The use of target sequences that occur in high copy number in the genome has led to PCR-based methods that can detect parasitaemias as low as 1–20 trypanosomes/ml (Desquesnes and Davila, 2002). These techniques are particularly useful for epidemiological analysis in terms of obtaining prevalence data at the species or subspecies levels. Further developments have been made so that the sample can be analysed by PCR without growth of the parasites in a susceptible host and analysis can be undertaken directly on blood samples collected on filter papers (e.g. Whatman FTA papers). PCR methods are now available for the detection of *T. vivax*, *T. brucei* and *T. congolense* subtypes and these are suitable for use directly with blood samples on filter papers. Developments in PCR strategies include sensitive detection even to levels of detection of a single trypanosome (MacLeod *et al.*, 1997). Recently, a new tool has been developed which can be used specifically to identify *T. b. rhodesiense* in East Africa. The *SRA* gene, discovered in 1989, has been shown to be the only gene (in East Africa) required to confer human serum resistance on sensitive strains and PCR analysis demonstrates that it is present in all *T. b. rhodesiense* stocks tested to date (Welburn *et al.*, 2001; Gibson *et al.*, 2002).

Dot blotting, touch blotting, *in situ* hybridization and the PCR-based diagnostic methods are all examples of the ways that hybridization can be used as a presence/absence detection system at the species level. In many cases, it is desirable to investigate the variation within species of trypanosomatids and to be able to identify specific strains that may be of epidemiological significance. Southern blotting hybridization techniques, based on restriction fragment length polymorphisms (RFLPs), provides a good method for achieving these objectives (Hide *et al.*, 1990). This technique essentially assays the variation in DNA sequence of a given gene (detected by hybridization with a specific probe) by identifying the presence or absence of the specific DNA sequence sites necessary for cleavage of the gene by restriction endonucleases. In general, repetitive DNA sequences are used for these types of approach because of their ability to generate large numbers of fragments which are observed as a ladder of bands on a Southern blot. These data can be interpreted using numerical taxonomic methods to develop dendrograms depicting the relationship between strains of trypanosomatids (Hide, 1996). Figure 5.1 shows a typical set of banding patterns using a repetitive probe on stocks of *T. brucei*. Figure 5.2 shows an example of a dendrogram that can be generated using the type of data shown in Fig. 5.1. This type of analysis has been described in different trypanosomes, using in particular the ribosomal RNA genes of *T. brucei* (Hide *et al.*, 1990; Hide, 1996) and other trypanosomatid species (reviewed in Hide, 1997).

Although a useful tool, hybridization with DNA probes has drawbacks. A large amount of parasite material is required for the extraction of DNA, with a consequent requirement for growth in rodents. PCR-based strategies that overcome this limitation are not so straightforward to design, as they need to generate a sufficient level of intraspecific variation to enable discrimination of subspecific groups.

The development of such tools has been the focus of recent and current research. A variety of PCR–RFLP-based analyses have

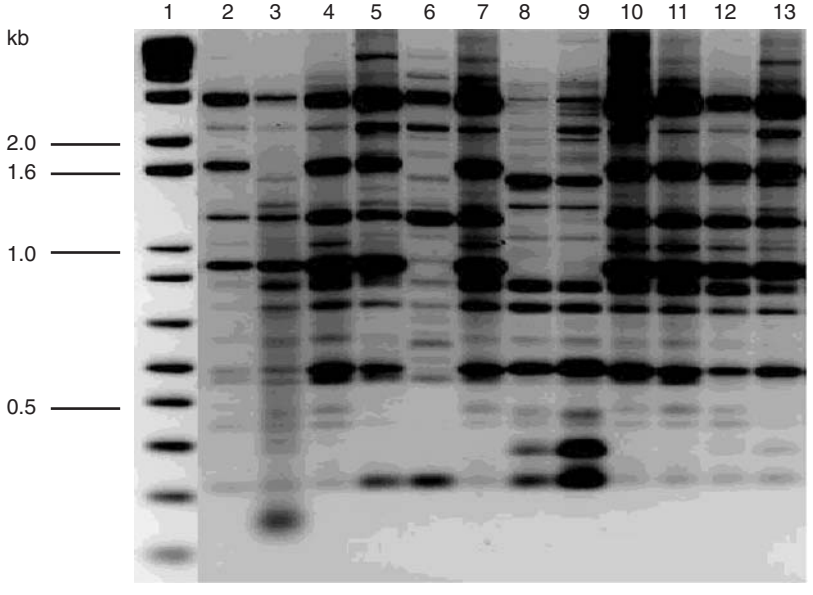

Fig. 5.1. An autoradiograph showing a Southern blot analysis of RFLP variation in trypanosome isolates. Lane 1 contains molecular size markers; lanes 2–9 contain DNA from trypanosome stocks isolated from cattle; and lanes 10–13 contain DNA from trypanosome stocks isolated from humans. All stocks were collected between 1988 and 1992 from the Tororo District of Uganda. Similarity co-efficients can be calculated between pairs of stocks based on presence or absence of bands at the same position and this data can be used to construct a dendrogram such as that shown in Fig. 5.2.

Fig. 5.2. A dendrogram of relatedness constructed from RFLP data such as that presented in Fig. 5.1. Similarity of banding patterns between pairs of stocks equates to a high level of similarity on the dendrogram. This dendrogram demonstrates that *T. b. gambiense, T. b. rhodesiense* (Busoga focus) and *T. b. rhodesiense* (Zambian focus) are all quite distinct groups. It also shows that *T. b. brucei* and *T. b. rhodesiense* from sympatric locations can be distinguished.

been developed, combining the variability of RFLP analysis with the power of PCR amplification, and these are known variously as riboprinting and intergenic region typing (IRT) (reviewed in Tilley and Hide, 2001). Although useful in the definition of subspecies groups in *T. cruzi*, these methods have generally detected too little variation for defining differences between and within populations, especially for *T. brucei* (Tilley and Hide, 2001). The randomly amplified polymorphic DNA (RAPD) approach, based on variable banding patterns generated by a single primer, has been used in a number of studies to define trypanosome populations (e.g. Mathieu-Daude *et al.*, 1995; Stevens and Tibayrenc, 1995). This approach, while identifying high levels of intraspecific variation, has the drawback that it can only be conducted on pure DNA samples, as contaminating host DNA will contribute to banding patterns. Amplified restriction fragment length polymorphism (AFLP), which has proved invaluable in the identification of strains in many microbial systems, has also been shown to be a powerful genetic marker for stock identification in *T. brucei* (Agbo *et al.*, 2002) although, as host DNA will also be amplified, it requires pure DNA. The identification of trypanosome variation directly from host or vector samples can be undertaken with three types of markers which are not subject to interference by host DNA. Hypervariable minisatellite DNA sequences, identified in *T. brucei*, have been shown to be very powerful tools for the detection of single trypanosomes (MacLeod *et al.*, 1997), the identification of parasite genotypes derived from the tsetse fly samples (MacLeod *et al.*, 1999) and the characterization of subspecific group trypanosomes (MacLeod *et al.*, 2001a,b,c). The application of these markers to defining differences between populations and subspecies is illustrated in Fig. 5.3; by combining the data from three minisatellite loci to define the multilocus genotype of each isolate, *T. b. brucei* can be distinguished from *T. b. rhodesiense* and the parasites from different foci can be defined. Microsatellite loci have also been recently identified in *T. brucei* and shown to be polymorphic between different stocks (Biteau *et al.*, 2000). MGE-PCR, a technique involving single primer PCR analysis of positional variation of mobile genetic elements (MGEs) in trypanosome genomes, shows that variation can be used to identify populations of *T. brucei* (Hide and Tilley, 2001; Tilley *et al.*, 2003).

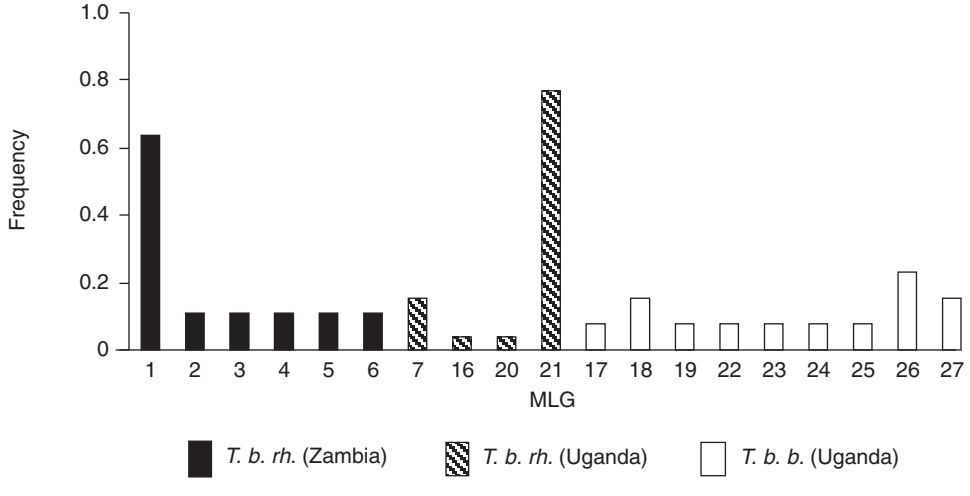

Fig. 5.3. Minisatellite multilocus genotypes (MLG) of isolates from the Busoga focus (*T. b. brucei* and *T. b. rhodesiense* – defined by their resistance/sensitivity to lysis by human serum) and isolates from the Luangwe Valley focus (Zambia). There is a clear distinction between the three groups of isolates in that they do not share any genotypes. (Data reported in MacLeod *et al.*, 2000.)

Based on the PCR tools outlined in this section, two types of molecular epidemiological analysis can be undertaken: (i) the definition of the range of species and subspecies infecting animals and humans to define prevalence and species interactions; and (ii) the analysis of subspecific variation using highly polymorphic markers within a particular species to define transmission, the spread of human-infective trypanosomes and other strain-specific questions. The sensitivity of these two types of analysis differ by several hundredfold but both can be applied to samples collected on filters. While markers for analysis of polymorphism within a species are available for *T. brucei*, they remain to be identified for the major species affecting livestock although, in principle, they could readily be developed.

Genera, Species and Subspecies

T. brucei complex

The subspecies status of *T. brucei* has been a matter for debate for many years. *T. brucei* has been classically defined in terms of three subspecies, *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense*, with distinctions being largely made in terms of convenience of description rather than strict taxonomic markers. *T. b. brucei* is defined as animal infective and can be found throughout the tsetse belt of Africa; *T. b. gambiense* and *T. b. rhodesiense* are both infective to humans, the former being associated with chronic disease in West Africa while the latter is associated with acute disease in East Africa. The problem with these definitions is that the boundaries are often blurred. As an example of this, *T. b. rhodesiense* was also found in a variety of domestic and wild animals and furthermore was found to be associated with asymptomatic, low-virulence disease in some foci (reviewed in Hide and Tait, 1991). The first clear indications of the relationships of these subspecies were provided by MLEE analysis. Using MLEE to measure the genetic distances between populations, it was concluded that *T. b. gambiense* behaved as a true subspecies (Tait *et al.*, 1984) while *T. b. rhodesiense* was found to be a host range variant of *T. b. brucei* (Tait *et al.*, 1985). As more molecular analyses have been carried out, however, it has become clear that the situation is not as straightforward as this. Considering *T. b. gambiense* first: more than one type of *T. b. gambiense* was identified and described variously as 'non-*gambiense*' (Tait *et al.*, 1984) or 'type 2 *gambiense*' (Gibson, 1986). The 'type 1' stocks represented the classical *T. b. gambiense* as judged by association with chronic disease and distinction by other molecular markers such as the absence of the *Antat1.1* antigen gene and RFLPs in the *Antat1.8* antigen gene. Furthermore, these 'type 1' *T. b. gambiense* stocks fall into a distinct group by virtually all genetic markers used (e.g. Gibson *et al.*, 1980; Tait *et al.*, 1984; Hide *et al.*, 1990; Mathieu-Daude *et al.*, 1994, 1995) and by differences in susceptibility to the trypanocidal drug difluoromethylornithine (DFMO) (Bacchi and Yarlett, 1993). There is little dispute that these 'type 1' *T. b. gambiense* stocks represent a separate subspecies.

The origins of the 'type 2' *T. b. gambiense* stocks are much less clear. Using RFLP analysis, these stocks group together with cattle stocks from Nigeria (Hide, *et al.*, 1990) and a limited analysis of disease profiles associated with these stocks (Hide, 1998) showed that at least some were associated with acute sleeping sickness. Thus, current evidence would suggest that these 'type 2' Gambian stocks may have an analogous relationship to *T. b. brucei* as does *T. b. rhodesiense* (i.e. a host range variant). Further work needs to be conducted on these isolates to establish their identity and, importantly for treatment, the susceptibility to DFMO should be established as it is unclear whether they have a similar susceptibility to that drug as the 'type 1' Gambian stocks.

When *T. b. rhodesiense* is considered, it is also apparent that this taxon may consist of more than one distinct group. It has long been recognized that human sleeping sickness due to *T. b. rhodesiense* in Zambia has been associated with low virulence (Rickman, 1974), which is in stark contrast to the acute disease that characterizes the Busoga focus in Uganda. Characterization of *T. b. rhodesiense* stocks from each of these

regions has been carried out using a number of molecular approaches, including RFLP (Hide *et al.*, 1991), minisatellite variant repeat mapping (MacLeod *et al.*, 2001c) and MGE-PCR (Hide and Tilley, 2001; Tilley *et al.*, 2003). These independent methods all demonstrate that the causative agent of human sleeping sickness is quite distinct in these two foci, suggesting that human infective trypanosomes in East Africa have arisen at least twice. One proposed model (Hide, 1999; Gibson, 2002) suggests that a different human-infective variant of *T. brucei* exists in each focus and that the range and transmission of that variant may determine the geographical extent of each focus. Such a model could explain the distribution of human sleeping sickness as a series of distinct foci within the tsetse fly belt where widespread animal trypanosomiasis is present. Furthermore, this model could explain the origins of the 'type 2' *T. b. gambiense* stocks and, albeit in a more distant evolutionary step, the origins of 'type 1' *T. b. gambiense*. Future work is required to characterize the human-infective isolates from other foci to establish the generality of this model.

One question generated by the concept of multiple origins of *T. b. rhodesiense* (and *T. b. gambiense*) concerns the mechanism of human infectivity. From the absence of the *SRA* gene, it is clear that the mechanism employed by *T. b. gambiense* is probably distinct from that operating in *T. b. rhodesiense*. Recent evidence, based on the presence of the *SRA* gene in *T. b. rhodesiense* stocks from throughout East Africa (Gibson *et al.*, 2002), suggests that *T. b. rhodesiense* isolates may have a similar mechanism for determining human infectivity. This poses a dilemma – there seems to be conservation of the *SRA* gene between *T. b. rhodesiense* stocks that are genetically distinct by all other markers (Gibson *et al.*, 2002). One possible explanation is that genetic exchange has introduced the *SRA* gene into populations from different genetic backgrounds (i.e. different foci). Another possibility is that the rates of mutation of the two types of markers (*SRA* versus others) are different. If mutation rates in minisatellites and RFLP markers are higher than the *SRA* gene, the trypanosome strains in different foci may have diverged (as judged by these markers) subsequent to *SRA* spread. In other words, *T. b. rhodesiense* may have a common origin but then divergence occurred subsequently in each focus. These questions still need to be resolved.

In summary, the current data generally support the concept that *T. b. gambiense* is a separate subspecies while *T. b. rhodesiense* comprises a complex of host range variants of *T. b. brucei*. These molecular approaches bring into question our current nomenclature and classification of the *T. b. rhodesiense* 'complex'.

T. evansi

Morphologically, *T. evansi* is similar to *T. brucei* and is non-cyclically transmitted by blood-sucking flies. In a number of studies of *T. brucei*, using MLEE, ribosomal genes or the repetitive probe pBE2, one or two stocks of *T. evansi* have been included and shown to be closely related but distinct from the *T. brucei* group (Gibson *et al.*, 1980; Hide *et al.*, 1991). The analyses of isoenzyme variation in *T. evansi* define a few different zymodemes within a fairly large number of isolates. The similarity with *T. brucei* has created some difficulties in defining *T. evansi* in infected cattle within the tsetse belt, but specific minicircle DNA probes have been developed that allow the species to be unambiguously identified, with an additional criterion being the lack of maxicircle DNA.

T. congolense and *T. simiae*

The salivarian trypanosomes (*T. congolense* and *T. simiae*) are major pathogens of livestock in sub-Saharan Africa and, by both classical and sequence-based (Stevens *et al.*, 1999) phylogenetic analysis, are placed in a separate subgenus to the *Trypanozoon* containing *T. brucei*. Compared with the extensive analysis of the *T. brucei* group in relation to molecular epidemiology, *T. congolense* has been relatively less studied and, as a result, a number of questions still remain to be fully answered. An extensive analysis of isoen-

zyme variation in a large number of *T. congolense* isolates (Gashumba *et al.*, 1988) led to the proposal that there were subspecific groups within the classically defined *T. congolense.* The numerical analysis of this data has led to the definition of five discrete groups of *T. congolense* isolates that show substantial differences in isoenzyme pattern. A focus of the subsequent research using a range of molecular markers (RAPDs, molecular karyotype, kDNA and repetitive sequences) has been to define subspecific groups rather than extensively analyse variation within a group. These studies have led to the isolation of specific repetitive satellite DNA probes that are able to distinguish four groups of *T. congolense* isolates: (i) savannah; (ii) forest; (iii) Kilifi; and (iv) Tsavo (for review see Tait, 2000), as well as *T. simiae*. It is not completely clear how these relate to the groupings defined by MLEE data except in the case of the forest and Kilifi groups.

T. vivax is a significant pathogen of domestic animals in sub-Saharan Africa. Because of its ability to be transmitted by biting flies other than tsetse it is now widespread in South and Central America, probably as a result of importing infected cattle from Africa. Studies on *T. vivax* have been limited by the lack of available culture techniques and the low parasitaemias observed in laboratory rodents and so the available data are derived from the analysis of relatively few isolates. MLEE analysis has shown significant differences between West and East African *T. vivax* and a small sample of South American isolates was shown to be more similar to the West African isolates. A similar conclusion can be reached from the results of DNA hybridization studies using a repetitive DNA probe isolated from a West African isolate: the probe hybridizes to both West African and Colombian isolates but does not hybridize with DNA from East African isolates. While the data suggest a distinct subdivision between East and West African isolates, with the latter being more closely related to South American isolates, it should be pointed out that the number of isolates examined is small. Additionally, specific primers have been defined that allow the PCR detection of *T. vivax.*

Genetic Exchange

One of the major controversies since the 1980s has been the clonality versus genetic exchange debate. Two apparently conflicting observations prevailed. Genetic exchange could be demonstrated in trypanosomes but considerable linkage disequilibrium in field isolates suggested that it did not occur at a high frequency in natural populations.

The first evidence for genetic exchange in trypanosomes showed, using MLEE, that genotype frequencies in *T. brucei* populations conformed to the Hardy–Weinberg equilibrium (Tait, 1980), suggesting that trypanosomes were both diploid and undergoing random mating. Furthemore, recombinant genotypes, suggestive of genetic exchange, could be detected in some trypanosome stocks (Gibson *et al.*, 1980). Jenni *et al.*, 1986) demonstrated that *T. brucei* could be crossed in the laboratory and that genetic exchange took place in the tsetse fly. Further crosses, using three stocks, demonstrated that there were no mating barriers and that segregation followed a Mendelian process. The process of genetic exchange is now well characterized and accepted (Tait and Turner, 1990; Gibson and Stevens, 1999). Studies on field isolates, after the initial observations by Tait (1980), however, showed that considerable linkage disequilibrium could be detected in trypanosome populations and this led to the notion of infrequent genetic exchange in the field, leading to the clonal theory (Tibayrenc *et al.*, 1990) which proposed that most protozoan parasites exist as clonal populations where genetic exchange is rare. Using Hardy–Weinberg analyses and measures of linkage disequilibrium, several studies in *T. brucei*, using MLEE and RAPDs, supported the clonal hypothesis (e.g. Mathieu-Daude *et al.*, 1995). Another analytical approach, allele association (Maynard Smith *et al.*, 1993; Stevens and Tibayrenc, 1995), measures the degree of coinheritance of alleles at pairs of loci in a population: a high degree of association of alleles indicates little recombination while the dissociation of alleles indicates frequent recombination. This analysis generates another category of population structure: the 'epidemic' population

structure, which refers to a situation where genetic exchange is occurring within the population but that the population does not conform to Hardy–Weinberg analysis because of the rapid expansion of a single or small number of genotypes within the population. A reanalysis of *T. brucei* MLEE data showed that the epidemic structure was applicable and therefore provided some evidence that genetic exchange was important in field isolates (Maynard Smith *et al.*, 1993; Stevens and Tibayrenc, 1995). How could these various conflicts be resolved?

One possible reason for the discrepancy could have been the existence of barriers to mating which could preclude genetic exchange between some individuals within the populations studied. Such barriers could be either geographical or host range. Many of the analyses of linkage disequilibrium relied on a 'population' of isolates pooled from data sets collected at different times and different places, thus there might not be the opportunity for random mating to occur in such a 'population' and therefore linkage disequilibrium would be detected. Evidence that this might be the case was obtained from a study carried out during a sleeping sickness epidemic in Tororo, south-east Uganda, in 1988–1990. Sympatric *T. brucei* isolates were collected from cattle, humans and tsetse and analysed by MLEE and allele association (Hide *et al.*, 1994). Using RFLP markers and sensitivity or resistance to human serum, two groups of *T. brucei* were detected – human (*T. b. rhodesiense*) and non-human (*T. b brucei*) infective isolates – and were analysed separately. Allele association showed that both populations had an index of association that was not significantly different from zero, showing that there was little association between alleles and therefore recombination was occurring. However, the human-infective isolates were characterized by a predominance of a few genotypes which placed them in the 'epidemic' population category. Thus the data from this sympatric study were consistent with the occurrence of genetic exchange in field isolates, though in the human-infective isolates this was masked by the epidemic expansion of a small number of genotypes. The implications of this study were that different population structures could exist within a single sympatric population.

This finding that different population structures exist within *T. brucei* has been confirmed for other populations (Stevens and Tibayrenc, 1995). More recently, this question has been addressed with minisatellite markers (MacLeod *et al.*, 2000), using this set of sympatric samples from the Tororo epidemic. The high levels of polymorphism shown by these markers and the ability to interpret the banding patterns in genetic terms has allowed isolates to be distinguished more readily than with MLEE. The data obtained with these markers using the isolates from the Tororo focus has also shown that there are different population structures in human and non-human isolates. However, the results differ slightly in that the non-human isolates seem to have an epidemic population structure (i.e. genetic exchange masked by the expansion of individual genotypes) while the human isolates were found to be essentially clonal. Furthermore, both of these studies show that, at least in this focus, there is little gene flow occurring between human and non-human isolates. The question of clonality seems to have been reconciled for isolates collected from this focus, but further work is required to determine whether this is the case for other disease foci.

While considerable data have been accumulated for *T. brucei*, there is a lack of extensive data in *T. congolense* and *T. vivax*. In particular, little is known about the population structures of the four subdivisions of *T. congolense* (savannah, forest, Kilifi and Tsavo) and whether there is any genetic interaction between or within the groups. Further research effort needs to be directed at these important animal pathogens.

Molecular Epidemiology

Introduction to molecular epidemiology

The epidemiology of African trypanosomes has been studied at a number of levels, including analyses of prevalence, the use of geographical information system (GIS) data and the generation of predictive models with

the overall aim of defining the key factors that determine the spread and transmission of the disease in both humans and livestock. These studies have used the established approaches of classical epidemiology and have made significant contributions towards control programmes. Since the early 1980s, scientists have used biochemical and molecular methods to characterize populations of trypanosome isolates in order to address a range of questions of relevance to the epidemiology, speciation and evolution of the African trypanosomes; this is termed molecular epidemiology. Until very recently, classical and molecular epidemiology followed separate routes but both are now coming together, although there is a substantial need to integrate these areas if the key factors that lead to outbreaks of disease are to be fully understood. Much of the molecular epidemiological analysis has been applied to the study of the speciation and population biology of human rather than animal trypanosomiases. Additionally, limited molecular research has been undertaken on the host or vector component of epidemiology, which could address a number of important questions in relation to susceptibility and resistance. This section will detail the advances made in the understanding of the molecular epidemiology of African trypanosomes, focusing specifically on the questions that have been addressed, the available approaches and an analysis of some of the outstanding issues that require analysis.

The application of molecular and biochemical markers to the analysis and definition of the genera, species and subspecies of the salivarian trypanosomes is particularly important in the investigation of their epidemiology, because of the broad host range of a number of species and the ability of the same vector species to transmit different genera and species of trypanosome. It is clear that a range of epidemiological and phylogenetic questions are raised by the biological variation observed both within and between classically defined species and these are particularly suited for analysis by molecular and biochemical tools aimed at the analysis of genotypic variation. The range of tools available has been discussed previously and each has advantages and disadvantages. However, there are no 'good' or 'bad' genetic markers, rather a set of markers that differ in their suitability for addressing particular questions (Tibayrenc, 1995).

Epidemiological questions

There are a number of different levels at which epidemiological questions have been addressed within the *Trypanosoma* and while some questions are context dependent, most are general and have been addressed in each of the genera studied. The availability of an increasing amount of genome sequence information and molecular markers, coupled with the application of polymerase chain amplification, offers a wide range of tools and methodologies for undertaking epidemiological analysis of the trypanosomiases. Potentially these technologies, coupled with the ability to detect and characterize host, vector and parasite genes from crude small volume samples collected simply on filters or other media, allow a full range of epidemiological questions to be addressed. It is clear that the power of these types of analysis remains to be fully exploited in extensive field-based studies but the reasons for this are beyond the scope of this chapter. In this context, it is important to distinguish between what questions could be addressed and those that have. Potentially the prevalence, host distribution, host genotypes, vector genotypes, blood meal analysis, species and subspecies identification, strain variation and associated interactions between these factors could be analysed. This would build up a full picture of where specific pathogens occur, the role of different host and vector species in disease transmission, the role of different genotypes (host, vector and parasite) in virulence, transmission, pathogenesis and, in the future, markers for drug resistance. However, such broad-ranging studies have not been undertaken and the primary focus of research has been on species definition, methods of field sample analysis and the population genetic analysis of primarily *T. brucei* spp. These studies form the main subject of this section.

In broad terms, the following questions have been addressed using a range of molecular and biochemical marker systems:

- Can the classically defined species and subspecies be clearly defined and do some of the species constitute one or several subspecies?
- What is the population structure and role of genetic exchange in this group of parasites?
- Are particular disease and biological phenotypes associated with specific parasite genotypes?
- Within species infecting multiple mammalian hosts, does population substructuring occur in relation to host or geography, leading to the potential for the evolution of different biological and disease characteristics?
- Can sensitive methods be developed to detect and characterize trypanosomes without the need for amplification in laboratory animals?
- Can species-specific probes be developed for application in prevalence studies?
- What is the role of different host species in the spread of disease?

These questions have been addressed primarily in relation to the human-infective species (*T. b. rhodesiense* and *T. b. gambiense*). In our view, the ultimate goal of molecular epidemiology is to be able to define the key factors that determine the spread, maintenance and infectivity/virulence of the different parasite species. There is a significant need for further research that should incorporate an integrative approach with more classical epidemiological analysis. As a detailed example of the applications of molecular epidemiology to trypanosome research, the recent human sleeping sickness epidemics in the Busoga focus of Uganda will be considered.

Busoga epidemic

Epidemics have flared up on numerous occasions in the Busoga focus (Fig. 5.4) since the Great Epidemic of 1900 (Hide, 1999). Most recently an epidemic occurred in the Tororo District in 1988 and peaked in 1990, with cases still continuing to this day. In December 1998 a case of sleeping sickness was reported in the Soroti District of Uganda, some 60 miles north of Tororo, and by June 2000 a further 118 cases were known. This area had never had a case of sleeping sickness, apart from a single report in the 1960s. One of the long-standing puzzles in sleeping sickness epidemiology is why human disease is located in specific foci and why there are long periods of endemicity (low-level disease) interspersed with occasional epidemics. These epidemics in Busoga have created an opportunity to use molecular methods to address these issues.

A large collection of *T. brucei* isolates was made during the 1988 Tororo epidemic, from humans, cattle and tsetse, and isolates were amplified in culture or in laboratory rodents. The isolates were tested for human serum resistance (a measure of human infectivity), DNA was extracted and analysed by RFLP (Hide *et al.*, 1994), minisatellites (MacLeod *et al.*, 2000, 2001a,b,c) and MGE-PCR (Hide and Tilley, 2001; Tilley *et al.*, 2003). These analyses showed that human and non-human isolates could be clearly distinguished and that the human isolates appeared to be a rather homogeneous group that was genetically distinct from the non-human isolates. Furthermore, these human isolates were genetically similar to isolates from previous epidemics, dating back as far as 1960, in the Busoga focus but genetically dissimilar to human isolates from another focus (Luangwa Valley, Zambia). This indicated that human-infective strains from different foci have distinct origins, yet are conserved within a focus. This may explain the geographical conservation of human disease foci: each focus is defined by the presence of a human-infective strain. Analysis of cattle isolates from Tororo showed that 23% of isolates were harbouring human-infective trypanosomes, indicating that cattle were a reservoir for disease in this area. Using other demographic and epidemiological data collected at the time, it was calculated that the probability of transmission of human-infective trypanosomes by the tsetse–cattle–tsetse route

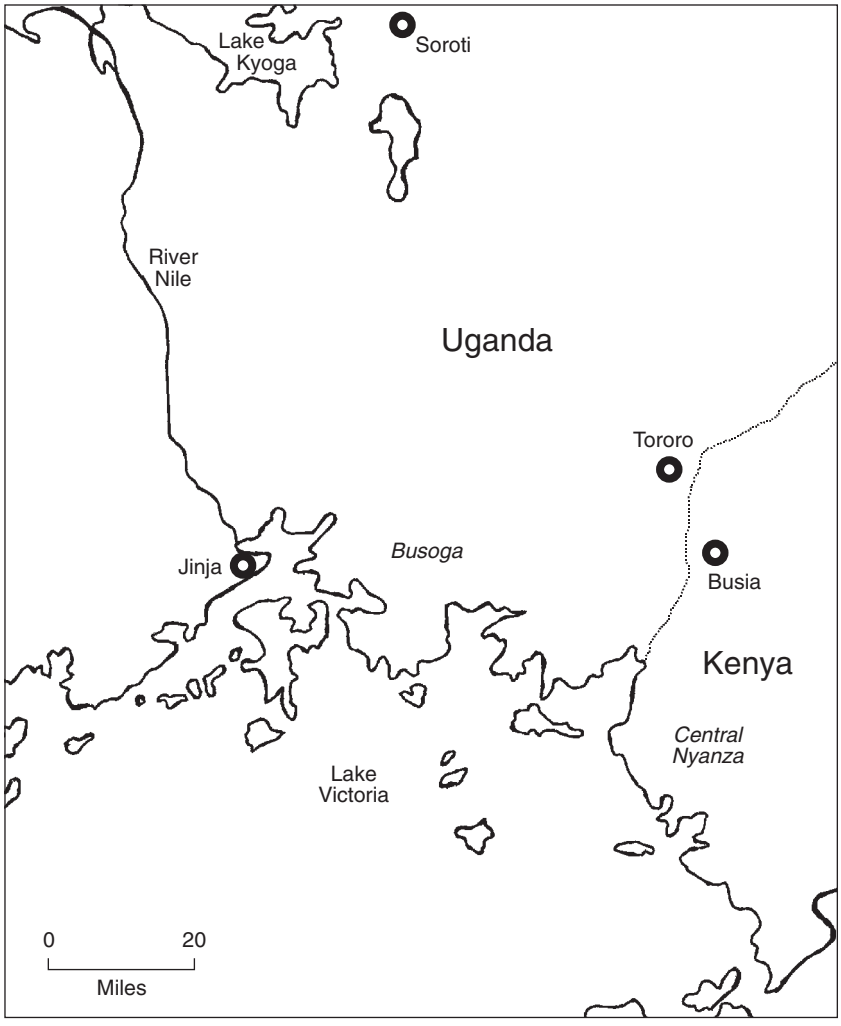

Fig. 5.4. Map of the Busoga focus of Uganda and western Kenya. This area has been an active sleeping sickness focus since the Great Epidemic of 1900 (and probably before). Recent epidemics in Tororo (1988–) and Soroti (1998–) are the subject of a number of epidemiological studies discussed in this chapter.

was five times higher than from the tsetse–human–tsetse route (Hide *et al.*, 1996). Thus cattle contributed very significantly to transmission in this epidemic and the close interaction between the local population and their livestock may explain the maintenance of human-infective trypanosome strains during the epidemic. This is supported by MGE-PCR analysis, which shows that isolates collected from Tororo in 2000 have identical genotypes to those present in the early stages of the epidemic (Tilley *et al.*, 2003).

Soroti epidemic

The Soroti epidemic of 1998 has offered some opportunities to explore the factors that cause the generation of human sleeping sickness epidemics. In Soroti, prior to 1998, there had been a major cattle restocking programme following a period of considerable social unrest. Based on the knowledge that cattle were an important reservoir for sleeping sickness, Fèvre *et al.* (2001) analysed cattle movements into and

around Soroti. They conducted spacial analysis of the timing and distribution of sleeping sickness cases with respect to cattle markets around Soroti. They concluded that Brooks Corner cattle market was the epicentre of the epidemic and proposed that sleeping sickness was imported into the region by means of cattle infected with human-infective trypanosomes from other epidemic areas (possibly Tororo). Confirmation that the cattle imported to Soroti harboured human-infective trypanosomes was achieved by the detection of the presence of the serum resistance associated (*SRA*) gene in trypanosomes isolated from these cattle (Welburn *et al.*, 2001). Thus there is compelling evidence suggesting that epidemics can be generated by the import of human-infective strains by means of cattle. Future work in this study should be aimed at using methods for tracking trypanosome isolates (minisatellites, MGE-PCR) to determine genetic identity between isolates from Soroti and those from Tororo or other possible source sites. Furthermore, this phenomenon should be investigated in other foci to determine whether this mechanism of generation of sleeping sickness epidemics is universal. Clearly, this model suggests attractive, cost-effective methods of prevention of epidemics: monitoring the movement of infected cattle and targeted treatment of high-risk animals.

Conclusions

The population biology of trypanosomes has been revolutionized since the 1980s by the advent of molecular epidemiology. While significant advances have been made in the development of molecular tools and their application to specific problems, many unanswered questions still remain. In particular, studies have been aimed at investigating specific areas or small groups of samples. Future research should be aimed at establishing the generality of these studies and considering how they might be effectively applied to disease control.

The availability of PCR-based analysis that defines the species of trypanosomes infecting livestock should allow more detailed data on distribution and prevalence to be collected. Molecular analyses have defined at least four subtypes of *T. congolense* (savannah, forest, Kilifi and Tsavo) and subtype-specific primers are available for identification. Despite these tools, there is a very fragmentary knowledge of the distribution of these subtypes, their transmission and their significance in relation to virulence and pathogenesis. There are few markers for *T. vivax* and *T. congolense* that allow the analysis of population structure and strain characterization. A similar gap in the repertoire of molecular tools for these important livestock parasites is the absence of markers for drug resistance; such markers could be very valuable in monitoring and defining the spread of drug resistance and informing control programmes of local drug susceptibility.

Current PCR-based methods for defining the major species of trypanosome, while effective and sensitive, involve multiple PCR amplifications to characterize a given blood sample. Thus there is a need for a single PCR-based method for detecting all key species, such as the reverse line blot technique developed for tick-borne cattle disease (Gubbels *et al.*, 1999). As one role of epidemiological analysis is to inform control programmes, there is also a need to develop diagnostic methods that define the range of pathogens infecting a particular host in a particular region. An example of this need would be to combine detection of tick-borne diseases and trypanosomiasis within East Africa and such tools will become even more relevant as integrated control programmes are considered. The potential to develop PCR-based systems for analysing the questions raised in this chapter is significant and, by adapting some of the technology for high throughput 'chips' developed for human genetic analysis and functional genomics, it is possible to envisage cheaper, simpler and more informative methodologies for molecular epidemiology.

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PART 2.
VECTOR BIOLOGY

6 Tsetse Genetics: Applications to Biology and Systematics

Ronald H. Gooding and Elliot S. Krafsur

Introduction

Although tsetse are difficult and expensive to culture, knowledge of their genetics is remarkably good when compared with that of most other arthropods of medical and veterinary importance. There is one comprehensive review of early work on tsetse genetics (Gooding, 1984) and several additional reviews that focus on specific aspects of tsetse genetics: hybrid sterility (Gooding, 1990a); quality control in tsetse colonies (Gooding, 1990b); tsetse–trypanosome interactions (Welburn and Maudlin, 1999) and paratransgenics for control of pathogen transmission (Beard *et al.*, 1998). Most recent papers on tsetse genetics provide reasonable coverage of relevant earlier work. Because of space constraints, we take advantage of this by citing only the most recent or comprehensive paper, leaving the reader the 'leg-work' of searching out earlier papers.

The study of tsetse genetics was facilitated by rearing centres from which puparia were distributed, at little or no cost to investigators. It is unfortunate that the Tsetse Research Laboratory at Langford, UK was closed but several species are still maintained at Centre International de Recherche-Dévelopment sur l'Elevage en Zone subhumide (CIRDES) Bobo-Diouasso, Burkina Faso, Centre de Coopération Internationale en Recherche Agronomique pour le Développement – Département d'Elevage et de Médecine Vétérinaire (CIRAD-EMVT) Montpellier, International Atomic Energy Agency (IAEA) Seibersdorf Laboratory, International Livestock Research Institute, Kenya Trypanosomiasis Research Institute, Prince Leopold Institute of Tropical Medicine (Antwerp), Tsetse and Trypanosomiasis Research Institute at Tanga, Yale School of Medicine, and University of Alberta (RHG).

Because of their low reproductive potential, tsetse are ideal targets for genetic control. Realization of this goal will be aided by knowledge of tsetse genetics but, despite recent advances, much further work is indicated, particularly in applying the techniques of molecular biology. Modern concepts of area-wide control require a more thorough understanding of population and ecological genetics than we now have. Research on the genetics of vector–parasite and tsetse–symbiont relationships is needed, particularly coadaptation of tsetse taxa and demes to trypanosome strains and to the three prokaryotic symbionts that infect some (but not all) tsetse. Tsetse are unusual among insects in that they reproduce by adenotrophic viviparity; their evolutionary history and phylogenetic relationships bear further investigation.

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This chapter reviews the major features of tsetse genetics, concentrating on the classical areas of cytogenetics, formal genetics and population genetics. It reviews current thrusts in these areas and in ongoing studies at the molecular level. It concludes by mentioning applications of genetic technology to control programmes and makes suggestions for further research. Because of space limitations and readability, only a few simple formulae are given for the genetic indices. The mathematics of population genetics can seem formidable at first and a good place to begin is Hartl and Clark (1997).

Genetic Variation

Kinds of genetic variation

Genetic variation arises from mutation and may be detected from the behavioural to the molecular level. Morphological mutants, numerous in *Drosophila*, are extremely rare in tsetse. Moreover, their usefulness is limited to laboratory work because they usually confer a loss of fitness. Chromosomal variation includes inversions and these are most readily detected by cytological examination of polytene chromosomes present in the trichogen cells of the thorax of pharate adults in puparia. Relatively few preparations give satisfactory spreads, however. Mitotic and meiotic chromosomes are also useful and more easily prepared than polytene chromosomes.

Biochemical variants, allozymes and isozymes, have been examined in representatives of all tsetse species groups (Gooding *et al.*, 1991; Gooding and Rolseth, 1992, 1995; Krafsur and Griffiths, 1997; Elsen and Roelants, 1999; Gooding and Challoner, 1999). These genomic markers offer convenient and reproducible means to study linkage, sex determination and population genetics. Their drawbacks include the need to work with fresh material or to freeze specimens quickly in order to preserve enzyme activity. Population genetics studies must assume that the genetic markers employed are selectively neutral, and evidence accumulates that balancing selection acts upon some allozyme loci (Li, 1997), including those in *Glossina pallidipes* (E.S. Krafsur, unpublished).

Selective neutrality, however, obtains for simple sequence repeats, a class of repetitive DNA also termed microsatellite DNA. Microsatellite loci offer many advantages in theory but require sophisticated and time-consuming methods of molecular biology to find and develop them for routine use. Only a fraction of such loci in tsetse flies have proved to be useful for genetic studies and further work is required.

Abundant variation occurs at mitochondrial loci in *morsitans* group (Krafsur and Wohlford, 1999; Wohlford *et al.*, 1999). Variants in mitochondrial DNA do not recombine and, with rare exceptions, are inherited matrilineally. Such diversity is particularly useful for examining maternal lineages and testing for bottlenecks in population size.

Genetic variation in tsetse flies

There is much chromosome diversity within and among tsetse species. Historically, the species have been divided into three groups: *fusca*, *palpalis* and *morsitans* (see Chapter 8). The *morsitans* group of seven species and subspecies have karyotypes of $2n = 4 + XY$ plus heterochromatic, telocentric supernumerary chromosomes that vary in number within and among the taxa (Southern, 1980). Supernumerary chromosomes (i.e. B chromosomes) occur also in at least one member of the *fusca* group, *G. brevipalpis*, which is also polymorphic for the number of chromosomes (two or three) that have rDNA loci, demonstrated by fluorescence *in situ* hybridization (Willhoeft, 1997). Paracentric inversions have been detected in tsetse polytene chromosomes (Jordan *et al.*, 1977). There is sex chromosome aneuploidy in *G. palpalis palpalis* among which phenotypic females may be XX, XXY and XXYY; phenotypic males may be XY, XO or XYY (Maudlin, 1979).

Allozyme diversity, or heterozygosity, can be expressed as the chance that a randomly chosen insect is heterozygous at an average locus. Compared with common Diptera such as house flies, *Drosophila* or stable flies, tsetse show rather low levels of diversity, about

6%, the level demonstrated by many mammals (Table 6.1). Heterozygosities at only polymorphic loci, however, are much greater at a mean of 27% in field-collected specimens of the *morsitans* group and 19.5% among colonies of *G. p. palpalis* and *G. p. gambiensis* (Table 6.2). For selectively neutral alleles, the expected heterozygosities are directly related to the effective population sizes and the prevailing mutation rate. Mutation rates are very low, approximately 10^{-6} per gamete per generation. Thus the rather low allozyme diversities in tsetse are best explained by their small population sizes when compared with many other Diptera. Nevertheless, there is sufficient allozyme diversity in tsetse to allow research on the breeding structure of natural populations.

Microsatellite loci, simple sequence repeats, are a class of repetitive DNA in which a sequence of two to five nucleotides is repeated many times. They tend to occur throughout the nuclear genome and are untranscribed, hence they are selectively neutral. Co-dominant alleles have different numbers of repeat units and may be distin-

Table 6.1. The levels of allozyme heterozygosity in natural populations of *Glossina* spp., expected on the basis of Hardy–Weinberg criteria, compared with estimates from natural populations of other Diptera. The proportions of tsetse loci polymorphic are homogeneous ($\chi^2 = 2.75$, 4 d.f., $P = 0.60$).

	Species	No. loci	Percentage polymorphic	Alleles per locus	Mean heterozygosity, H_E
House flies	*Musca domestica*	68	53.4	2.5 ± 1.9	18.3 ± 3.0
Face flies	*Musca autumnalis*	50	62.0	2.4 ± 1.6	18.6 ± 3.1
Stable flies	*Stomoxys calcitrans*	38	52.6	1.8 ± 1.0	9.6 ± 2.6
Tsetse	*G. m. morsitans*	45	20.0	1.4 ± 0.2	6.6 ± 2.8
	G. m. centralis	31	32.3	1.4 ± 0.6	6.0 ± 2.5
	G. m. submorsitans	31	21.9	1.4 ± 0.2	4.6 ± 2.4
	G. swynnertoni	34	17.6	1.2 ± 0.6	7.1 ± 3.0
	G. pallidipes	38	26.3	1.5 ± 0.9	6.8 ± 2.4

Table 6.2. Genetic statistics at polymorphic loci among various muscomorph Diptera.

Species	Number of polymorphic loci	Mean heterozygosity H_E ± SD %[a]
M. domestica	39	36.7 ± 5.7
M. autumnalis	31	30.5 ± 3.8
S. calcitrans	20	18.2 ± 4.5
Haematobia irritans	11	32.8 ± 8.2
G. m. morsitans	9	29.9 ± 8.4
G. m. centralis	10	18.7 ± 6.4
G. m. submorsitans	7	20.8 ± 8.6
G. pallidipes	10	25.4 ± 5.8
G. swynnertoni	6	40.5 ± 8.4
G. palpalis palpalis[b]	14	19.1 ± 5.8
G. palpalis gambiensis[c]	14	20.0 ± 5.6

[a] $H_E\,\% = [\Sigma(1 - \Sigma p_i^2)/n] \times 100$, where p_i is the frequency of allele i at a locus and n is the number of loci. SD = $\Sigma[(H_E - h_e)^2]/[n(n - 1)]$.

[b] From two laboratory cultures (Elsen and Roelants, 1999).

[c] From three laboratory cultures (Elsen and Roelants, 1999).

guished by size, after amplification by the polymerase chain reaction. Amplification requires primers that anneal to conserved regions flanking the repeated sequence. Acrylamide electrophoresis will separate the alleles. Microsatellites have been isolated in the *palpalis* (Solano *et al.*, 1999; Liangbiao Zheng, 2000, personal communication) and *morsitans* groups (M.D. Baker and E.S. Krafsur, unpublished data).

Formal Genetics

Linkage groups

A linkage group is a group of genes that are known to be linked, i.e. they are located on the same chromosome. Although cytological studies have not been carried out on all tsetse species, our present understanding is that members of the *fusca* group have the largest number of chromosomes ($2n$ = *c.* 12–22, including six to 16 B chromosomes), whilst members of the *morsitans* group have two pairs of autosomes, a pair of sex chromosomes and from zero to 12 B chromosomes; members of the *palpalis* group have only two pairs of autosomes and a pair of sex chromosomes. Females are normally XX and males XY, in those taxa in which sex chromosomes have been identified. The loci for biochemical markers can be assigned to autosomes in those cases in which heterozygous males are found, or to the X chromosome in cases in which there are heterozygous females, but not males (Gooding, 1984).

Linkage maps have been established for only three taxa: *G. morsitans morsitans* (16 loci, Gooding and Rolseth, 1992), *G. m. submorsitans* (eight loci, Gooding and Challoner, 1999) and *G. p. palpalis* (eight loci, Gooding and Rolseth, 1995). During this work it was established that intrachromosomal recombination occurs at a very low rate in, or is absent from, males. Linkage among those biochemical loci common to all species suggest that the linkage groups within the *morsitans* and *palpalis* groups have been conserved during evolution of these taxa, but that the linkage groups in tsetse differ from those in the other higher flies (Gooding and Rolseth, 1995). A composite linkage map for the *palpalis* and *morsitans* groups is provided in Fig. 6.1. One complication not depicted in Fig. 6.1 is that within the *G. m. submorsitans* colonies (at least those in Gooding's laboratory) there are two types of X chromosomes in which there is a segment of at least 24 map units (i.e. 24% recombination during meiosis) in which there is no recombination between the two types of X chromosome. This non-recombining section carries four loci: *Pgm* (phosphoglucomutase), *wht* (locus for the white eye mutation), *Est-X* (a thoracic esterase) and *Sr* (sex ratio distortion locus) (Gooding and Challoner, 1999). The lack of recombination may be due to an inversion or to a general suppression of recombination in females that are heterozygous for the two types of X chromosomes.

Unfortunately the linkage groups described above have not been associated with polytene chromosome banding patterns (Southern and Pell, 1974), C-banding patterns (Southern, 1980; Willhoeft, 1997), or the locations of rDNA loci, deduced from fluorescence *in situ* hybridization (Willhoeft, 1997).

Sex determination

Although it is well established that males are the heterogametic sex, at least in the *morsitans* and *palpalis* groups, details of the sex determination mechanism are not known. Two types of visible abnormalities, found in *G. morsitans*, suggest that sex determination is based upon the relative numbers of autosomes and X chromosomes, within each cell, as in *Drosophila*. These abnormalities are gynandromorphs (individuals in which one side of the body is male and the other is female) and mosaics (females in which the apical scutellar bristle is very long, as in males, rather than short). The number of Y chromosomes has no effect upon the sex phenotype: females may be XX, XXY or XXXY and males may be XY, XYY or XO; the Y chromosome is apparently required only for production of motile sperm (Southern, 1980). XO males have normal meiosis but do not produce functional sperm, because

Fig. 6.1. A composite linkage map for *morsitans* and *palpalis* group tsetse. Loci are: *Alkph*, midgut alkaline phosphatase; *alpha-Gpd*, α-glycerolphosphate dehydrogenase; *Ao*, aldehyde oxidase; *Apk*, arginine phosphokinase; *brk*, brick eye colour; *Est-1*, a thoracic esterase; *Est-2*, a thoracic esterase; *Est-t*, a testicular esterase; *G6pd*, glucose-6-phosphate dehydrogenase; *Hex*, hexokinase; *oc*, ocra body colour; *Mdh*, malate dehydrogenase; *Odh*, octanol dehydrogenase; *Pgi*, phosphoglucoisomerase; *Pgm*, phosphoglucomutase; *sabr*, scutellar apical bristle length; *sal*, salmon-colour eyes; *tan*, tan-colour eyes; *To*, tetrazolium oxidase (= superoxide dismutase); *wht*, white eyes; *Xo*, xanthine oxidase. Taxa abbreviations: (m), *G.m.morsitans;* (p), *G. p. palpalis;* (s), *G. m. submorsitans*. Map based upon Gooding and Rolseth (1992, 1995) and Gooding and Challoner (1999).

their mitochondria derivatives degenerate, thus depriving sperm of their energy-producing organelle.

Sex ratio distortion occurs in *G. m. submorsitans* from Nigeria and Burkina Faso, and perhaps elsewhere in west Africa. The gene for sex ratio, *Sr*, is located on the X chromosome and is expressed in males: distorter males sire very few male offspring (Gooding and Challoner, 1999); those that are produced are almost invariably sterile. To date, strains of tsetse in which there is a genetically determined preponderance of males have not been established. There is a paucity of information on the molecular genetics of sex determination in tsetse. There is, however, evidence that dosage compensation occurs among *morsitans* and *palpalis* groups (Gooding and McIntyre, 1998). No work has been done on tsetse to identify the mechanism of dosage compensation: inactivation of genes on one X in females; down-regulation of transcription of both X chromosomes in females; or doubling the rate of transcription of X chromosome genes in males. Since the latter is used in *Drosophila melanogaster* (Mukherjee and Beerman, 1965), it is the most likely candidate.

Population Genetics

Objectives of population genetics

The objective of population genetic research is to obtain a 'snapshot' of the breeding structure of populations. The Hardy–Weinberg rule states that gene frequencies will be homogeneous among sampled populations if matings are random, the genetic variation examined selectively neutral, the mutation rate negligible, and the sampled populations large in numbers. Careful choice of variation can assure that selective neutrality and mutation rates are generally negligible and may safely be ignored. Most deviations from these initial assumptions are caused by departures from random matings within and among populations and population sizes that have not been very large. Thus, we can test hypotheses about gene flow and population sizes by sampling a series of populations and estimating the amounts and spatial components of gene diversity. Genetic distances between populations can be estimated by several measures. All measures rely on the assumption of a common ancestor; individuals that share most of their variation are considered to be more closely related than individuals that share less variation. The concept of shared variation can be extended to higher levels of hierarchy, as between populations, populations in regions, and so forth.

Gene flow

With no barriers to gene flow – that is, with free exchange of reproducing flies and random mating – gene frequencies in subpopulations will approach homogeneity and variance in gene frequencies will be small. Variance in gene frequencies will increase with greater divergence in gene frequencies. The standardized variance in gene frequencies among subpopulations is termed F_{ST} and this statistic is also defined as the correlation between two randomly chosen gametes in a subpopulation, relative to those in the population as a whole. F_{ST} is the departure from random mating among subpopulations. Departures from random mating within subpopulations is F_{IS} and this statistic will normally be very close to zero. F_{ST} can be related to migration and dispersal by various theoretical models. According to Wright's island model, the number of breeding migrants, N_em, in a generation can be related to F_{ST}. The relationship is $F_{ST} = (1 + 4N_em)^{-1}$. Thus N_em is the number of migrants exchanged among subpopulations that would give the observed value of F_{ST}. In principle, numerically little gene flow among populations will prevent genetic differentiation by drift. Moreover, the amount of gene flow is virtually independent of the sizes of the populations and thus the 'critical' level of gene flow is about one reproducing fly per generation. Below this number genetic differentiation will increase by random drift to fixation and above it further differentiation, as a result of drift alone, will not occur (Wright, 1978).

Until recently, little work had been done on gene flow in tsetse, but substantial ecological work with the *morsitans* group suggests a great capacity for dispersal and mixing of populations (Rogers, 1977). It was therefore surprising that the spatial patterns of allozyme diversities in 11 *G. pallidipes* populations from Kenya, Zimbabwe and Mozambique indicated a high level of population structuring (mean F_{ST} = 0.25) and correspondingly low average rate of gene flow (N_em = 0.75) among the populations sampled. Moreover, mating was random within populations (Krafsur *et al.*, 1997). The F_{ST} estimate of 0.25 was supported by further allozyme work on Kenyan and Ethiopian *G. pallidipes* samples and by diversities at microsatellite loci (Krafsur, unpublished data).

Mitochondrial variation was studied in *G. pallidipes* and *G. morsitans*. Diversities (the probability that two randomly chosen tsetse have *different* haplotypes) were substantial in each taxon (Table 6.3). The proportion of unique haplotypes varied from zero in *G. m. centralis* to 5% in *G. m. morsitans*. The most frequent haplotype varied from 26% in *G. m. morsitans* to 70% in *G. m. submorsitans*. *G. m. centralis* in Namibia and Botswana showed very little diversity (heterozygosity h_e = 0.03), suggestive of an earlier stringent bottleneck in population densities. An index of 0.67 was obtained in samples from Zambia. *G. m. submorsitans* in The Gambia showed h_e = 0.27, much less than populations sampled in Ethiopia (h_e = 0.84). Estimates of gene flow for these taxa are given in Table 6.3.

The picture of genetic differentiation in *G. pallidipes* afforded by allozyme loci was confirmed with mitochondrial loci in 18 populations from Ethiopia, Kenya, Zambia, Zimbabwe and Mozambique. Large differences were observed in haplotype diversities in *G. pallidipes* from East Africa and southern Africa (Krafsur and Wohlford, 1999). Mean diversity in the East African populations was 0.55 ± 0.25 whereas that in the southern African populations was 0.15 ± 0.19. When the population from Mozambique was dropped from consideration, diversity became only 0.09 ± 0.13, averaged over the Zambia and Zimbabwe samples. These data are consistent with an earlier catastrophic diminution in southern African *G. pallidipes* populations.

The north–south contrast in *G. pallidipes*, however, was very different when allozyme loci were considered. Allozyme diversity among southern populations was 0.21 ± 0.08 compared with 0.16 ± 0.08 in the northern. This is strong empirical evidence for balancing selection that favours heterozygotes. This cannot occur with mitochondrial loci because mitochondrial variation is selectively neutral, single copy and maternally inherited. Mitochondrial alleles are therefore more subject to loss than are alleles at diploid loci when populations undergo reductions in size.

The bottlenecks detected in southern *G. m. centralis* and *G. pallidipes* populations are consistent with the historical record that indicates a great loss of mammalian hosts to a rinderpest epizootic that began in 1887

Table 6.3. Mitochondrial variation and gene flow in *morsitans* group tsetse flies.

Taxon	No. populations	No. haplotypes	Diversity		Equivalent no. migrants (N_em)
			H_T	F_{ST}	
G. m. morsitans	6	23	0.630	0.088	5.2
G. m. centralis	7	7	0.543	0.866	0.1
G. m. submorsitans	7	26	0.895	0.348	0.9
G. pallidipes	20	26	0.630	0.485	0.5

H_T is the probability that two randomly chosen flies have different haplotypes.
F_{ST} is the correlation of haplotypes in populations relative to the total. It measures departures from a random distribution of haplotypes among populations.
$N_em \approx (1 - F_{ST})/(2F_{ST})$

(Ford, 1971). Data on tsetse genetic variation would suggest that mammalian mortality was less extensive in northern equatorial Africa than in the south.

Significant genetic variation in tsetse occurs also in western Africa: two X-linked microsatellite loci in *G. p. gambiensis*, sampled in Senegal and Burkina Faso, indicated genetic differentiation on macro- and micro-geographic scales, i.e. significant departures from random mating at all levels of hierarchy (Solano *et al.*, 1999).

Work needed on population genetics

The foregoing genetic evidence suggests that many *morsitans* group and *palpalis* group populations are strongly localized. How contradictory are estimates of gene flow and the substantial ecological evidence that tsetse flies, particularly the savannah group, are highly vagile? An estimated root mean square displacement of 200 m/day suggests a rate of advance of about 7 km/year and there is good experimental and empirical evidence to confirm the latter figure (Williams *et al.*, 1992). Estimates of N_em do not contradict the ecological estimates, because the two indices measure different things. N_em is a number that satisfies an estimated level of F_{ST} among idealized populations of equal and constant size. Of course, tsetse populations are not equal and constant in size. Moreover, N_em is a non-linear function of F_{ST} and so its average value can be quite misleading if gene flow differs greatly among the various populations as, indeed, it does.

If N_em cannot legitimately be used as an instantaneous measure of dispersal, what does it really tell us? Genetic differentiation at selectively neutral loci (such as many allozyme and probably all microsatellite and mitochondrial variants) occurs largely because genetic drift within populations is greater than gene flow among them. The effects are cumulative over time. Dispersal without a high rate of per capita reproduction will have no detectable genetic effects and the genetic differentiation of populations will be unimpeded. Thus the seeming disparity between high ecologically measured rates of dispersion and indirect measures based on gene frequencies can be the result of dispersion without reproduction. Reproductive failure of dispersed flies may have many causes, one of which may be natural selection in which immigrants are at a selective disadvantage.

Clearly, further research into gene flow among tsetse populations is indicated. In particular, we need to learn in some detail the relative strengths of selection, drift and dispersal in establishing the gene frequency patterns observed among tsetse populations.

Systematics

Systematic position of tsetse

The systematic position of tsetse and their close relatives in the higher Diptera is summarized as follows (based upon McAlpine, 1989):

Order Diptera:

- Suborder Brachycera:
  - Infraorder Muscomorpha (= Cyclorrhapha):
    - Section Schizophora:
      - Subsection Calyptratae (three superfamilies as listed below):
        - Superfamily Hippoboscoidea (Glossinidae, Hippoboscidae, Strebleidae, Nycterbiidae.)
        - Superfamily Muscoidea (several families, including the Muscidae)
        - Superfamily Oestroidea (several families, including Calliphoridae, Sarcophagidae and Oestridae, in which McAlpine includes the Cuterebridae, Gasterophilidae and Hypodermatidae).

The above placement of Glossinidae is based mainly upon an extensive study of the morphology of higher flies (McAlpine, 1989) and is substantially supported by DNA sequence data for the 28S rRNA gene (Vossbrinck and Friedman, 1989). We consider this placement of tsetse to be more reasonable than alternative placements summarized by Jordan (1993).

Relationships among *Glossina* species

Keys for identification of tsetse have been published by Potts (1970, 1973) and Jordan (1993). These authors and Dias (1987) present information on the systematics of tsetse, based largely upon morphological, ecological and zoogeographical data. Most tsetse workers accept that, on the basis of genital structure, tsetse may be divided into three species groups (or subgenera): *fusca* group (*Austenina* Townsend), *palpalis* group (*Nemorhina* Robineau-Desvoidy) and *morsitans* group (*Glossina* sensu stricto Wiedemann). *Austenina* males have sharply pointed superior claspers that are not joined medially by a membrane; females have five genital plates (sclerites). *Nemorhina* males have sharply pointed superior claspers that are joined medially by a membrane; females have six genital plates. *Glossina* s. str. males have superior claspers that are rather blunt, fused distally and joined medially in the proximal regions by a membrane; females have two genital plates (a medio-sternal plate and the fused anal plates). In general this arrangement of *Glossina* spp. is supported by ecological data (discussed in Chapters 7 and 8), but *G. austeni* has often been referred to as an aberrant member of the *morsitans* group for several reasons: male genitalia are typical of the subgenus *Glossina* s. str., female genitalia resemble those of *Austenina*, and its habitat preferences and some external structures are similar to those of the *Nemorhina* (Dias, 1987). This unusual combination of characters led to erection of the subgenus *Machadomyia* Dias. Genetic data generally support a classification in which there are four subgenera (Gooding *et al.*, 1991; Chen *et al.*, 1999).

Potts (1970) and Jordan (1993) summarized the proposed evolutionary relationships among the subgenera of *Glossina*. The arguments revolve around the assumptions about the habitat in which the ancestors of tsetse lived (lowland rainforest or savannah), and whether complexity of genital structures is an indication of advanced evolution or of an ancient lineage in which complex structures have accumulated. Potts (1970) and Jordan (1993) outlined the groupings for some *Glossina* species, but no phylogenetic tree encompassing all tsetse taxa has been published. Figure 6.2 partially redresses this but the relationships shown are tentative and raise questions that are discussed below. *Austenina* is considered the oldest subgenus because the greatest genetic divergence between sister taxa occurs in it (Gooding *et al.*, 1991), because molecular genetics data show the greatest divergence between members of this subgenus and other tsetse (Chen *et al.*, 1999) and because interspecific copulation does not occur in this subgenus, but hybridization occurs within *Nemorhina* and *Glossina* s. str. and mating attempts occur between *Nemorhina* and *Glossina* s. str. species (summarized by Gooding, 1990a).

Markers shared among species

Almost all of an organism's features (morphological, physiological, behavioural and biochemical) have a genetic basis, but only variation that is genetically based is useful for systematic studies. Tsetse have a remarkably uniform body plan and this ought to make it easy to use structural features to define the relationships among tsetse species and subspecies. Unfortunately, there is no universally accepted sister family for tsetse that can be used to define the ancestral or primitive characters in tsetse and thus define the point from which other character states have been derived. This problem is at least partially responsible for the divergent views on the question of which tsetse subgenus is most like the hypothetical ancestor.

Genetic information provides a useful supplement for understanding the evolutionary relationships among organisms. The central dogma of modern biology is that the information defining a species is stored as DNA and that the information flow is from DNA to RNA and then to proteins (and thus other characters of the organism). There are, therefore, three levels at which genetic information may be obtained about an organism (and the taxon or population that it represents): DNA, RNA and protein. The problems are to select appropriate levels of information, and appropriate methods to obtain and

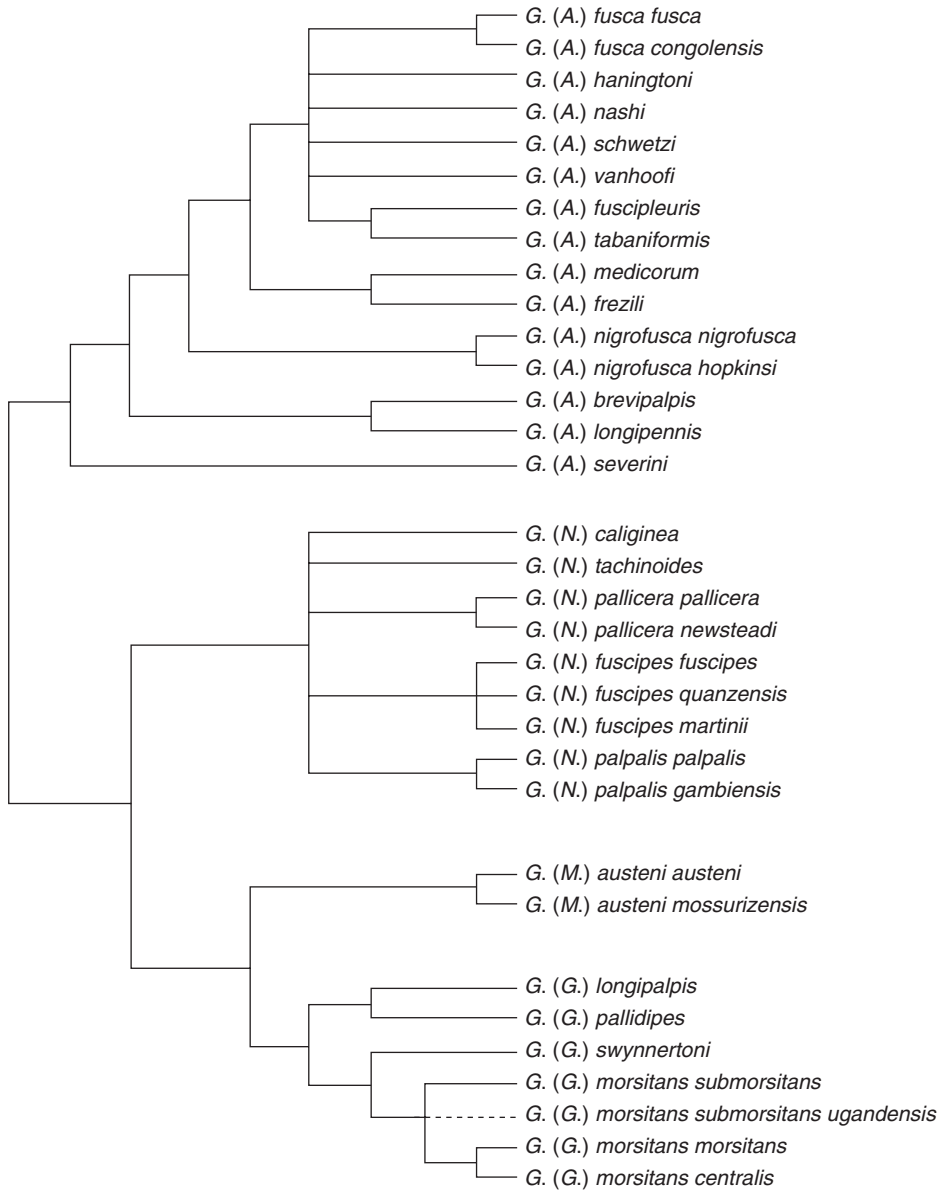

Fig. 6.2. Hypothesized relationships among species and subspecies of tsetse, based upon Potts (1970), Dias (1987) and Jordan (1993) and modified by chemical (Carlson and Sutton, 1999), genetic (Cross and Dover, 1987; Gooding *et al.*, 1991) and hybridization data (Gooding, 1990a, 1993, 1997). The status of *G. m. s. ugandensis* is problematical; it may be a geographical variant or it may warrant subspecies status, as discussed in the text. Subgenera are: (*A.*) *Austenina* Townsend; (*G.*) *Glossina* s. str. Wiedemann; (*M.*) *Machadomyia* Dias; (*N.*) *Nemorhina* Robineau-Desvoidy.

to analyse data. The solutions to these problems are not trivial and ultimately depend upon the questions being asked and the known or hypothesized differences between the populations and taxa being compared. (Unfortunately, financial and other mundane considerations often limit the choices.) For higher levels of phylogeny (such as the ques-

tion of where to place the family Glossinidae within the order Diptera), the most useful are slowly evolving (conserved) sequences, with relatively low levels of variation; for population and subspecific levels, rapidly evolving genes in which there are significant amounts of variation are appropriate. Some nuclear DNA sequences (e.g. rDNA, the ribosomal DNA repeat unit) combine these features and have been particularly useful in systematic studies ranging from higher-level classification to recognition of cryptic species (Hoy, 1994; Caterino *et al.*, 2000).

Low molecular weight biochemicals, which are generally the products of a series of enzymatic processes and which may have more than one biosynthetic or accumulation route, provide information that is usually too far removed from the genome to be useful for systematic studies. Other unsuitable characters include those that yield different phylogenetic groupings when analyses are performed on different stages or sexes of the same species. For these reasons, cuticular hydrocarbons contribute little to the understanding of the evolutionary relationships among tsetse, although they raise questions about relationships among populations within at least one taxon, *G. f. fuscipes* (Carlson and Sutton, 1999).

Genetic information that is applicable to tsetse systematics has been obtained using allozymes (Gooding *et al.*, 1991; Krafsur and Griffiths, 1997; Krafsur *et al.*, 1997), microsatellites (Solano *et al.*, 1999) and rDNA sequences (Cross and Dover, 1987; Vossbrinck and Friedman, 1989; Chen *et al.*, 1999). Summaries of molecular techniques, their biological foundations, their appropriateness for addressing systematics problems ranging from mating systems to higher level phylogenies, and methods of analysis are given in Hoy (1994) and Caterino *et al.* (2000). In addition to answering questions of interest to those working with tsetse, studies of tsetse systematics may be of interest to a broader audience, if the data can be readily integrated into existing information about other animals. DNA sequences for four loci (*cytochrome oxidase I* and *16S rDNA* in mtDNA, and *18S rDNA* and *elongation factor-1 alpha* in nuclear DNA) have been successfully and widely used in insect systematics and Caterino *et al.* (2000) argued that insect systematics would be best served if these loci were emphasized in future systematics studies. Other candidate loci, and the taxa in which they have been studied, were summarized by Caterino *et al.* (2000); those studied in dipterans may be useful in elucidating phylogenetic relationships among tsetse.

Further work necessary

Hypothesized relationships among tsetse species (see Fig. 6.2) reveal several polychotomies, i.e. points at which several taxa arise, and these polychotomies reveal a need for a thorough systematic revision of *Glossina*. The revision should include information on tsetse genetics, as well as morphological and ecological information. The most reliable genetic information will be molecular (DNA sequence) data.

In addition to the polychotomies (Fig. 6.2), there are several other phylogenetic questions that need to be resolved. A longstanding controversy has been the question of which is the oldest species group, *fusca* or *morsitans*. The arguments centre upon assumptions made about the tsetse ancestor and the significance of complex genitalia. Genetic information provides a more objective approach to solving this problem since genetic diversity is more readily quantified and analysable than morphological, behavioural or ecological characters. With a genetic approach based upon many independent loci, it may be hypothesized that the species group in which the genetically most divergent taxa occur is likely to be the oldest group. This argument has been used (Gooding *et al.*, 1991) to suggest that the *fusca* group is the oldest group, but the data are limited. Recent data provided by maximum parsimony analysis of the ribosomal DNA internal transcribed spacer-2 (ITS-2) regions indicate that the *fusca* group is the most ancient, and that *G. austeni* is the sister clade of the *morsitans* group (Chen *et al.*, 1999). Although the genetic evidence seems convincing, it relies upon few taxa within

the *fusca* group. To resolve the problem, significantly more taxa must be examined.

The taxonomic status of four taxa has been questioned on several occasions. Is *G. pallidipes* a subspecies of *G. longipalpis*? Is *G. austeni mossurizensis* a valid subspecies? Is the east African form of *G. m. submorsitans* a valid subspecies, *G. m. ugandensis*, or just a variant, *G. m. submorsitans* var. *ugandensis*? Is *G. swynnertoni* a subspecies of *G. morsitans*? All of these questions are amenable to genetic and hybridization studies. In the absence of such studies, the first two questions revolve around the significance of what appear to be relatively minor morphological and ecological differences between putative taxa, and it is hard to reconcile accepting these differences as indicative of species status for one combination but not supporting subspecific status in the other. The question about the status of *'ugandensis'* is strengthened because of different sex ratios in the eastern and western populations of *G. m. submorsitans*, and differences in the hybridization characteristics of these populations (Gooding, 1993; Gooding and Challoner, 1999), yet examination of mitochondrial variation in *G. m. submorsitans* from The Gambia and from Ethiopia indicated a fairly low level of divergence between populations (Krafsur *et al.*, 2003). Sex ratio distortion appears to be due to a single locus or block of loci, and hybrid sterility may be due to only a few loci. How much weight are these loci and mitochondrial loci to be given in defining taxonomic status? The status of *G. swynnertoni* has been re-examined recently. Analyses of cuticular hydrocarbons from both males and females place *G. swynnertoni* as a subspecies of *G. morsitans* sensu lato (Carlson and Sutton, 1999). Similarly, allele frequencies at ten biochemical loci place *G. swynnertoni* within the cluster of *G. morsitans* subspecies (Gooding *et al.*, 1991). However, the small number of loci used may have produced an inaccurate phenogram, since *G. pallidipes* was also placed within the *G. morsitans* subspecies cluster, a placement that is not supported by hybridization patterns (Gooding, 1990a, 1997). The above questions raise the fundamental question of what the criteria are for valid taxa of tsetse. Are subspecies and species to be defined on the strength of their reproductive (i.e. genetic) isolation under natural conditions, or by geographical isolation and morphological differentiation? These questions must be addressed in a rigorous manner in any systematic revision of tsetse.

Finally, there is the question of whether there are cryptic species of tsetse. To answer this question, extensive sampling over wide geographical areas will be required. To date, this has not been done and most studies are unreplicated comparisons of a few populations. Hybridization of nominally conspecific tsetse from a few widely separated geographical localities has not revealed cryptic species. None the less, comparisons of geographically distant populations within the same nominal taxon have revealed significant differences: colonies derived from a northern and from a southern sample of *G. m. morsitans* differ by an X chromosome inversion and some bionomic traits (Jordan *et al.*, 1977); a *G. pallidipes* colony from Uganda and a colony from Zimbabwe differ in bionomic traits but are interfertile (Langley *et al.*, 1984), yet there is restricted gene flow, as indicated by allozyme and mitochondrial genetic markers, between *G. pallidipes* populations (Krafsur *et al.*, 1997; Krafsur and Wohlford, 1999); East African and West African populations of *G. f. fuscipes* differ sufficiently to make this subspecies appear to be paraphyletic in phenograms based upon cuticular hydrocarbons (Carlson and Sutton, 1999); and colonies of *G. m. submorsitans* from West Africa have different sex ratio and hybridization patterns than do the East African form (*G. m. submorsitans* var. *ugandensis*) (Gooding, 1990a, 1993). Overall, these reports indicate that considerable genetic variation exists throughout a species' range and that a systematic search for cryptic species may be warranted, particularly in species that exist in discontinuous populations over a wide geographical range (see Chapter 8).

When considering the contributions of genetic and hybridization studies to the understanding of tsetse systematics it must be borne in mind that relatively few tsetse colonies have been established, and that these colonies originate from very few localities. In some cases relatively small samples

were used to establish the colonies, and colonies may have subsequently differentiated further from the field population by drift and (or) selection. However, there is little empirical evidence that colonies of even modest size are significantly different from the natural populations from which they were derived (Gooding, 1990b; Krafsur and Wohlford, 1999). This may be the result of breeding regimes used to maintain tsetse colonies. Hopefully, in the future, more extensive sampling of the genomes of natural populations will contribute more to the knowledge of tsetse systematics.

Genetic Methods of Control

Sterile males as control agents

The most promising methods for genetic control of tsetse are introduction of sterile males into a population and use of semi-sterility that arises from certain chromosome rearrangements. The object of the sterile insect technique (SIT) is to reduce the fertility of target populations to the extent that they cannot replace themselves, i.e. 'birth control'. The object of using semi-sterile mutants is twofold: to introduce desirable genes into natural populations while simultaneously inducing a high degree of sterility. The release of radiosterilized tsetse is the subject of Chapter 32. We deal here with analogous methods.

Vanderplank (1947) suggested the use of sterile hybrids that obtain when reciprocally crossing two related species or subspecies. Hybrid inviability or sterility commonly result when crossing closely related taxa and is most clearly expressed in the heterogametic sex (i.e. the sex with an X and a Y chromosome: females in birds and Lepidoptera, males in most other taxa). Such hybrid sterility or inviability characterizes speciation in animals and is known as Haldane's Rule. The underlying reasons why the heterogametic sex is most strongly affected are subtle and complex and were recently reviewed by Orr (1997). Whatever the molecular basis, sterility among tsetse hybrid males is most often associated with the presence of sex chromosomes from two taxa; occasionally autosomes are involved and maternally inherited factors (probably *Wolbachia*) may be involved in hybrid male sterility in the *morsitans* group (Gooding, 1990a, 1993, 1997).

Vanderplank (1947) described an experiment in which he eliminated *G. swynnertoni* in a region of Tanzania that had experienced a human trypanosomiasis epidemic. *G. swynnertoni* is allopatric to the closely related *G. morsitans*, in that area. Vanderplank released field-collected *G. m. centralis* puparia into an isolated *G. swynnertoni* population. Hybrid females are semi-sterile and the males are completely so. Presumably a large genetic load had been introduced into the *G. swynnertoni* population and it greatly declined. At the end of the trial, *G. swynnertoni* had been replaced by *G. m. centralis*, but *G. m. centralis* could not survive long in the region's aridity. The region then became essentially tsetse free and was reinhabited by the people who had been driven out by the earlier trypanosomiasis epidemic. This was the first field trial of a genetic control method, preceding by about 7 years the first practical work on screwworm flies.

Possibility of non-vector tsetse

Vanderplank's success notwithstanding, hybrid sterility has not played a role in tsetse control, and the only genetic control method in use is release of radiosterilized males (see Chapter 32). There are, however, more conceptually sophisticated genetic tools for tsetse control than sterile male release. Reciprocal interchanges ('translocations') between non-homologous chromosomes cause semi-sterility in heterozygotes. About one-half of the normal egg hatch is observed in simple interchanges between only two linkage groups. This is because heterozygotes produce duplication-deficiency gametes. One-half of the progeny of a heterozygous $\times$ wild-type arrangement or homozygous rearrangement are themselves interchange heterozygotes. Thus, semi-sterility is heritable. Higher levels of sterility obtain where more than two linkage groups are involved. Interchange homozygotes, on

the other hand, should be normally fertile. Curtis (1968) suggested that the release of strains of tsetse made homozygous for one or more interchanges could be used to induce high levels of sterility in target populations. Because interchange heterozygotes are less fertile than either homozygous arrangement (karyotype), an unstable equilibrium will obtain between the two, leading to a frequency-dependent increase in the favoured karyotype. Genetic recombination between karyotypes is reduced and thus Curtis (1968) suggested that interchange homozygotes could be used as a transport mechanism to drive and fix desirable genotypes into a target population of tsetse. Such 'desirable' genes might include insecticide susceptibility, conditional lethal traits, and refractoriness to trypanosome infection or maturation. Numerous chromosome interchanges in *G. austeni* were produced but no viable homozygotes were obtained and it seems that the majority of radiation-induced rearrangements are lethal or semi-lethal when homozygous.

Curtis and Adak (1974) suggested the use of cytoplasmic incompatibility (CI) to enhance SIT and the use of CI as a mechanism to drive desirable genotypes into a target population. CI is the sterility caused by *Wolbachia* bacteria – intracellular parasites that typically reside in the ovaries, testes and other tissues of infected insects. The organisms are maternally transmitted. Infected females usually are normally fertile and fecund, but uninfected females inseminated by an infected male will produce eggs that do not undergo embryogenesis. This asymmetry in fertility will result in the proportion of *Wolbachia*-infected insects increasing in the population, if the fidelity of vertical transmission and strength of the CI are adequate. Therefore, a novel *Wolbachia*-infected strain carrying a maternally inherited factor such as an anti-parasitic gene could be 'driven' into a natural population, diminishing greatly its vector potential. Such a possibility is afforded by engineering certain gut symbionts characteristic of tsetse (Beard *et al.*, 1993). *Wolbachia* has been detected in *G. swynnertoni*, *G. brevipalpis* and *G. austeni*, but not in *G. longipennis*, *G. pallidipes*, *G. fuscipes* or *G. tachinoides* (Cheng *et al.*, 2000).

Population genetics and the 'reinvasion' problem

Assuming that mass-produced, genetically modified flies are compatible and competitive with those in the target populations, the chief technical problems in achieving tsetse eradication, by using a genetic control method, would seem to be producing enough tsetse for release against natural populations, and estimating accurately the likelihoods and rates of recolonization by tsetse from unchallenged populations. The subject of tsetse dispersal has a rich literature of observational, experimental and analytical components. Random diffusion models (Rogers, 1977; Williams *et al.*, 1992) indicate that movement of savannah and forest tsetse fly populations may advance at a mean rate of approximately 7 km/year, a value consistent with root mean square displacement rates derived from a large number of independent studies in the past 70 years. Reinvasion of areas from which tsetse have been eradicated is therefore a most serious prospect. Indeed, it may be amplified by density-dependent factors (Rogers and Randolph, 1985). Inferences based on gene flow estimates in *G. pallidipes*, *G. morsitans* and *G. palpalis* suggest, however, that many tsetse populations are fairly localized. This localization could, in principle, be explained by demographic histories and by genetically based adaptation to local conditions. If natural selection were to account in large measure for the low rates of gene flow, the question of reinvasion may be less acute than ecological studies indicate. Additional genetic research is needed. In any case, it is necessary for genetic control advocates and their customers to decide how much time a treated region should remain free of tsetse to justify application of genetic control measures.

Summary

This chapter began with the claim that the field of tsetse genetics compares favourably with that of most other arthropods of medical and veterinary importance. No attempt was made to document this claim, but rather the chapter concentrated on provid-

ing information on the current state of tsetse genetics and its contribution to knowledge of tsetse systematics and biology, and its potential to contribute to these fields. Questions that need to be addressed in the near future were also indicated. Many of these will be addressed best through molecular techniques but other approaches should not be ignored. Because of their relationships with other organisms (particularly pathogenic trypanosomes and three species of symbiotic prokaryotes) and because of their unusual reproductive biology (adenotrophic viviparity), tsetse offer unique opportunities to study evolutionary biology through studies of the genetics of intraspecific interactions and of transgeneration interactions that affect fitness and other aspects of animal biology.

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7 Tsetse Population Dynamics

John W. Hargrove

Introduction

Mosquitoes and tabanids deposit their eggs in water; stable flies and horn flies deposit them in wet dung. Most biting flies, in short, lay their eggs in a moist environment in which their larvae feed and develop. In the tsetse fly (*Glossina* spp.), by contrast, a single fertilized egg is retained in the uterus during each pregnancy; when it hatches, the female feeds it until she deposits it as a late third-instar larva. The larva, which may weigh more than the female that has just deposited it, burrows into the ground and forms around itself, within minutes, a hard puparial case. The adult fly develops within this case and emerges at least 3 weeks later, having not fed since its deposition as a larva.

Freedom from a requirement for a moist external environment in the larval stage is matched in the adult stages by the fact that both sexes feed exclusively on blood, which provides for the fly not only the nutritional requirements but also its water needs. While vegetation is required for shelter, and as food for the fly's hosts, tsetse are well suited to survive in dry conditions. The behaviour, physiology and population dynamics of the genus *Glossina* are entirely dominated and conditioned by the consequences of these adaptations in both juvenile and adult stages.

Seasonal variations in numbers are much smaller than in blood-sucking insects such as mosquitoes, stable flies and many tabanids, which depend on surface water or other moist media for breeding. On the other hand, the massive inputs of energy and raw material required by the larva mean that only one larva can be produced every 7–12 days. This is a much lower birth rate than in almost all other insects and means that death rates must also be low if the species is to survive. The larvae and pupae, which spend virtually their entire existence either in the uterus or under the ground, are less prone to predation than their aquatic counterparts. Losses in the larval and pupal stages are generally small, both in absolute terms and in comparison with other blood-sucking flies.

The remaining, serious problem for the fly is to minimize mortality in the adult stages. Complete reliance on blood means that adult tsetse must regularly make flights to contact host animals and feed off them. Indeed, for female tsetse, life consists of little other than a cycle of finding a blood meal, sitting somewhere safe turning that blood into a larva, depositing said larva and flying for the next meal. But flight results in an order-of-magnitude increase in the rate of energy consumption – and feeding, moreover, carries its own risks. Optimal feeding

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behaviour in females thus involves balancing the requirement of getting enough blood to produce a larva, whilst using as little energy as possible and avoiding being killed while feeding.

The imperative for males differs in that optimal behaviour consists entirely of mating with as many virgin females as possible. Rates of energy replenishment must nonetheless be balanced against the risks associated with feeding and the high costs of flight. This balance for both sexes is modified by climate. Thus, when temperatures rise, metabolic rates increase in poikilotherms such as tsetse. If flies are to maintain condition and, in the case of females, the size of the pupae they produce, they must feed more frequently and/or modify their behaviour so as to reduce body temperature below ambient. Failure to do so will lead to increases in mortality and consequent decreases in population growth rate.

Population growth rates are determined by the interplay of rates of dispersal, birth and death, as summarized by the equation:

Growth rate = (birth rate + immigration rate) − (death rate + emigration rate).

This chapter is accordingly concerned with a consideration of each of the components of this equation. How well do tsetse manage to minimize mortality in different environments? How do the rates of gain and loss vary with climate and with population density? Which environmental factors cause the greatest problems? And at what stages are the flies most vulnerable? Of overriding concern is the problem of how we find the answers to these questions and what confidence we have in them.

Birth Rates

The reproductive rate in tsetse depends on the rate of production of larvae and on the rate at which those larvae develop, via the pupal phase, into adults. Both elements are temperature dependent but the functions involved are quite different and the processes need to be considered separately.

Rates of larval production

The massive load of the late third-instar larva on its mother would be greater yet but for the fact that, while the nutritional content of the pupa is sufficient to produce an adult, the young fly which emerges has smaller fat reserves and a less well developed flight musculature than the mature fly. Before the emerging adult female embarks on reproduction she uses the first three or so blood meals to rectify this situation. As a consequence, the time (I_0) to production of the first larva is longer than the time (I) between the production of subsequent larvae.

Early laboratory estimates of the effect of ambient temperature on I_0 and I were unsatisfactory in that, at low temperatures, I_0 appears to be less than I (Anonymous, 1955). The only extensive field estimates of I (Hargrove, 1994) were always lower than predicted by the laboratory equation, particularly at low temperatures (Fig. 7.1a). The discrepancy at high temperatures is probably larger than apparent from Fig. 7.1a since tsetse utilize microenvironments such that they experience temperatures in the field that may be 2–6°C lower than ambient (Hargrove and Coates, 1990).

Data from Rekomitjie Research Station in Zimbabwe suggest that females generally produce their first larva when 14–17 days old and at 8–10-day intervals thereafter. For flies introduced to Redcliff Island, Lake Kariba, Zimbabwe the periods were even shorter and suggest that I may approach a minimum of 6 days under optimal conditions. The trend of the data for Rekomitjie indicates, similarly, that I may take a maximum value of at least 12 days at low temperatures. While such values of I are readily observed in the laboratory, they have not been measured in the field. This is doubtless due, in part, to the fact that tsetse behave in such a way as to minimize the effects of extremes of temperature – by using refuges when temperatures exceed 32°C and by sitting in direct sunshine at low temperatures. Extrapolations beyond the range of field measurements should therefore be treated cautiously.

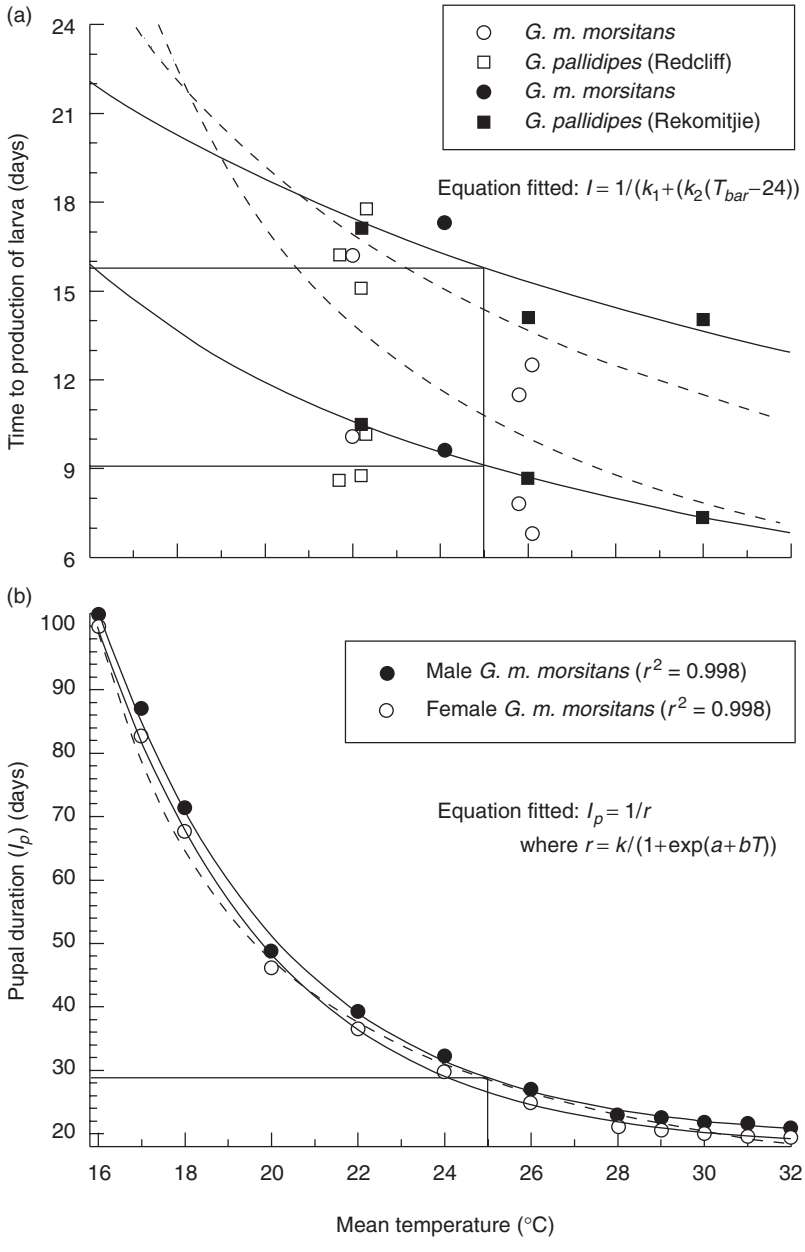

Fig. 7.1. The relation between temperature and development times for immature phases of tsetse. (a) The observed and predicted times (I_0) to production of the first larvae and the duration (I) of subsequent inter-larval periods. Bold lines fitted to the data for flies collected at Rekomitjie Research Station, Zimbabwe (Hargrove, 1994). Estimated values, and standard errors, of the coefficients for the equation in the body of the graph were: for time to production (I_0) of the first pupa, $k_1 = 0.061 \pm 0.002$, $k_2 = 0.0020 \pm 0.0009$; for subsequent inter-larval periods (I), $k_1 = 0.1046 \pm 0.0004$, $k_2 = 0.0052 \pm 0.0001$. Dotted lines show the predicted values from a laboratory study in Tanzania (Anonymous, 1955). (b) Pupal durations (I_p) of *G. m. morsitans* in the laboratory (Phelps and Burrows, 1969a). Estimated values, and standard errors, of the coefficients for the equation in the body of the graph were: males, $k = 0.053 \pm 0.001$, $a = 5.3 \pm 0.2$, $b = -0.24 \pm 0.01$; females, $k = 0.057 \pm 0.001$, $a = 5.5 \pm 0.2$, $b = -0.25 \pm 0.01$.

Rates of pupal development

Phelps and Burrows (1969a) produced precise measurements of the effect of temperature on the pupal period (I_p) for field-deposited larvae kept in the laboratory at constant temperatures. While their model did not predict I_p accurately outside the temperature range 20–30°C, analysis of their data shows that this was only because they attempted to fit the rates of development over the entire temperature range (8–32°C) of the experiment. If attention is restricted to the range 16–32°C, over which flies actually emerged from the pupae, then the resulting fit is near perfect with all pupal durations predicted to within 2.5 days (Fig. 7.1b).

Phelps and Burrows used their data and averages of maximum and minimum Stevenson screen temperatures in Zimbabwe to produce predictions of I_p that generally followed the seasonal changes in ambient temperature in the field. The observed variation in I_p was high, however, as a consequence of the variation in mean temperature between deposition sites. Pupae deposited under logs and rocks during winter tended to have shorter developmental times than expected, while the reverse was true for pupae deposited in ant-bear holes during the hot season. This accords with findings that temperatures in the former situation were often higher than ambient and those in ant-bear burrows were lower. Recent measurements during the hot season at Rekomitjie, in artificial ant-bear burrows where tsetse pupae had been deposited, showed that 24 h mean temperatures were 2.2°C lower than ambient.

Mortality Rates

Since tsetse reproductive rates are so closely dependent on temperature, growth rates could be predicted quite simply if the population's mortality were also known – but mortality is generally not as easily estimated. It is not even generally agreed which abiotic factors are most important in determining density-independent mortality, nor where and how density-dependent effects act to regulate tsetse numbers.

Since mortality varies with developmental stage, the situations in the pupal, teneral (i.e. adults that have not yet taken their first meal) and post-teneral adult stages are considered separately. Mortality can vary even within the post-teneral phase, particularly in flies that have taken at least one meal but have not yet completed the maturation of their thoracic musculature.

Mortality *in utero*

Ovarian dissection of large numbers of tsetse indicate that *in utero* losses are not generally a major source of mortality (references in Hargrove, 1999) though at the hottest times of the year at Rekomitjie the abortion rate rose as high as 3%. Moreover, the proportion of *Glossina pallidipes* found with empty uteri rose exponentially with temperature and there is some suggestion that the abortion rate at high temperatures may be higher than indicated by current methods of estimation (Hargrove, 1999).

Pupal mortality

Pupae are generally not easily found in the field and the estimation of their numbers in a population is a problem that has so far defeated tsetse biologists. There are, consequently, few estimates of the natural pupal death rate, but parasitism and predation may none the less be severe. In Zimbabwe, Heaversedge (1969a,b) and Hargrove and Langley (1993) recorded maximum parasitoid frequencies of 20–40%. The maximum values overestimate the greatest losses per pupal cohort, because the duration of the intrapuparial stages of tsetse parasitoids is greater than that of their host and parasitized pupae therefore accumulate in favoured deposition sites. None the less, losses can clearly be high in the hot, dry season in habitats such as the Zambezi Valley of Zimbabwe. For the remainder of the year losses due to parasites in this environment appear to be only *c.* 1%. For a 30-day pupal period this is a daily loss rate of *c.* 0.03%, which is inconsequential compared with adult losses and even estimated pupal losses due to predation.

Jackson (1937) marked pupae of *G. morsitans morsitans* in field deposition sites and found that 26% (49/188) were lost or taken by predators during the whole pupal period. Using similar techniques, Challier (1973) estimated pupal losses of 0.2–1%/day in *G. palpalis gambiensis* Vanderplank, and Rogers and Randolph (1990) 1%/day in *G. pallidipes* at Nguruman, Kenya.

The effect of climatic parameters on pupal mortality is poorly understood but Phelps and Burrows (1969b) found that repeated exposures of pupae to temperatures of 36°C for up to 4 h/day did not raise mortality above normal levels. More than half of the pupae died, however, when they were subjected to the same temperature for 6 h/day, or to temperatures of 38°C for anything over 4 h/day. Laboratory studies also show that high relative humidities are required for good pupal survival; moreover, temperatures > 28°C in the laboratory led to male sterility in *G. pallidipes* (Leegwater-van der Linden, 1983). It is not known whether the same effects of high temperature occur in the field.

Teneral mortality

Given the low fat levels and poorly developed flight muscle of teneral tsetse, it is to be expected that this class will be particularly at risk from starvation. This was first inferred from field data by Jackson (1948), who released *G. m. morsitans* and *G. swynnertoni* Austen in the field on the day of their emergence. Wing-vein lengths of post-teneral females of both species, and of male *G. swynnertoni*, were significantly greater than those of recaptured tenerals. He concluded that small teneral flies were selectively eliminated, but that there was no size selection once flies had taken their first meal. Several other studies have produced similar conclusions. The most complete of these (Phelps and Clarke, 1974) showed that, between April and December, flies emerging in the laboratory from field-collected pupae were significantly larger than young field-caught adult males estimated to have emerged at the same time. Field losses of small flies were estimated at 35% in the cool months and up to 75% in the hot dry season.

The relationships for pupae between temperature and development, and between temperature and fat consumption, are such that the size-specific fat contents of emerging tsetse are highest for temperatures in the region of 26°C. Phelps (1973) also showed that fat levels at emergence were relatively lower in smaller flies. At the hottest times of the year, small pupal size and high temperatures combine to produce a large proportion of small flies with low fat levels at emergence, thus explaining the particularly high losses of young tsetse in this season.

The same problem can occur at the coolest times of the year in certain habitats. This point is illustrated in Fig. 7.2 where annual variations in fly size are plotted for tsetse caught in the field in Zimbabwe. At Rekomitjie Research Station (altitude 510 m), where the temperature seldom declines below 10°C but maxima reach 42°C, fly sizes increase between January and August and only decline in the extreme heat prior to the rains. At Lusulu (altitude 990 m), by contrast, the temperature extremes were of the order of −1 and 37°C and fly sizes decreased between January and August. Mortality in young flies may therefore be expected to increase at both high and low extremes of temperature, as observed by Phelps and Clarke (1974).

Adult mortality

Since mortality changes with age, the age range over which the estimates are being made needs to be defined precisely. This range depends on the method used. Mark–recapture estimates generally depend on the flies coming to a sampling team and mortality estimates therefore exclude those teneral flies that die before they can appear in fly-round samples (Phelps and Clarke, 1974). The resulting mortality estimates are thus an ad hoc average of the mortalities of teneral, post-teneral immature and mature adult flies.

When the ovarian dissection technique is used to provide sample age distributions, female mortalities are most often estimated using only those flies that have ovulated at

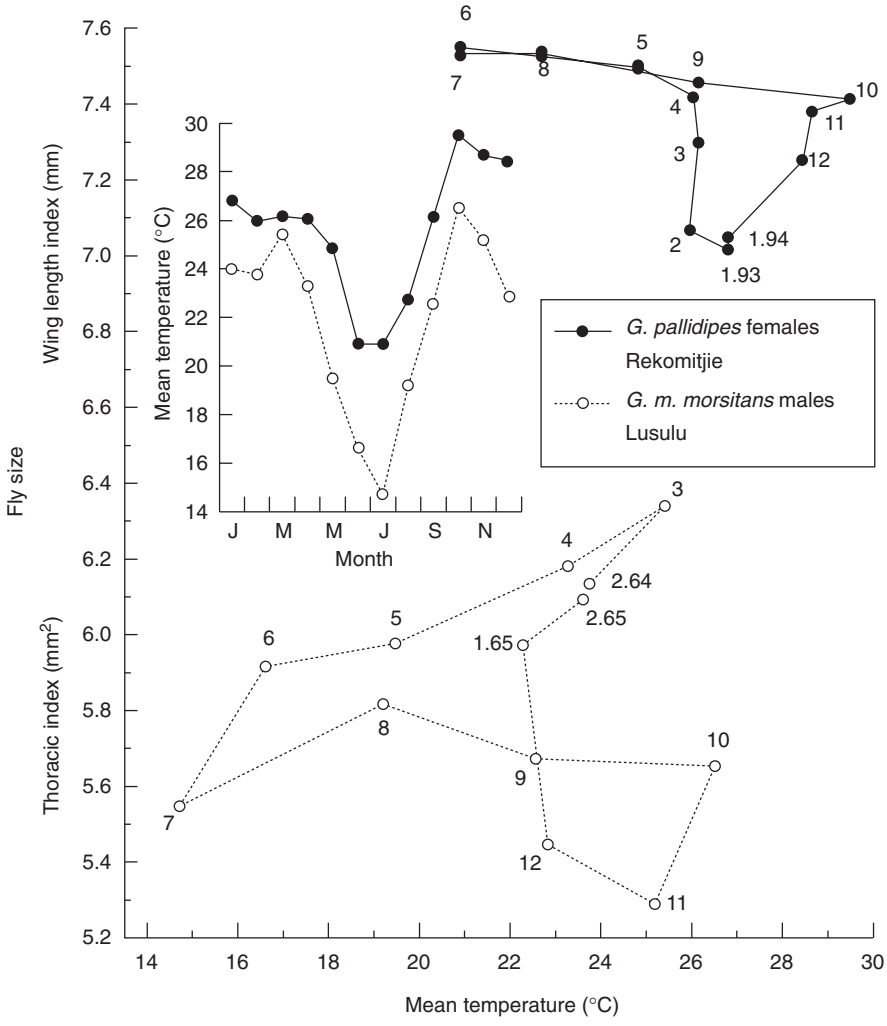

Fig. 7.2. Seasonal changes in the indices of size of tsetse flies in Zimbabwe. Mean monthly indices of wing length for female *G. pallidipes* at Rekomitjie Research Station and of thoracic area for male *G. m. morsitans* at Lusulu are plotted against mean temperature. Numbers in the body of the plot indicate the month and year to which the temperatures and fly sizes refer. Inset: mean monthly temperatures at the two sites over periods including the times of the two studies. Data from Hargrove (unpublished) and Phelps and Clarke (1974), respectively.

least once and that can therefore be considered to be mature adults.

Estimates of mortality can also be made from time series of catches of adult tsetse (Rogers, 1979). This method provides global estimates of mortality over all developmental stages. It is necessary to distinguish carefully between all these estimation methods if sense is to be made of the results of the field experiments.

Mark–recapture estimates

The most extensive and sophisticated estimates of mortality have involved the use of mark–release–recapture but, not surprisingly given the vast effort required, only a small number of long-term studies have been carried out. Jackson (1948), working in Tanzania, estimated mortality in adult male *G. m. morsitans* using this method. Early

demonstrations of a correlation between tsetse numbers and saturation deficit led him to look for an effect of this factor on adult mortality. The correlation was poor and recent analysis (Hargrove, 2001a) showed that mortality was no better correlated with saturation deficit than with maximum temperature (Fig. 7.3).

Mark–recapture experiments carried out on Antelope Island, Lake Kariba, Zimbabwe provided mortality estimates for the same sex and species of fly. Maximum and, more clearly, mean temperature (Figs 7.3, 7.4a,b) accounted for more of the variance in *G. m. morsitans* mortality than did saturation deficit. These results support the view of Bursell (1961) that mature adult tsetse are less likely to be stressed by water loss than they are by a shortage of food reserves. For *G. pallidipes*, however, differences between the effects of saturation deficit and of temperature were less clear and mortality appeared to increase with temperature only above a threshold temperature (Fig. 7.4c,d).

It is unclear why adult mortality increases with temperature. At extreme values there may be a direct effect but for *G. m. morsitans* the positive correlation between mortality and maximum temperature is already evident at < 25°C (Fig. 7.3) and here a direct effect seems unlikely. Since tsetse are poikilotherms, at least part of the increased mortality may stem from the greater number of risks taken in securing the more frequent meals required at higher temperatures. This problem would be most serious for teneral flies, since high temperatures imply that the flies emerge with sub-optimal fat levels.

The mortalities shown in Fig. 7.3 are estimated on the assumption that adult mortality is independent of age, though early laboratory and fieldwork suggested this was unlikely (Jordan and Curtis, 1972; Gouteux and Kiénou, 1982). In a field experiment designed to test this assumption, Hargrove (1990) released uniquely marked *G. m. morsitans*, on the day of their emergence, on Redcliff Island, Lake Kariba, and noted the marks of these flies every time they visited twice-daily ox fly-rounds. It was thereby possible to estimate capture probability and population (Fig. 7.5a,b) at regular intervals and to estimate the changing mortality during adult life. The population estimates are well fitted by a model that assumes that the mortality function is the sum of two exponentials (Fig. 7.5b). Female mortality declined during the first 10 days of life from an original rate > 10 to < 2%/day and increased slowly thereafter with age (Fig. 7.5c).

Estimates from ovarian age distributions

Ovarian dissection data (Challier, 1965) can be used to estimate female mortality. This is best done using a maximum likelihood method (Hargrove, 1993) making due allowance for the population growth rate (Van Sickle and Phelps, 1988). Rogers *et al.* (1984) provided a series of mortality estimates from ovarian dissection data obtained from *G. p. palpalis* (Robineau-Desvoidy) sampled at Degbézéré in the Ivory Coast using biconical traps. Daily mortality rates in any month were best correlated with mean temperature in the previous month. The estimates were not corrected for rates of population change, which were marked in the Ivory Coast study particularly because the population was subjected to two aerial spraying campaigns. When such allowance is made a number of the mortality estimates are then negative (Fig. 7.6). These impossible values occurred most frequently after attempts to control the population by aerial spraying and could result from post-spray invasion of the area by populations of predominantly older flies.

Gouteux and Laveissière (1982) estimated mortalities in female *G. p. palpalis* and *G. pallicera* Newstead Austen but their results are difficult to interpret because they used an inappropriate estimation method (Rogers *et al.*, 1984) and did not correct for growth rates. Jarry *et al.* (1996) later developed the maximum likelihood method such that both adult mortality and population growth rate could be estimated, as long as pupal mortality could be estimated separately. They applied the technique to Challier's (1973) ovarian dissection data for *G. p. gambiensis* (Fig. 7.6). Challier (1973) buried pupae in selected field sites and estimated pupal mor-

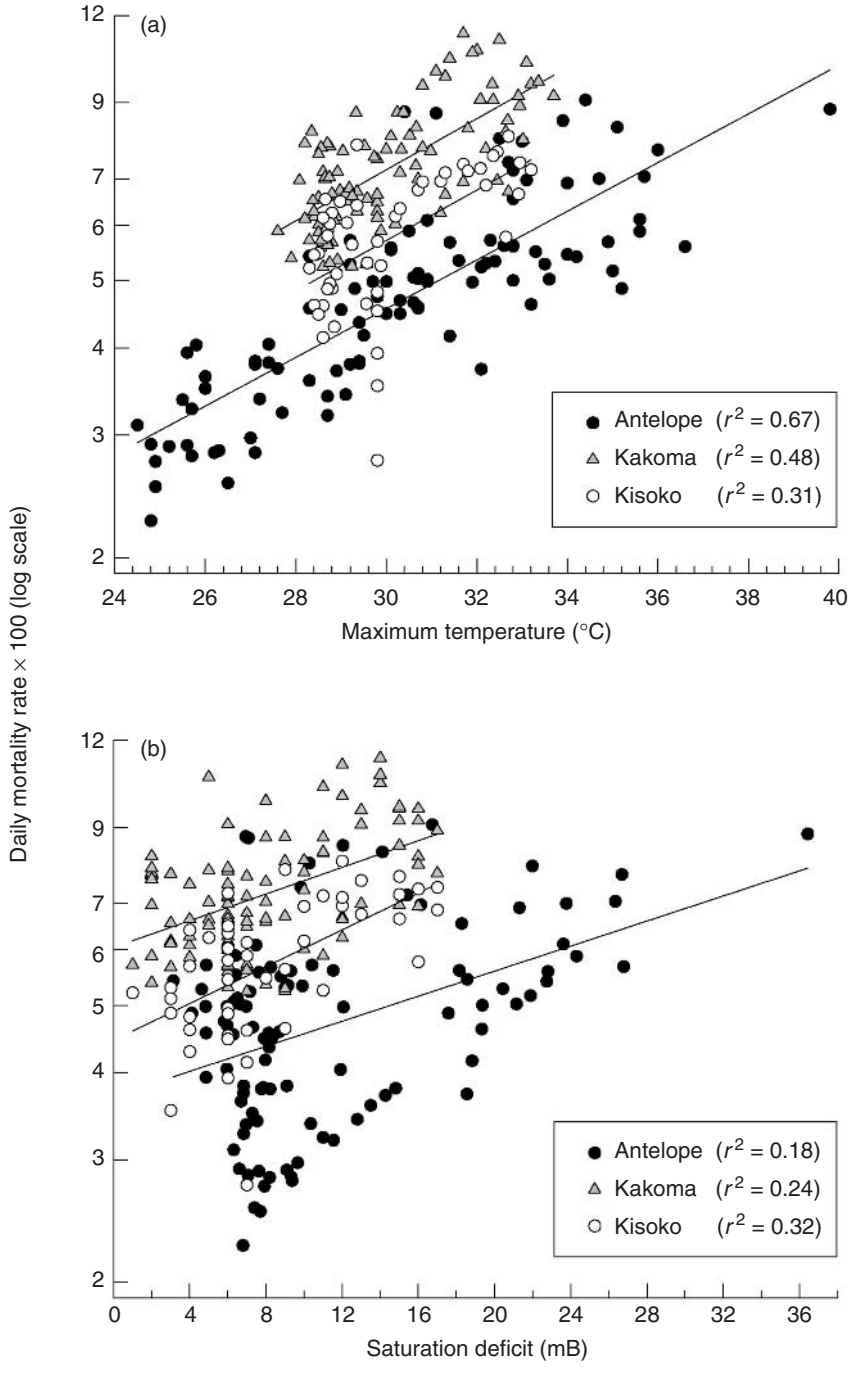

Fig. 7.3. Mean values of weekly mortality for male *G. m. morsitans* plotted against (a) maximum temperature and (b) saturation deficit (Hargrove, 2001a). Survival probability estimates from mark–recapture experiments carried out at Kakoma and Kisoko in Tanzania and on Antelope Island, Lake Kariba, Zimbabwe. Solid lines show the mortalities predicted by regression.

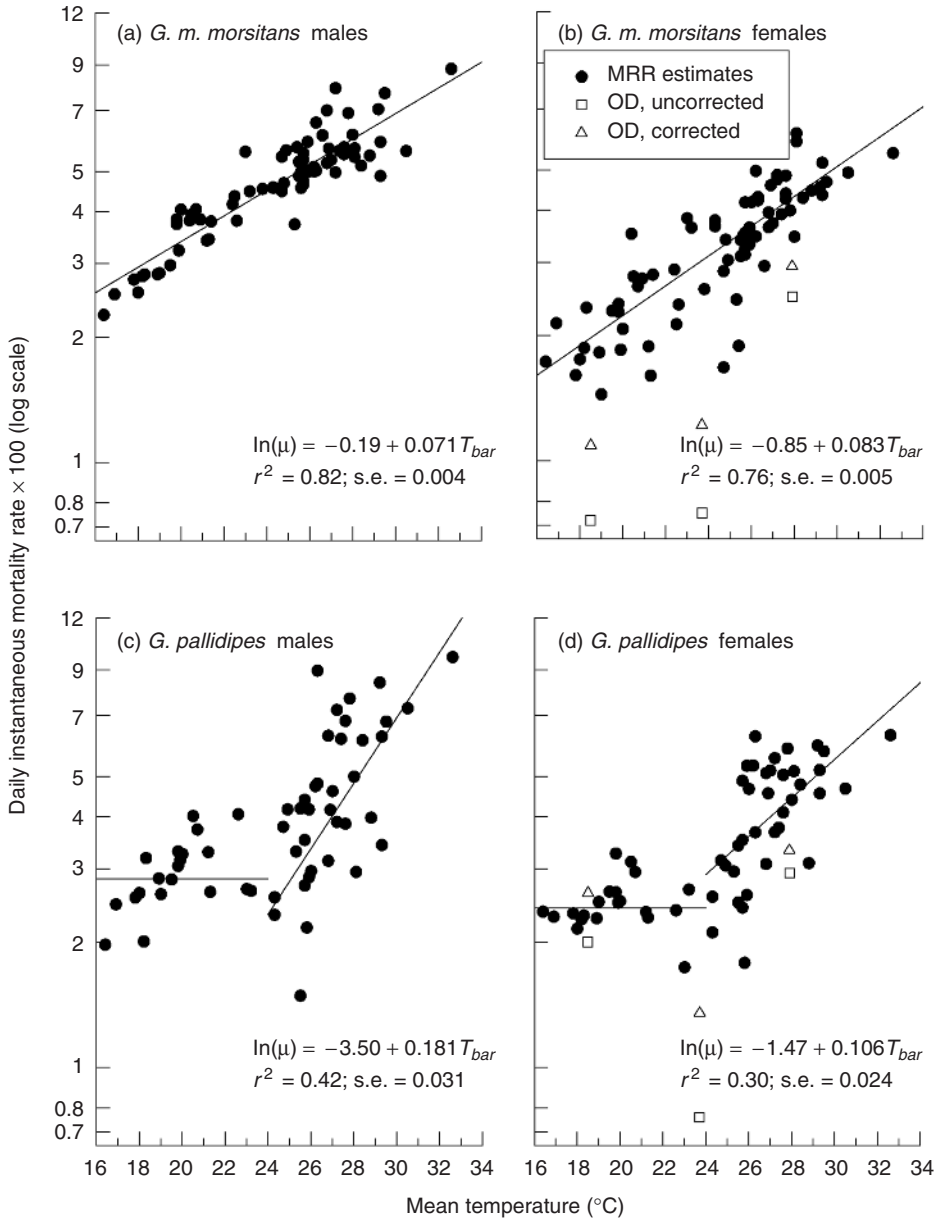

Fig. 7.4. The relationship between mean temperature (T_{bar}) and daily mortality rates (μ) for *G. m. morsitans* and *G. pallidipes* on Antelope Island estimated by mark–release–recapture (MRR) between February 1980 and November 1981 or by the analysis of ovarian dissection (OD) data collected in April, July and December 1981. The latter analysis was applied to raw ovarian age data or to data corrected for estimated biases in age-dependent probability of capture (Hargrove, 1993).

tality from the numbers subsequently emerging. It is not clear how closely these experimental loss rates approached the mean losses in all natural pupal sites. What is clear is that growth rates estimated in this way, being highly sensitive to sampling errors, have high variances (Van Sickle, 1988), the levels of which were not esti-

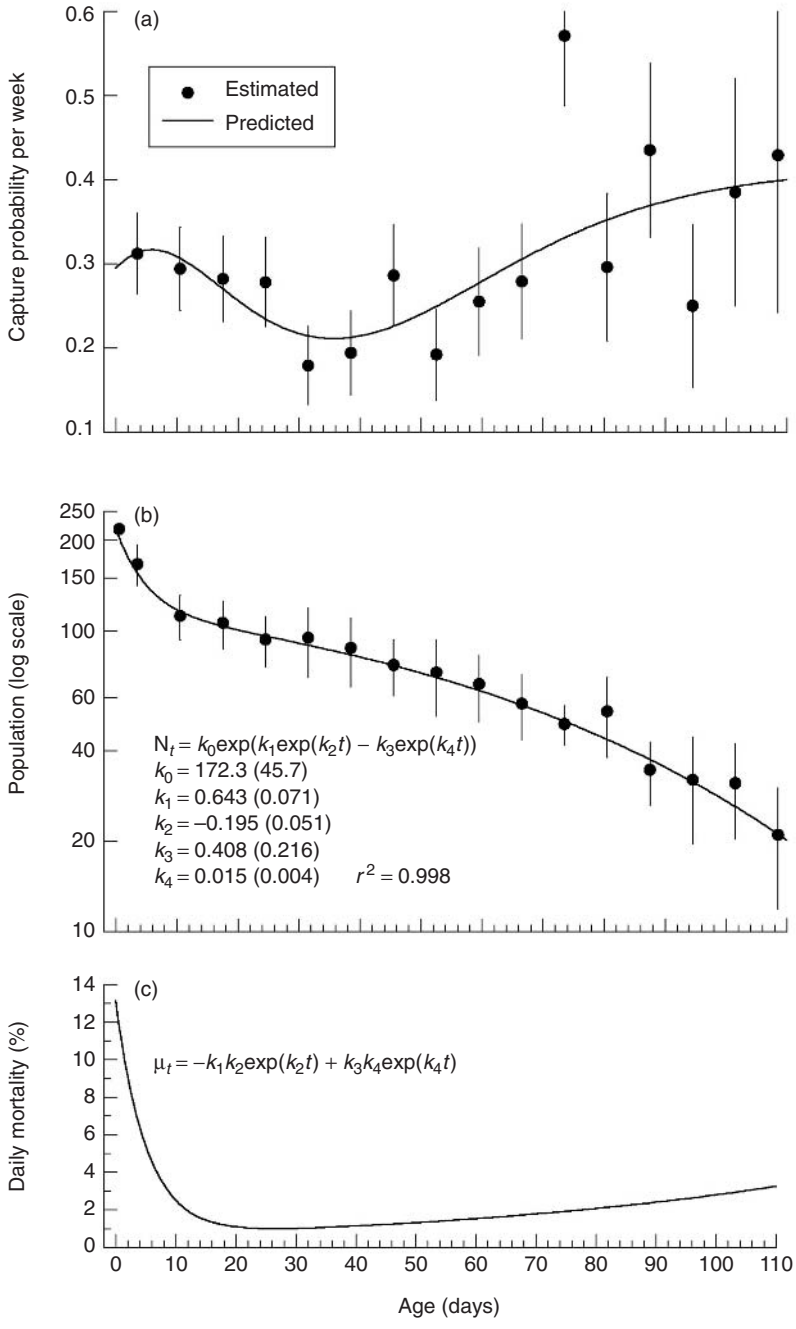

Fig. 7.5. Estimates of population parameters for a cohort of 218 female *G. m. morsitans* marked and released on the day of their emergence on Redcliff Island, Lake Kariba, Zimbabwe, in 1986. (a) Probability that a female is captured at least once during any given week of her life. (b) Population estimates. In the body of the graph are shown the function used to draw the fitted solid line and the parameter estimates, with standard errors in parentheses, for this function. (c) Age-dependent changes in mortality calculated from the parameter estimates in (b). Vertical bars in (a) and (b) denote the standard error of each estimate. New analyses applied to data from Hargrove (1990).

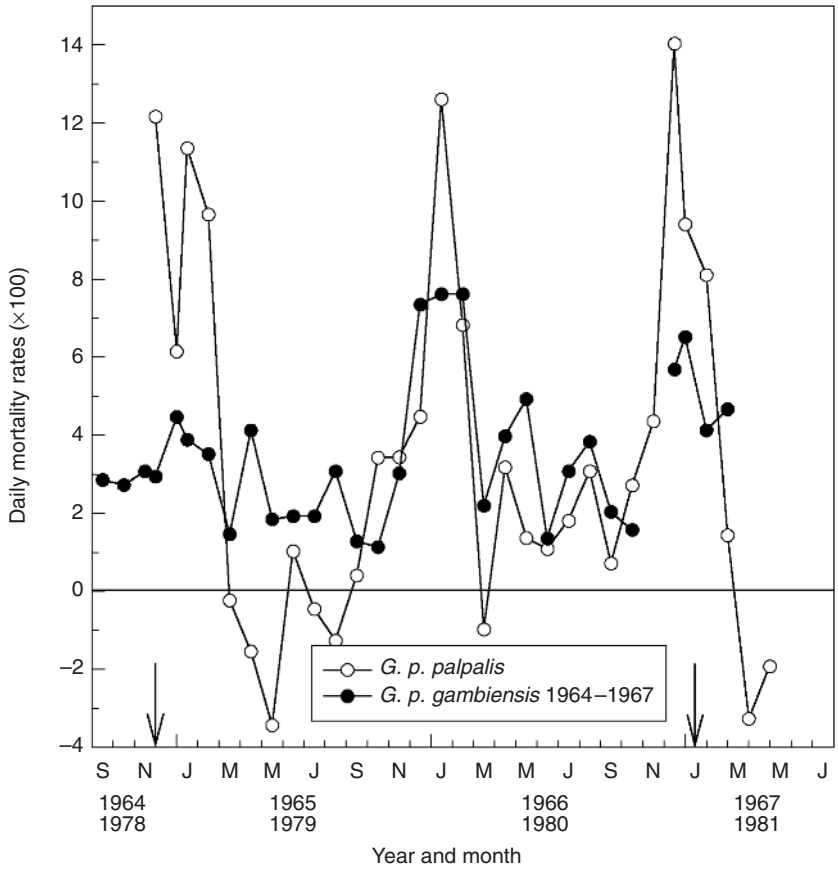

Fig. 7.6. Mortality of adult female tsetse estimated from ovarian age distributions. (i) *G. p. palpalis* captured in biconical traps at Degbézéré in the Ivory Coast. The arrows above the abscissa indicate the timing of aerial spraying operations. Data from Rogers *et al.* (1984). (ii) *G. p. gambiensis* caught on human fly-rounds in the forest of Kou, Burkina Faso. Graph redrawn using data from Challier (1973) and Jarry *et al.* (1996).

mated by Jarry *et al.* (1996). The resulting adult mortalities show, none the less, a pattern similar to those estimated from the data of Rogers *et al.* (1984) (Fig. 7.6).

Unpublished data for *G. pallidipes* caught in odour-baited traps in Zimbabwe provide the longest series of mortality estimates from ovarian dissection. The raw mortality estimates, uncorrected for growth rate, show a consistent cyclical pattern with well-defined minima in May–June and December. For most of the year the estimated mortalities, lagged by 1 month, follow the changes in mean temperature (Fig. 7.7a). Given the strong evidence that adult mortality increases with temperature (Figs 7.3, 7.4) the cool-season minimum for mortality makes sense. The apparent reduction in adult mortality in November–December, at the hottest time of the year, makes little sense, however, and is at variance with the contemporary decline in catches (Hargrove and Vale, 1980).

Inspection of the ovarian dissection raw data identifies the probable source of the paradox. At the end of each year there is a striking reduction in the proportion of young flies in field samples. In particular, the proportions of category zero flies, which are not used in the mortality estimation, show a rapid decline between September and the end of the year (Fig. 7.7b). The

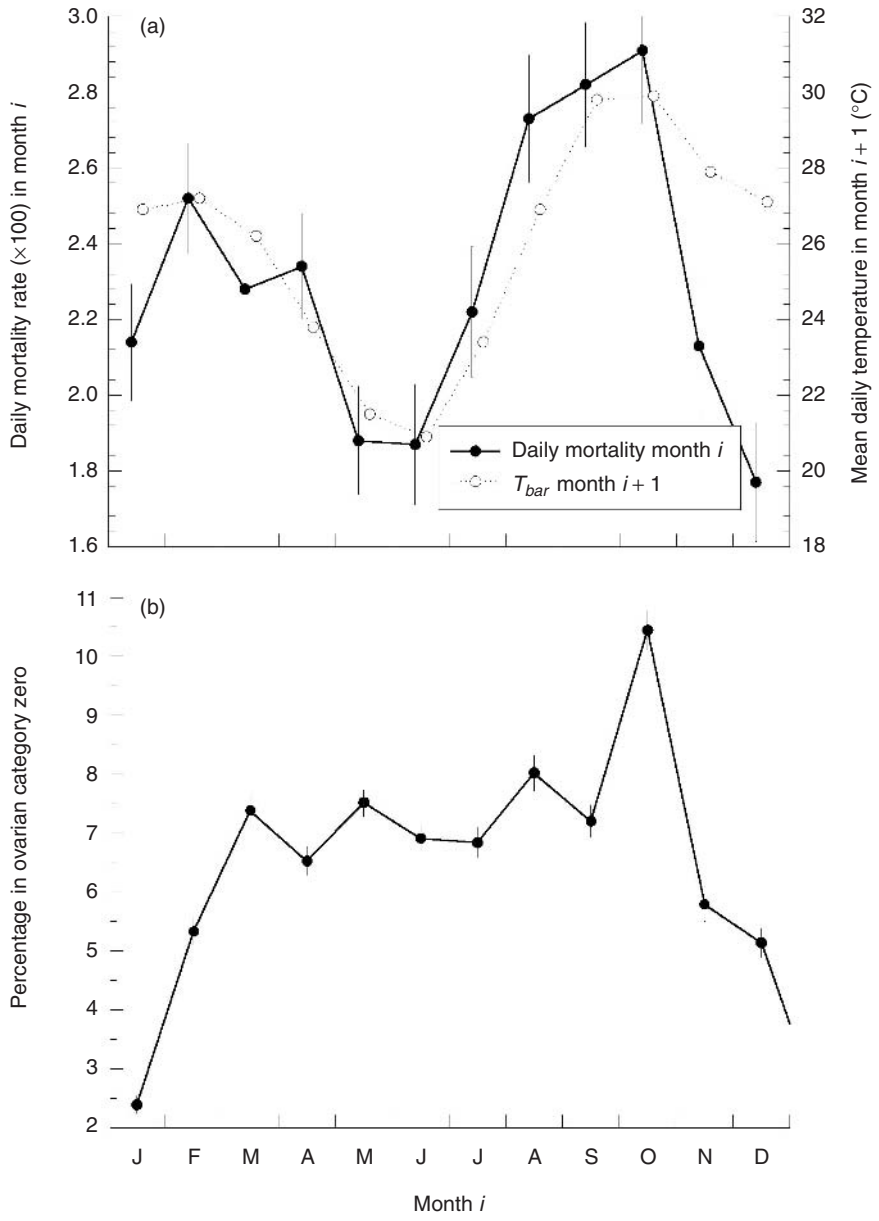

Fig. 7.7. Results of ovarian dissection of *c.* 110,000 adult female *G. pallidipes* caught in odour-baited traps at Rekomitjie Research Station, Zimbabwe, between September 1988 and January 1999 (J.W. Hargrove, unpublished). Means are for each month pooled over all years, with vertical bars indicating standard errors. (a) Daily mortality rates estimated from sample ovarian age distributions. (b) Percentages of flies sampled that were in ovarian category zero.

apparent decline in mortality is thus almost certainly an artefact consequent on a marked increase, during the hot dry season, in the losses of immature tsetse. This could be due to increased parasite levels (Hargrove and Langley, 1993) and/or to increased losses of teneral flies due to the combined effects of high temperatures and low fat at

emergence (Phelps and Clarke, 1974). These results provide a particular example of the problem raised by Van Sickle and Phelps (1988), who suspected that tsetse populations at Rekomitjie seldom exhibited the stable age distribution necessary for the reliable estimation of mortalities using ovarian dissection data.

The Antelope Island experiment provided a unique opportunity to compare contemporary mortality estimates on the same population using mark–recapture and ovarian dissection data. The former estimates, for female *G. pallidipes* and particularly *G. m. morsitans*, were almost always higher (Fig. 7.4b,d). Part of the difference could be due to age-dependent changes in capture probability (Hargrove, 1993) but correcting for this effect accounted for only part of the discrepancy. At least part of the remainder could have been due to the fact (see above) that the mark–recapture method estimated mortality in all adults that survived long enough to be captured at least once, whereas the ovarian dissection estimation procedure included only those that had already ovulated and that were generally at least a week old. The discrepancy between the mortality estimates from the two methods would thus be further reduced if the mortality were markedly higher in the first week of life than at later stages, and this is clearly the case (Fig. 7.5).

The elevated post-emergence mortality makes it important that category zero flies are excluded when estimating tsetse mortality from ovarian ages using any method that assumes that mortality is independent of age. Jarry *et al*. (1996) did not exclude the category zero flies in their analyses and they would thus have overestimated the mortality in all older flies. This tendency is further accentuated because their flies were caught in hand-nets. For female *G. m. morsitans*, at least, the probability of capture by human fly-rounds declines by an order of magnitude over the first 5 weeks of life (see below).

The results reviewed above provide a warning that mortalities estimated from ovarian dissection data can be seriously misleading. Standard errors, which have never been estimated in the past, are also often disturbingly high. Thus, even for the Zimbabwe data for 1991, where estimates were based on > 800 flies each month, standard errors averaged 23% of the mean; for Challier's (1973) data, based on only 20–160 flies, this figure rose to 74%.

Overall mortality in all developmental stages

Rogers (1979) used time series of field samples of adult tsetse to estimate mortality across all developmental stages, as opposed to the mark–recapture and ovarian dissection estimates, where measurements refer only to different subsets of adult flies. Various workers have found that the resulting mortality estimates are better correlated with some measure of dryness, such as saturation deficit or an index of vegetation cover, than with temperature (Fig. 7.8). Hargrove (2001b,c) contrasted these findings with mark–recapture estimates of adult mortality, which were generally better correlated with temperature (Fig. 7.3). If the results of both approaches are valid they imply, since the Rogers' method provides measures of mortality across a whole generation, that it must be the immature stages that are particularly sensitive to dryness and the mature adults to temperature. This is reasonable given that the only times during the fly's life when the exoskeleton is soft, and therefore particularly prone to water loss, are between larviposition and the formation of the puparial shell, and immediately after eclosion of the teneral adult. It is also consistent with the finding that mortality per generation between months i and i+1 is more highly correlated with the saturation deficit in month i, when it is affecting the immature stages, than in month i+1, when the effect is chiefly on the adults.

For the Antelope Island study, Hargrove (2001c) estimated post-teneral adult mortality from mark–recapture data, and overall mortality using Rogers' (1979) technique. It was thereby possible to estimate that the joint mortality in the larval/pupal and teneral adult stages was almost always markedly lower and less variable than in mature adults

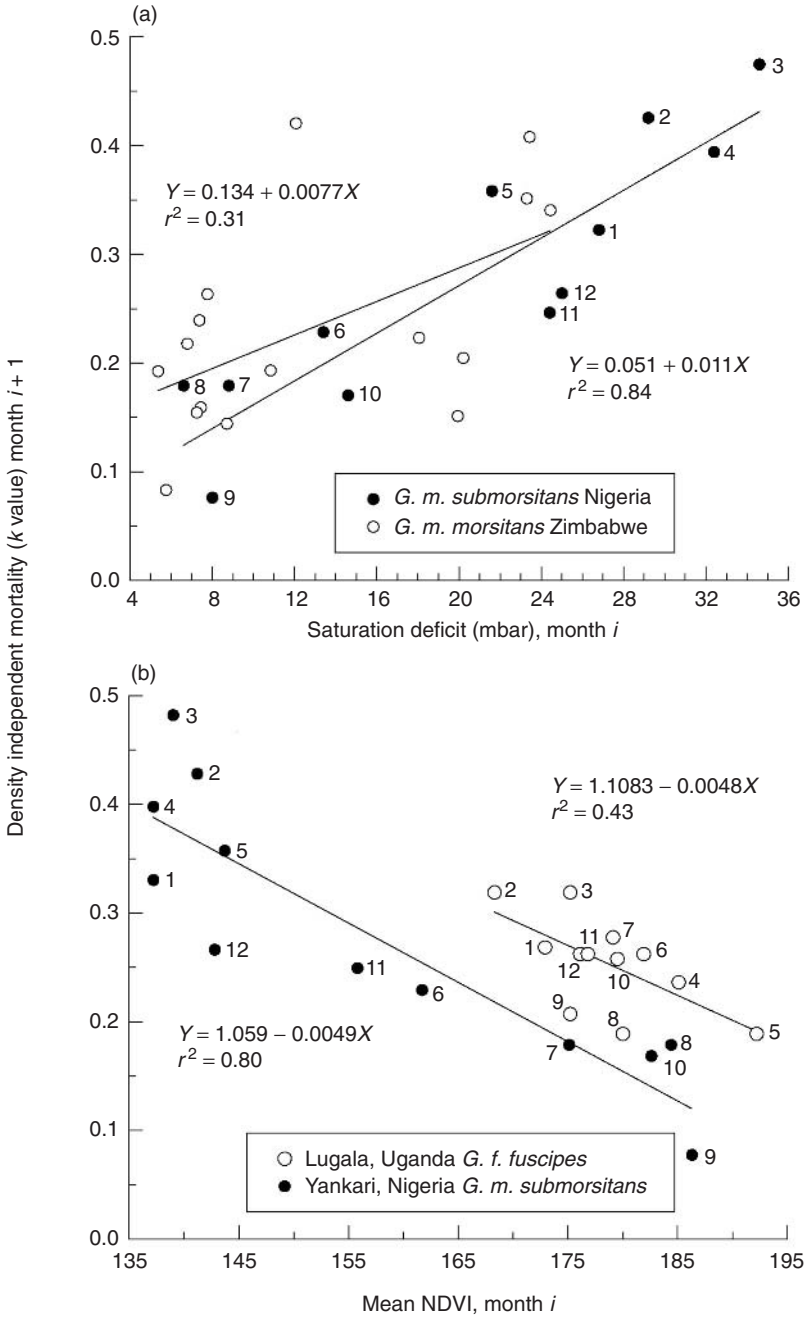

Fig. 7.8. Survival of generations of female tsetse flies, estimated from time series of catches using the method of Rogers (1979), plotted against (a) the prevailing saturation deficit or (b) mean normalized difference vegetation index (NDVI) in the preceding month. Redrawn using data from Rogers and Randolph (1991) and Hargrove (2001b).

(Fig. 7.9). Since there are good reasons for supposing that teneral adults are particularly at risk (see above) their mortality was presumably higher than the level estimated for post-teneral adults, which means that mortality in the pupal phase is lower yet than the joint estimate. At least on Antelope Island it may have been lower than the 1%/day estimated by Jackson (1937) and Rogers and Randolph (1990).

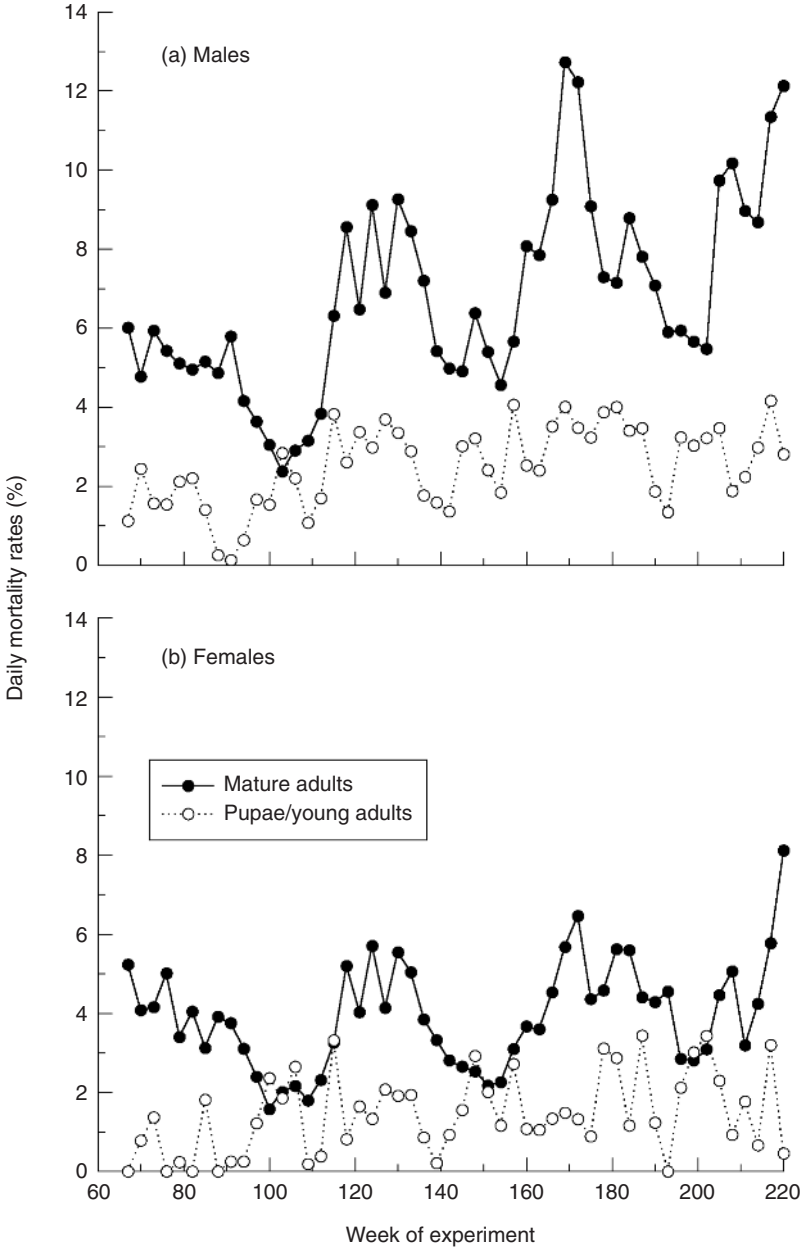

Fig. 7.9. Estimated mortalities of (a) male and (b) female adult and immature *G. m. morsitans* on Antelope Island, Lake Kariba, Zimbabwe. Redrawn from Hargrove (2001c).

Growth Rates of Small Closed Populations

Relationship between growth and mortality rates

For small closed populations, where density-dependent effects and migration can be ignored, Williams *et al.* (1990) provided a simple method for calculating the growth rate resulting from given age-dependent rates of mortality, larval production and pupal development. The latter two depend largely on the mean temperature, and the growth rate at a given temperature exhibits approximately a tenfold change for each 1% change in adult mortality. The level of this graph varies with the pupal mortality but the slopes are closely similar (Fig. 4 in Hargrove, 1988).

With zero mortality the population increases by a factor of *c.* 3500/year or nearly doubles each month. Observed growth rates are always smaller than this, because mortality is never zero, but short-term growth rates of *c.* 100/annum have been observed in the field (references in Hargrove, 1988). Predicted maximum growth rates increase with temperature for T_{bar} > 25°C but these predictions grow progressively less realistic, since death rates also increase with temperature (Fig. 7.3 and see below).

For T_{bar} = 25°C the functions in Fig. 7.1 predict values for I_p, I_0 and I of 29, 16 and 9 days, respectively. For these values, and assuming 20% losses during the egg, larval and pupal phases, the population would begin to decline if the adult mortality exceeded *c.* 3.5%/day. These are important results in practical terms because they imply that if a mortality of > 3.5%/day is imposed and maintained on the adult females, the annihilation of the population is assured, regardless of resilience due to density-dependent effects. Moreover, relatively small additional increases in this imposed mortality have large negative impacts on the population.

The sensitivity of the growth rate to changes in adult female mortality stems directly from the low natural birth rate and demands that tsetse behaviour, particularly the female's, must be tailored to minimize risk. Randolph *et al.* (1992) argued that this consideration requires female tsetse to take large meals at long intervals in order to maximize lifetime fertility. It also explains the fact that female *G. m. morsitans* and *G. pallidipes*, unless they are close to starvation, do not attempt to feed on high-risk hosts such as humans (Vale, 1974). Conversely, tsetse populations can be controlled and even eradicated using simple methods that attract a small proportion (4–8%/day) of the female population to point sources and kill them using traps, targets or treated livestock (references in Laveissière *et al.,* 1990).

Effect of temperature on population growth rates

Loss rates at various stages of a tsetse life are temperature dependent. Thus, the results of Hargrove (1999) suggest an effect of extremely high temperatures on reproductive losses, at least for *G. pallidipes* in Zimbabwe. Fat levels in emerging tenerals decline at extremes of both high and low temperature and survival probabilities in this group of flies presumably follow a similar function of temperature. Density-independent mortality in post-teneral adult *G. m. morsitans* (Fig. 7.3) increases exponentially with temperature.

Given these results, and the fact that reproductive rates are also temperature dependent, the interesting possibility arises that one can make reasonable predictions of the growth rate of a *G. m. morsitans* population in a given environment knowing nothing more than the prevailing mean temperature (T_{bar}). In doing so one needs to take account of age-related changes in female mortality (Fig. 7.5c) which suggest that adult mortality estimates in Fig. 7.4 provide only an average value biased towards the mortality of the most numerous young flies. Mortality will thus be overestimated in flies > 1–2 weeks old and underestimated in young flies. Increased mortality in very old flies is ignored for the present since relatively few survive to such ages.

Examples of the changes in mortality suggested by the above results, for $15 \leq T_{bar} \leq 33$°C, are shown in Fig. 7.10a. Reproductive losses are assumed to increase exponentially from *c.* 0 to 10% over the given range. Losses during the pupal phase are assumed to take a

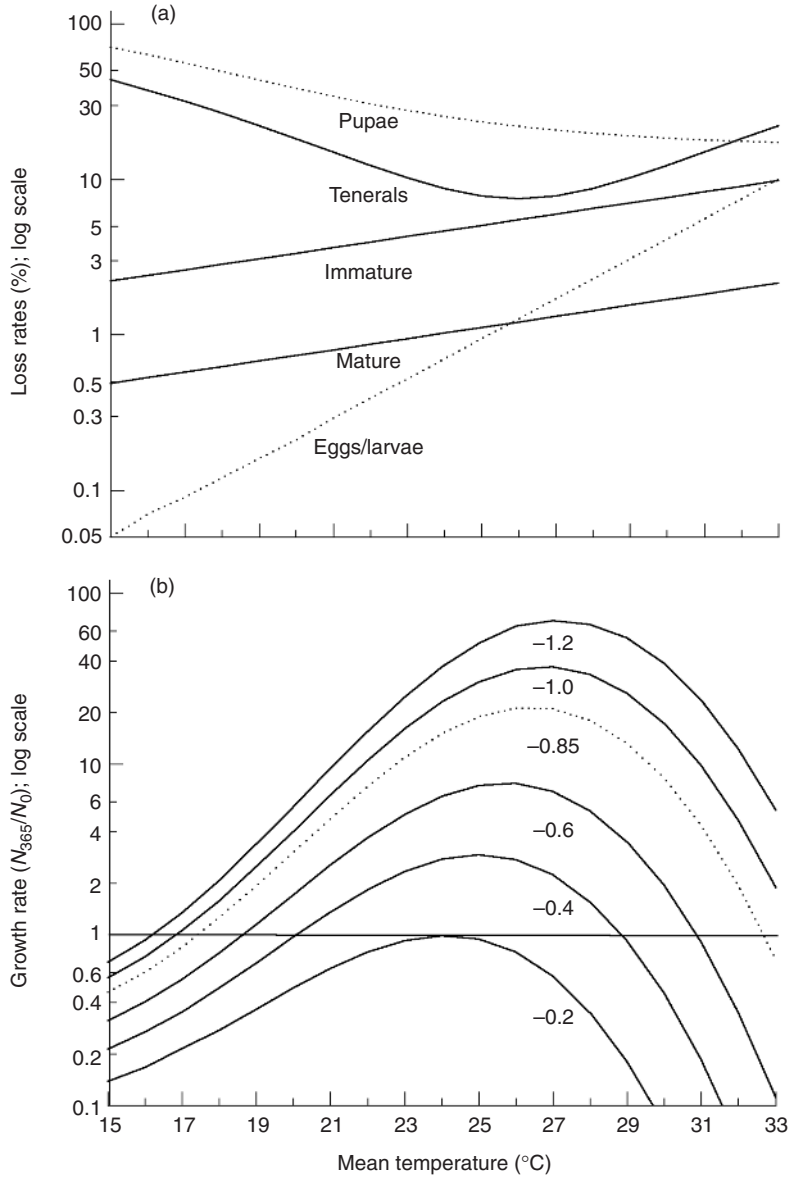

Fig. 7.10. The relationship between mean temperature and growth rate. (a) Mortality rates assumed for various stages. (b) Estimated growth rates. Developmental rates estimated using the equations in the legend for Fig. 7.1. All adult mortalities (μ) are assumed to be related to mean temperatures (T_{bar}) by the equation $\ln(\mu) = \alpha + \beta.T_{bar}$, where β is assumed to take the value 0.83 and α is treated as a parameter which, for female *G. m. morsitans* on Antelope Island (dotted line), took the value −0.85 (Fig. 7.4b). Pupal mortality was set at 0.5%/day. Growth rates calculated using equation 11 of Williams *et al.* (1990) for the different values of α shown in the body of graph. The horizontal line in (b) marks the boundary between areas of positive and negative growth.

constant value of 0.5%/day (cf. Rogers and Randolph, 1990) independent of T_{bar}. This means, of course, that the total losses during the pupal period increase with decreasing temperature (Fig. 7.10a). Losses consequent on the increased total fat consumption at extremes of temperature are also to be expected, but these are subsumed into the teneral phase. For the post-teneral adults it was assumed that, for the first week of post-teneral life, mortality was 1.5 times the value predicted by regression in Fig. 7.4b. For all older flies the mortality was assumed to be only one third of the predicted value.

The dotted line in Fig. 7.10b, which gives estimated growth rates for the Antelope Island population of female *G. m. morsitans*, predicts that positive growth will only occur for $17 \leq T_{bar} \leq 33$°C. A maximum rate of population increase of *c.* 20-fold per year is predicted for $T_{bar} \approx 25$–27°C. This is in good accord with the observed mean rate of increase of 23-fold per annum for the female *G. m. morsitans* population on Antelope Island between February and April 1981, for which period the temperature averaged 25.2°C.

For adult male *G. m. morsitans* the coefficient of increase in adult mortality with T_{bar} was similar for studies carried out in Zimbabwe and Tanzania (Fig. 7.3). Only the general level of mortality differed. In calculating the remainder of the trajectories in Fig. 7.10b it was assumed that the same was true for females. The figures in the body of the graph refer to the constant term (α) in the predictive equation for the mortality (see legend). As α increases, the mortality increases and the range of T_{bar} which supports positive population growth decreases. Regardless of the value of α, however, maximum growth rates are always predicted to occur for temperatures in the range $24 \leq T_{bar} \leq 27$°C.

The mortality functions used in constructing Fig. 7.10b are necessarily speculative but appear reasonable given published data. The assumed maximum abortion rate of 10% is less than the observed increase in the proportion of empty uteri (Hargrove, 1994) but greater than the 5% estimated by Williams *et al.* (1990) from Kenyan ovarian dissection data. The daily pupal mortality was set at 0.5% given the indications of low pupal mortality on Antelope Island (see above). Teneral losses are put at high levels at extremes of temperature, not unreasonably given Phelps and Clarke's (1974) estimate that up to 35% of undersized tenerals are lost in the cold season and up to 75% at the height of the hot season in Zimbabwe. The partitioning of mortality between different phases of post-teneral life is again speculative but is reasonable given the age-related changes in mortality estimated on the adjacent Redcliff Island (Fig. 7.5).

In Chapter 8 Rogers and Robinson consider the relationship between climate and tsetse distribution. The present approach provides a useful adjunct to their studies since it could be used to predict not simply the presence or absence of flies but also the potential growth rate of a tsetse population. The model involves several simplifications of reality; even for *G. m. morsitans*, the undoubted effect of saturation deficit or relative humidity on mortality, for some developmental stages, is ignored for the present. The qualitatively different relationship between adult mortality and temperature for *G. pallidipes* (Fig. 7.4) provides, moreover, a reminder that the situation will differ between species. Thus Rogers and Randolph (1986) plotted three-dimensional 'climograms' of overall mortality against temperature and saturation deficit and found that different species exhibit quite different climograms, indicative of their differing meteorological preferences.

For any tsetse species, population growth will, in any case, be affected by food availability. Where the fly's wild hosts have been removed by, for instance, a combination of hunting and rural development, potential growth rates will be much lower than suggested by the temperature data. The model indicates, none the less, the potential for reinvasion of these areas should domestic livestock or alternative hosts be introduced.

Estimating the Growth Rate from Field Data

Previous sections have been concerned with the problem of predicting the growth rate given estimates of mortality and birth rates,

or even perhaps given data on the mean temperature. Attempts have also been made to estimate the growth rate from ovarian age samples but Van Sickle (1988) showed that several such attempts were invalid because, in making the estimates, it was implicitly assumed that the growth rate was zero. The estimates are still used on occasion as if they were valid and this can lead to misleading results. As an example, Allsopp's (1985) growth rate estimates for *G. m. centralis* Machado in Botswana were negatively correlated with fly-round catches, and seemed therefore to be evidence in favour of density dependence (Rogers and Randolph, 1984). The later demonstration that the growth rate estimates were invalid makes it clear that the observed correlation was in fact serendipitous.

Van Sickle (1988), Williams *et al*. (1990) and Jarry *et al*. (1996) suggested methods whereby age distribution data can be used to provide *legitimate* estimates of the growth rate but, as Van Sickle (1988) pointed out, such estimates have unacceptably high variances. At present, therefore, growth rates can only be estimated satisfactorily from changes in population levels, and this normally means using changes in apparent density. This, in turn, involves the assumption that catch levels are, in general, proportional to absolute population levels and, in particular, that this proportion is independent of weather and season.

The Growth of Large Open Populations

Dispersal

Models of normal, wild populations of tsetse, which are generally large and open, must take into account the effects of movement, and the density-dependent effects that regulate the population around some characteristic level.

As with most ideas on tsetse population dynamics, early advances in the understanding of tsetse dispersal were due to C.H.N. Jackson, who concluded from mark–recapture experiments that tsetse dispersal involved to-and-fro movements between a home range and regular feeding grounds. Bursell (1970) argued that the data could as well be described by a random movement model, which had the advantage that it did not require tsetse to be able to navigate in what was apparently undifferentiated woodland. The model has been developed by several authors (references in Williams *et al*., 1992), most conveniently as the equivalent formulation of a diffusion process, which is currently used as the best available description of tsetse dispersal. Estimates of the daily step length for this model are generally in the range 0.15–1 km. Given that the mean distance moved is approximately the product of this figure and the square root of the time since dispersal began, the average fly moves no more than 10 km from its birthplace even if it lives for 100 days.

Density-dependent effects

The development so far has ignored the fact that, in a finite environment, population size has an upper limit, and when numbers approach that limit the growth rate must begin to decrease. In a closed population this means that the birth rate must decrease or the death rate increase, or both. In an open population there is the further option that the net rate of emigration can increase. The important features of the rate changes are that they are dependent on the density of the population, as opposed to the changes with temperature and saturation deficit that are density independent. Rogers and Randolph (1984, 1985) discussed density dependence, particularly as it applies to tsetse populations.

Density-dependent factors are essential for the regulation of population numbers but the effects are notoriously difficult to detect, let alone measure. Rogers (1974) placed puparia of *Glossina fuscipes fuscipes* Newstead at natural depths in the field near Lugala, Uganda, and found that losses were density dependent at densities > 4 puparia/m^2. In contrast, Rogers and Randolph (1990) found no such effect in similar experiments on *G. pallidipes* at Nguruman, Kenya.

Rogers (1974) tied adult *G. f. fuscipes* Newstead to branches of trees with pieces of

cotton and found that predation by vertebrates, but not invertebrates, was density dependent. Given the highly artificial nature of the experiment, and the fact that the observed losses were 10–30 times as high as those generally observed in the field, the results are of uncertain relevance to wild populations of tsetse. In fairness, however, no other worker before or since has attempted to provide experimental evidence on predation of adult tsetse. All of the other evidence on density dependence is indirect.

In modelling tsetse populations, Rogers (1990) found it necessary to include density-dependent mortality in both the adult and pupal stage in order to achieve good fits to the data (see below). Data from Zimbabwe support this indirect evidence in favour of density-dependent losses of pupae. At Rekomitjie, tsetse pupae are most easily collected during the hot dry season, presumably because, as this season proceeds, female tsetse increasingly concentrate their larviposition activities in sites such as ant-bear burrows, under leaves on the edges of dried up river beds, and under fallen logs. This behaviour has the effect of greatly increasing the density of pupae in these larviposition sites and there is a simultaneous increase in the proportion of parasitized pupae (Hargrove and Langley, 1993) and a marked decrease in the proportion of young flies in the population (Fig. 7.7b). It is reasonable to think that parasites, like humans and probably also other predators, find tsetse pupae more easily as pupal density increases and the pupae-rich sites are more easily identifiable. But the increase in pupal density is purely local. Trap catches at this time of the year suggest that adult populations, and hence presumably overall pupal density, peak in September and decline rapidly thereafter (Hargrove and Vale, 1980). Increased pupal loss rates would thus depend directly on local pupal density but would be *inversely* correlated with total population density. The 'density-dependent' effect would thus actually work in concert with increased density-independent losses due to the increasing temperature and both would serve to drive the population below optimal levels. This interesting situation clearly merits further study.

There is some evidence that feeding success is density dependent, though Rogers and Randolph (1984) pointed out that this need not necessarily lead to increased mortality and, indeed, nobody has yet demonstrated such an increase. Vale (1977) found that the feeding success of *G. m. morsitans* in some field experiments in Zimbabwe, but not others, decreased as the numbers of flies visiting the host increased. Other workers, using Vale's methods, find that the observed irritability of host animals increases with fly density but that this is not always translated into reduced feeding success (references in Schofield and Torr, 2002).

Indirect evidence for density-dependent effects comes from estimates of changes in growth rate with estimated changes in population size. The estimates have always been based on time series of sample catches, rather than absolute population estimates, so that an important untested assumption is that the capture probability is independent of time. For *G. p. palpalis* at Katabu in Nigeria, Rogers and Randolph (1984) found that the log of the change in fly-round catch between December and January was negatively correlated with the log of the catch in December. Similarly, the maximum rate of increase in trap catches of *G. p. palpalis* in the Ivory Coast was inversely related to catch size at the time when that increase was occurring. In this case catches increased at a rate greater than could be explained by birth processes alone and it was concluded that density-dependent rates of invasion were involved.

Modelling Population Changes

Birth and death models

Changes in tsetse population reflect the integrated effects of numerous exogenous and endogenous factors, not all of which are fully understood and many of which have delayed effects. These complexities make it difficult to derive analytical formulae for predicting population changes. Instead, modelling of population data has so far involved the use of computer simulation.

Rogers (1990) developed the first such model and used it to provide good fits to time series of catches of tsetse, assumed proportional to actual population levels. Birth rates were estimated from ambient temperatures and overall density-independent mortalities from observed changes in the population. The important result of this approach was that it was always found essential, for population stability, to include density-dependent mortality in the model.

Hargrove and Williams (1998) developed the simulation approach further in modelling time-series of population estimates of *G. m. morsitans* on Antelope Island. By linking a simulation procedure, similar to that of Rogers (1979), to a non-linear optimization routine it was possible to estimate, for any given model, the parameter values that produced the best fit to the data. Variables thought likely to affect mortality were entered one at a time and it was thereby possible to keep in the model only those variables that significantly improved the fit. The model was used to provide a successful fit, without any need for scaling, to estimates of changes in the absolute population of male and female *G. m. morsitans* over a period of 240 weeks. This was achieved without reference to estimated mortalities, though there was a good correspondence between their predicted and observed values (see Fig. 4 in Hargrove and Williams, 1998). Adult mortality was modelled as a linear function of maximum temperature (T_{max}) whose coefficients were estimated by the optimized simulation procedure. No other meteorological variable was required for a good fit; indeed, once T_{max} had been included no other meteorological variable could be included that improved the fit significantly. This result supports indications from other work (see above) of the overriding importance of temperature in controlling the density-independent growth rates of tsetse populations.

Models including migration

Dransfield *et al.* (1990) modelled the outcome of an attempt to use odour-baited traps to control tsetse at Nguruman, Kenya, and the same study gave rise to the only serious attempt at modelling the growth of large, open populations (Williams *et al.*, 1992). They assumed diffusive movement and logistic growth, making no assumption about the mode of operation of the density-dependent processes except that they applied only to the birth and death processes, not to dispersal rates. In simple terms it was assumed that each population has a characteristic carrying capacity and that the growth rate slows as this level is approached.

The resulting differential equation has no analytical solution but a solution is easily approximated using a numerical technique. The approach has been used only by Hargrove (2000, 2003a,b) and Hargrove *et al.* (2003) to model the outcome of various tsetse-control campaigns. It could be used more generally for modelling observed changes in tsetse populations, both natural and as a consequence of human intervention. Having obtained adequate fits to existing data it could then be used to predict the progress of other operations, needing only as input data realistic estimates of the levels of imposed mortality and rates of fly movement. As such the model provides a powerful weapon for the analysis of past, and the planning of future, control operations. It also has the potential, therefore, of providing a much-needed *objective* measure of the cost effectiveness of different approaches to tsetse control under different circumstances.

Caveats

In the interests of brevity, simplicity and readability the above development has glossed over numerous difficulties associated with the estimation of the rates of birth, death, movement and growth. The scale of these difficulties is evident in an early elegant experiment in which Jackson (1946) allowed teneral *G. m. morsitans* to emerge from pupae introduced to the habitat of *G. swynnertoni* in Tanzania and captured the adults on a spiral fly-round

centred on the release point. Any reasonable assumption of rates of loss of flies, by death and emigration, from the sampling area leads to the prediction of an approximately exponential decline in the numbers of flies still alive and still within the boundaries of the spiral. If the probability of capture were independent of the time after release (i.e. the fly's age in this case) then captures should have shown a similar decline with time.

This expectation was not realized in either sex. Male catches did not decrease consistently until the 7th week after release. Females, in contrast, always showed a lower capture probability and were only caught in any numbers during the first week after emergence. Making, again, reasonable assumption of rates of loss, one can estimate the probability of capture during each week of the experiment. For males, this increased by a factor of between five and eight during the first 6 weeks of life. Conversely, for females, the capture probability declined by a similar factor over the same period.

If one assumes unbiased sampling in Jackson's experiment, one is led to the erroneous conclusions that male tsetse have sub-zero mortality rates and that mortality in females is higher than in males. Jackson made the point that this was not the result of random sampling error but rather was due to consistent sampling biases, specific to the fly-found system, which are functions of the age and the sex of the flies being sampled. This was only clear because of the sophisticated nature of the experiment, which served as an early warning about the dangers involved in the interpretation of tsetse sampling data.

There is no a-priori reason to suppose that the biases inherent in other sampling systems are less severe than those seen in the fly-round method. The difficulty lies in measuring the bias, as is clear from Jackson's results. While male capture probability clearly increased with age, it is not possible to provide quantitative estimates of the bias. Estimates of bias depend entirely on *assumed* rates of mortality and dispersal, the very rates we would like to estimate from the data. But those estimates can only be made if the bias is known.

Jackson had, in fact, carried out a prodigious series of mark–recapture experiments prior to his discovery of the age-dependent sampling bias. Subsequent analysis of these data suggests that the anomalies which puzzled Jackson were due in part to age-related changes in capture probability (Hargrove, 1981). In order to provide a good fit to the data it also helped to assume that death and dispersal rates changed with age. Independent evidence has since been presented that such changes do occur throughout adult life (Fig. 7.5). Taking these changes into account complicates the analysis of data on tsetse population dynamics. Nonetheless, a complete understanding of the subject demands that we ultimately make sense of the reason behind, and the effect of, these interesting changes with age in the biology of the tsetse fly.

Conclusions

The preceding review and the next chapter on tsetse distribution suggest that it is now possible to predict with some confidence the behaviour of tsetse populations under a wide variety of circumstances. In particular, on the practical level of fly and disease control, it can be predicted how quickly fly populations grow under different levels of imposed mortality, how quickly they can reinvade cleared areas and what needs to be done in order to prevent this happening. In short, advances in the understanding of population dynamics have led to the ability to predict the outcome of various approaches to tsetse and trypanosomiasis control. As pointed out in a recent review of tsetse control methods and operations (Hargrove, 2003b), it should therefore be possible to move rapidly towards a situation where this knowledge can be used to select the most sensible, cost-effective approach to combating a given disease situation.

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8 Tsetse Distribution

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Introduction

Classification of tsetse

The historical classification of tsetse, based on morphological criteria, divides the species into three groups (Newstead *et al.*, 1924), which have different habitat requirements that are thought to reflect their evolutionary history. The *fusca* group flies (subgenus *Austenina*), with supposedly the most primitive male genitalia, tend to occur in the lowland rainforests of West and Central Africa; *palpalis* group species (subgenus *Nemorhina*) occupy similar forest habitats throughout Africa and also extend into riverine and lakeside forests or the moist areas between such forests; and finally the *morsitans* group of flies (subgenus *Glossina* s.s.) occurs in a variety of savannah habitats lying between the forest edges and deserts.

In each species group there is at least one exceptional species showing structural features or habitat preferences quite different from the others. For example, *Glossina austeni*, a member of the *morsitans* group, has, according to some workers, genetic characteristics (Chen *et al.*, 1999) and genitalia (Dias, 1987) sufficiently distinct from the other members of this group to warrant its separation into a fourth, monospecific subgenus, *Machadomyia*. It shows a resistance to desiccation similar to that of other *morsitans* group species and apparently far in excess of that required in the moist coastal areas of East Africa where it occurs (Bursell, 1958). This and other exceptional species provide challenges to taxonomists, physiologists and evolutionary theorists alike.

After comparing the water relationships of a number of tsetse species, Bursell (1958) concluded:

> … it is not unreasonable to envisage the evolution of tsetse flies in terms of an invasion of progressively more arid environments, concomitant with the regression of tropical rain forest in Central Africa. A retreat of the ancestral habitat, whether occasioned by long-term climatic changes, or brought about by the activities of man, or both, would leave outrunners of gallery forest along major drainage lines, thus enormously extending the front between primitive and prospective habitats, and increasing the opportunity for invasion. The available evidence suggests that conquest of the surrounding semi-arid environment may have occurred independently in at least two of the groups of tsetse flies, namely, in the *fusca* group (*G. longipennis*) and in the *palpalis* group (*G. tachinoides*). In the *morsitans* group all except *G. austeni*, which it is suggested has made a secondary return to the primitive habitat, occupy arid or semi-arid environments; whether this situation has arisen by independent invasion from the ancestral type

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> of habitat, or whether it represents a branching from a common *palpalis–morsitans* stem which took place after the initial spread into semi-arid regions cannot be decided; but in view of the similarity between the male genitalia of the two groups the latter alternative is not unlikely …

More recently a variety of genetic techniques has been employed to illuminate tsetse phylogeny, and these are discussed by Gooding and Krafsur in Chapter 6 of this volume.

Traditional tsetse fly mapping

Tsetse live in habitats that provide shade for developing puparia and resting sites for adults. Their development, like that of many invertebrates, is temperature limited. These factors together enabled the early map-makers to 'guesstimate' the distributional limits of many species of tsetse based on vegetation type, meteorological records and altitude.

The first continental estimates of the distribution of tsetse were compiled and published in 1954 by W.H. Potts, based on individual records dating back to Austen in 1903 and on territorial maps of colonial surveys prepared between 1947 and 1951 (Ford and Katondo, 1975; Katondo, 1984). In 1973 the Inter-African Bureau of Animal Resources (IBAR) began to update these early distribution maps and each African country was invited to submit its most recent *Glossina* distribution maps. These maps, and other information arising between 1951 and 1973, were combined with Potts's original maps to generate new tsetse distribution maps (Ford and Katondo, 1977b) for each of the three subgeneric tsetse groups at a scale of 1:5,000,000. The maps were presented as provisional because of the uncertain quality of the data on which they were based. Ford and Katondo (1975) recommended that they should be used as a foundation for a programme of intensive revision leading to a third edition that never appeared.

Ford and Katondo (1977b) stressed that many records used in the distribution maps were very old; in many cases recorded absences indicated that surveys had not been conducted. Furthermore, map reliability varied between the groups, the *morsitans* and *palpalis* group species being better surveyed and understood than the *fusca* group. In the *morsitans* group, distributions of some species tend to follow the limits of certain vegetation types (e.g. *G. morsitans* and *G. swynnertoni*), whilst those of others are based on spot records and do not necessarily indicate continuity of habitat on the ground (e.g. *G. pallidipes* and *G. longipalpis*). Flies of the *fusca* group are poorly understood in terms of their distribution; with the exception of *G. brevipalpis* and *G. longipennis* in East Africa, which were relatively well surveyed, the distributions of most species within this group were also based on drawing outlines around groups of spot records.

Katondo (1984) provided some details of changes in tsetse distribution reported for a number of countries between 1973 and 1981 and gave revised maps (diagrams only) of the distribution of *G. morsitans* in southern and western Africa, and of *G. tachinoides, G. palpalis* and *G. longipalpis* in western Africa. Updates for species in other countries have also been reported: for southern Africa (Lovemore, 1996), Sudan (Abdel Razig, 1973), Botswana (Davies, 1981), Ethiopia (Fuller, 1978; Hadis *et al.*, 1995), Mozambique (Takken, 1988), Mali (Ashton *et al.*, 1979) and The Gambia (Rawlings *et al.*, 1993).

Since the construction of all of the above maps, remotely sensed satellite data have become available that are able to identify many features of climate, vegetation and altitude, and it should therefore be possible to use such data to produce maps of habitats suitable for flies. This chapter describes the satellite data that are available for such studies, and how they are used to describe tsetse distributions.

The potential of remotely sensed satellite data

All satellite sensor designs show trade-offs between spectral, temporal and spatial resolutions that are determined by the con-

straints of the earth's atmosphere (which is opaque to radiation in many parts of the electromagnetic spectrum) and otherwise reflect the original requirements of the commissioning agencies. The data they provide are thus rarely ideal for entomological or epidemiological studies. Most biological applications to date have used passive satellite sensor data (i.e. reflections or emissions ultimately arising from the sun's activity) though there is increasing interest in radar satellites with active sensors that can produce images even under cloudy conditions. Only passive sensor data were used for the present study.

Spectral resolution

Passive satellite sensors detect reflected sunlight or infrared radiation emitted by all bodies above absolute zero. Data are generally available in three to seven wavebands or channels in the human-visible and near-to-thermal infrared parts of the electromagnetic spectrum (0.3–14 μm wavelengths) and newer types of instruments have many more data channels, e.g. 36 in the MODIS instrument (see below).

Temporal resolution

Higher spatial resolution satellites have a repeat frequency of 11 (Ikonos), 16 (Landsat) or 26 (Satellite pour l'Observation de la Terre, SPOT) days. Orbiting meteorological satellites produce two images per day (National Oceanographic and Atmospheric Administration, NOAA), whilst the Earth Science Enterprise (ESE) project of NASA includes as one component the Terra spacecraft with five different instruments, one of which is the Moderate Resolution Imaging Spectroradiometer (MODIS), providing a 2-day repeat global coverage. Geostationary satellites, such as the European Meteosat and the recently launched Meteosat Second Generation (MSG) satellite, maintain a constant position above a fixed point on the rotating earth and produce images every 30 min (Meteosat) or 15 min (MSG) to monitor weather systems.

Spatial resolution

It is usually convenient to talk of 'high' and 'low' resolution when referring to satellite imagery, with individual picture elements or 'pixels' representing ground areas of less than or at least 0.25×0.25 km, respectively.

High-resolution earth observing satellites produce data with spatial resolutions of 1–4 m (Ikonos-2), 10–20 m (SPOT), 30–120 m (Landsats 1–5) or 15–60 m (Landsat 7). Images have swath widths of ~11 km (Ikonos), ~60 km (SPOT) and 185 km (Landsat). The 'vegetation instrument' on SPOT-4 has a spatial resolution of 1 km and a 2250 km swath width. The potential for using high spatial resolution Landsat imagery to describe tsetse habitats was first realized by Giddings (Giddings and Naumann, 1976). Subsequently, tsetse and land cover or land use studies using either Landsat or SPOT data have been carried out in Nigeria (Bourn, 1983), Zimbabwe (Pender and Rosenberg, 1995; Pender *et al.*, 1997), Kenya (Kitron *et al.*, 1996) and Burkina Faso (De la Rocque, 1997). Studies using such high-resolution imagery tend to identify the habitat types more or less directly and from these infer the likely presence of tsetse flies. Problems arise when habitat classifications derived from the spectral characteristics of known vegetation types in one image are applied to other imagery. If these images were taken at different times of the year, or even at the same time of year but in different years when the seasons are different, the resulting classifications can be quite inaccurate for many land cover types (Pender and Rosenberg, 1995; Pender *et al.*, 1997).

Environmental and meteorological satellites have lower spatial resolutions of 0.25–1.00 km (channel-dependent, MODIS), or 1.1 km (NOAA Advanced Very High Resolution Radiometer, AVHRR) and a correspondingly wider swath width of ~2300–2400 km. Geostationary satellites produce images of the entire earth half-disc with spatial resolutions of 1–8 km (GOES for the Americas), 2.5–5 km (Meteosat 4–6 for Europe/Africa) or 1–3 km (MSG).

Image processing

The quality of all imagery from passive sensors is adversely affected by atmospheric contamination, the most obvious example of which is clouds, although other aerosols are also important. The relatively low revisit frequency of the higher spatial resolution satellites means that they are unable to record cloud-free seasonal changes that are often important determinants of vector-borne disease transmission rates. Despite the poor spatial resolution of data from meteorological satellites, the images are available sufficiently frequently to capture habitat seasonality in a way not possible with the higher spatial resolution satellites. Each image from NOAA-AVHRR or Meteosat is, of course, as contaminated by clouds as are each of the higher spatial resolution images, but there are more of them per month and so they can be combined by selecting for each pixel in turn the highest value from all of those recorded for that pixel in each period of time. This is believed to be the most cloud- or contaminant-free value. The resulting images are called maximum value composites (MVCs) (Holben, 1986).

Data from each of the satellite channels may be used directly in their 'raw' form or may be processed to produce various indices that are related more directly to ground-based meteorological or other, more familiar variables such as soil surface temperatures. Commonly used products include the middle infrared (MIR) band from AVHRR Channel 3 and the land surface temperature (LST, derived from AVHRR channels 4 and 5), both related to the temperature of the earth's surface; the normalized difference vegetation index (NDVI) derived from AVHRR channels 1 and 2 and related to plant photosynthetic activity; vapour pressure deficit (VPD), also derived from AVHRR channels 4 and 5 and effectively a measure of atmospheric drying power; near-surface air temperature (TvX) derived from Land Surface Temperature (LST) and vegetation index measurements; and the Cold Cloud Duration (CCD) from Meteosat that is correlated with rainfall in convective precipitation systems (all reviewed in Goetz *et al.*, 2000; Hay, 2000).

Data processing and application

Monthly composite imagery usually shows strong serial correlations and therefore data redundancy, which may be eliminated in two different ways. Either the data are subjected to principal components analysis (PCA), and the resultant significant principal components are used in analyses (Lillesand and Kiefer, 2000), or the data are subjected to temporal Fourier analysis that describes the cycles of temperature, vegetation growth etc., in terms of annual, biannual, triannual and other cycles with shorter or longer periods (Rogers, 2000). The great attraction of temporal Fourier processing is that it removes data redundancy and produces a set of orthogonal (i.e. uncorrelated) outputs whilst retaining a description of seasonality (lost in PCA) that is of vital interest in vector and disease mapping (Rogers and Williams, 1994; Rogers *et al.*, 1996; Rogers, 2000). One disadvantage of both PCA and temporal Fourier analysis is that they assume stationarity (i.e. constant mean and variance) of the data over time. Trends in data can first be removed by differencing the time series from a moving average spanning a number of annual cycles, and then analysing the detrended time series.

The series of maximum value composite satellite data, either in their original form or transformed using PCA or temporal Fourier methods, form one source of data used in various ways for mapping tsetse distributions. Other data are derived from satellite data either directly (e.g. recent digital elevation surfaces) or indirectly (e.g. an increasing number of land-cover maps are produced by classifying AVHRR data) or else arise from a combination of satellite and ground-based meteorological data (e.g. vapour pressure deficit; Goetz *et al.*, 2000) or ground-based data alone (e.g. the length of growing period (LGP), derived from temperature, rainfall and evapotranspiration meteorological records). The next section outlines the ways in which these data sets may be used to describe tsetse distributions.

Predicting tsetse distributions – theory

Rogers (2000) describes two broad approaches to modelling tsetse distributions: the biological (predictive) approach and the statistical (descriptive) approach. Predictive (sometimes called 'process-based') models aim to capture the population dynamics of a species in terms of the biotic and abiotic mortalities that cause changes around the species' equilibrium value: at least some of these mortalities are driven by environmental conditions that may be detected by satellites. If the combined (biotic plus abiotic) mortality is excessive, then the species cannot survive in that area and its absence is predicted. When the combined mortality is less than the potential fecundity (ignoring immigration and emigration) the species can survive, at a level of abundance determined at least in part by density-dependent processes that do not necessarily have satellite variable proxies. Thus we expect species' distributions to be more easily predicted by satellite data than are species' abundances.

Because of the labour involved in gathering data for the process-based approach, predictive models are generally derived from intensive studies covering only relatively small geographical areas (Rogers, 2000). Descriptive models, on the other hand, look for direct correlations between species presence or absence and a set of predictor variables, and are usually developed over large geographical areas that exhibit a wide range of environmental conditions allowing the definition of the limits of the species in a statistical sense. This section is concerned with the distribution of tsetse over the whole African continent and so statistical modelling is the more appropriate.

Methods for statistical modelling of tsetse distributions are derived from an extensive literature for predicting the distributions of other animal species or of areas of high biodiversity or conservation importance. They can be divided into two broad categories: one in which no assumptions are made about the underlying statistical distributions of the predictor data around their sample means; and the other in which such a statistical distribution (usually multivariate normality) is assumed.

Logistic regression is a commonly used example of a distribution-free method, and is able to describe binary data such as the presence or absence of a species at representative sample sites (the 'training set') that span as much as possible of the entire range of environmental conditions experienced by the species in question, or characterizing its absence (Augustin *et al.*, 1996; Manel *et al.*, 1999). Simple logistic regression assumes, for any single predictor variable, that there is a range of values defining vector absence and a lower or higher range of values defining vector presence: only one change (e.g. presence to absence, or absence to presence) is allowed between these two states for gradual increases in each predictor variable in the analysis. Gaussian logistic regression allows a species to be present (or absent) over intermediate values of each predictor variable and absent (or present) at values both above and below this range: hence two changes are allowed per predictor variable, from absence to presence and back again, or from presence to absence and back again. The output from a logistic regression fit of presence/absence data is probabilistic and the user must choose a threshold probability defining the borderline between predicted presence and absence in each analysis; this is generally done in such a way as to maximize the goodness of fit (or some other, more desirable outcome, such as to optimize either the sensitivity or specificity). Probability thresholds selected in this way appear to vary with the relative sample sizes of presence and absence data in the training set. Logistic regression does not easily allow descriptions of predicted variables with more than two classes (e.g. multiple categories of presence and absence, or more than two categories of abundance) but can be very successful within these limitations (Cumming, 1999). Other examples of distribution-free approaches, all allowing for $n > 2$ categories, include k-nearest neighbour, tree-based classification or neural network methods (all briefly reviewed with respect to tsetse mapping in Williams *et al.*, 1992).

Another approach uses the observed distribution of presence/absence data on a continuous environmental variable to select an optimum threshold value which most

accurately distinguishes presence from absence (this is very similar to a one-dimensional tree-based classification). Training set observations for presence and absence are separately cumulated from the highest or lowest value of the environmental variable and an appropriate threshold is selected giving the greatest predictive accuracy: for obvious reasons this is called the method of optimal threshold distribution functions (OTDF) (Robinson *et al.*, 1997b).

When multivariate normality (or some other easily defined statistical distribution) can be assumed for the training sample data, both linear and non-linear single and multiple regression methods may be applied, though these often give disappointing results because they are frequently unable to describe the most extreme (highest or lowest) values in a data set. An alternative method, usually giving better results, is based on the family of discriminant analysis techniques where, in its simplest form, presence is assumed to be defined by one multivariate normal distribution and absence by another. The technique then defines an appropriate equiprobability dividing line or plane between the two distributions. These techniques are easily extended to multiple categories of both presence and absence (or abundance) in a single analysis. The output of discriminant analysis models may be either a definite statement of presence or absence (dependent upon the environmental conditions in the area concerned) or else the probabilities of these two outcomes; the latter is obviously preferable since it also identifies areas of only marginal suitability for species' occurrence. Discriminant analytical methods are often criticized for their assumptions of statistical normality – assumptions that are seldom met in real-world situations across large areas of any species' distributions – but these criticisms can generally be overcome by subdividing the training set before analysis using clustering algorithms applied to the predictor variable data set. These algorithms give a user-specified number of clusters, each of which is more likely to be normally distributed than is the unclustered data set. Discriminant analysis can then be applied to the multiple-cluster data set (Rogers and Randolph, 1993; Rogers, 2000).

Mapped outputs record the similarity of each pixel in an entire set of satellite images to the satellite-determined environmental characteristics of the training set sites. Obviously for this to be successful the training set should have captured the entire range of conditions present throughout the area for which predictions will eventually be made. This is not always the case, and it is then preferable to identify in the output image a separate category of 'no prediction' for those areas where the environmental conditions are some specified minimum distance (in multivariate space) away from any of the training set clusters.

It is important to stress that the methods outlined in this section do not give predictions of the actual distribution of any species but simply of places where environmental conditions are similar to those from which the species has actually been recorded. Such areas are deemed to be climatically suitable for the species but are not necessarily occupied by it. They should be regarded as potentially at risk of invasion by the species, or could support the species if it arrives there somehow and as long as other necessary conditions for existence (e.g. availability of host animals) are met.

Predicting tsetse distributions – practice

Methods for analysing tsetse distribution using AVHRR satellite data were developed in a series of studies of *G. morsitans* and *G. pallidipes* in Kenya, Tanzania and Zimbabwe (Rogers and Randolph, 1993), of *G. morsitans, G. longipalplis, G. palpalis, G. tachinoides, G. pallicera, G. fusca, G. nigrofusca* and *G. medicorum* in Burkina Faso and Côte d'Ivoire (Rogers *et al.*, 1996) and of the two subspecies of *G. morsitans* and *G. pallidipes* in Zambia (Robinson *et al.*, 1997a,b). Most of these studies relied on one form or other of discriminant analysis, but Robinson *et al.* (1997a) used optimal threshold distribution functions, which have also been used more recently to distinguish the habitats of the three major groups of tsetse (i.e. *fusca, palpalis* and *morsitans*) (McDermott *et al.*, 2004) using as the predictor variable the LGP,

which defines a period when crop production is possible, and is thus a good composite indicator of 'greenness'.

Linear discriminant analysis, using a single cluster each for presence and absence, and equal prior probabilities during classification (see below), gave predictive accuracies of 80% or greater in East, West and southern Africa. These studies revealed several important features of local and regional fly distribution. Firstly, only a single climatic variable appears to determine tsetse distribution at the edge of its continental range (e.g. the maximum of the mean monthly temperature for *G. morsitans* in Zimbabwe). Secondly, more than one climatic variable is required to describe distributions well within the continental range (e.g. in Kenya and Tanzania). In such areas, one variable excludes the flies from some places and other variables are more important elsewhere. Thirdly, the average temperature difference between areas of fly presence and absence may be less than 1°C (Rogers and Randolph, 1993). Fourthly, in West Africa thermal variables tend to be more important in describing distributions than are either vegetation index (NDVI) or rainfall (CCD) variables (Rogers *et al.*, 1996). Fifthly, again in West Africa, rainfall (i.e. CCD) data were relatively more important in describing the 'abundance' of flies (i.e. flies per trap per day) than they were for describing their distributions, suggesting that, in this region, distributions are limited by temperature and abundance by rainfall (Rogers *et al.*, 1996). Finally, the two southern African subspecies of *G. morsitans* in Zambia appear to respond very differently to climatic variables, but the distribution of each is described well by multivariate methods (Robinson *et al.*, 1997a,b,). Analyses allowing the use of different covariance matrices for each cluster (in parameter space) of presence and absence (i.e. the maximum likelihood solutions), and variable a priori probabilities, in general provided better fits than those that did not (Rogers *et al.*, 1996; Robinson *et al.*, 1997a). This suggests that flies show adaptations to regional climate (deduced from the increased accuracy obtained with clustered data) (see also Rogers, 1990; Baylis and Nambiro, 1993) and that, in different regions, different sets of variables appear to be important in determining fly distributions.

Habitat signatures in the thermal, vegetation and rainfall (CCD) channels were used in conjunction with ground data in the interpretation of a unique data set from Togo that included contemporary observations on flies, disease and cattle at a spatial resolution of 0.125 degrees across the entire country (Hendrickx *et al.*, 1999a,b, 2000, 2001) In selected sites, fly populations and cattle disease were monitored monthly, so that data on both spatial and temporal patterns of vectors and disease were recorded. This research confirmed and extended the relationships previously found elsewhere in West Africa, between mean trap catches of *G. tachinoides* and *G. palpalis* and remotely sensed AVHRR NDVI (Rogers and Randolph, 1991), with further relationships between fly abundance and both CCD and surface temperature estimates. The countrywide distributions of these two species and of *G. m. submorsitans* and *G. longipalpis* were subjected to non-linear discriminant analysis involving Fourier processed AVHRR data and were described with accuracies exceeding 90%. Fly abundance, divided into three classes of low, medium and high, were described with accuracies > 70% for *G. tachinoides*, but only 56% for *G. palpalis* (the only two species for which abundance data were available).

The study also investigated the effects on accuracy both of using a subsample of the entire dataset and of changing the numbers of predictor variables used. As might be expected, accuracy tended to diminish with a smaller proportion (and therefore number) of observations in the training set but, within limits, increased with an increasing number of predictor variables. The accuracy of predicting samples not included in the training set was maximized with fewer predictor variables than were required to maximize the predictions of the training set itself; this suggests there is a danger of 'over-fitting' a training set so that the results are less reliably generalized to predict conditions in unsampled area (Hendrickx *et al.*, 2001). The study also compared the actual fly distribution in Togo with predictions previously made for Togo on the basis of less accurate tsetse distri-

bution maps for Côte d'Ivoire and Burkina Faso and satellite data for all three countries (Rogers *et al.*, 1996). These previous predictions for Togo were rather poor; whether this was a problem of a rather unsatisfactory training set (old and possibly outdated maps) or represents a genuine problem of extending such analyses from one place to another requires urgent investigation.

One criticism of the environmental envelope approach to mapping distributions is that it ignores the influence of biotic factors that are so important in determining the abundance of flies within their geographical limits (Davis *et al.*, 1998). We suspect that in most cases there are very few flies at the edges of distributions and therefore that biotic factors are much less important than are abiotic ones. The fact that we can produce such good fits without explicitly modelling biotic factors supports this idea, though it is also possible that some of the satellite data channels are acting as surrogates for un-quantified biotic factors.

The original Ford and Katondo (1977a,b) tsetse maps were recently described using logistic regression techniques and a set of temporal Fourier processed AVHRR satellite data (Wint and Rogers, 2000). To achieve better fits to the samples of 12,000 data points for each species, the analyses were carried out at four geographical levels – continental, regional, national and by a 50-category ecozone map (the latter determined by clustering the satellite data that were used in the model) – and the one for each species giving an accuracy of 90% or greater for the largest area was selected as the 'best'. Fifteen variables from each of five satellite data channels, and a further 15 ancillary variables, in total 90 variables, were used in the analyses, with no restriction on the numbers that were eventually selected for each model.

Assessing the accuracy of predictions

Assessing predictive accuracy is an important part of variable selection and model refinement. Of a wide variety of possible methods (well reviewed in Fielding and Bell, 1997) the method based on the kappa statistic, κ, is commonly used for situations with a variable number of observations in the presence and absence class(es) (Ma and Redmond, 1995). Kappa is calculated from the matrix of results where the number of observed presences and absences occupy the rows and the predicted presences and absences fill the columns. Thus all possible combinations of observed and predicted results are present in the body of this matrix, which is somewhat appropriately called the 'confusion matrix'. The marginal row and column totals are used to calculate the results expected assuming no skill in the prediction method, and the model outcome is compared with this result to calculate κ, which theoretically can vary between -1 and $+1$ but in practice tends to vary between 0 (no skill, results no better than random) and 1 (perfect skill, with all observations correctly classified). Landis and Koch (1977) suggest the following ranges of agreement for the kappa statistic: poor, $\kappa < 0.4$; good, $0.4 < \kappa < 0.75$; and excellent, $\kappa > 0.75$. Confidence intervals can be attached to the kappa statistic (Ma and Redmond, 1995), apparently making it suitable for hypothesis testing. It can be shown, however, that κ is sensitive both to overall prevalence and to the distribution of both correct and incorrect observations within their respective classes (Lantz and Nebenzahl, 1996), so that comparisons between models for species of very different prevalence, or with different error distributions, should be made with caution.

Ideally the training set should be divided, with half used to develop the covariance matrices and the other half used to test the accuracy of the predictions. Frequently, however, training data are scarce and the entire data set must be used in the training exercise. The resulting predictions will tend to inflate estimates of the accuracy of the techniques, though experience with large training sets suggests this inflation is slight.

Materials and Methods

Fly distribution data

The digitized Ford and Katondo maps were used in the present analysis (Ford and

Katondo, 1977a,b), with appropriate subdivision where necessary to separate subspecies for separate analyses (Table 8.1). Thus, for example, the paper maps show a single coverage for *G. palpalis* in West Africa, with no indication of the division into *G. p. gambiensis* and *G. p. palpalis*. We divided this map on the basis of a map in Challier's thesis (Challier, 1973) but excluding a rather broad band along the separation line indicated by Challier, because observations since 1973 suggest that this line has shifted as a result of environmental change (Hendrickx, 1999); thus none of the training set observations for either species was drawn from the excluded broad band. For the separation of subspecies of other species the geographical information given in Jordan (1986) was used. The original Ford and Katondo paper maps were drawn in the Miller Oblated Spheroid projection (once commonly used for maps of Africa) and reprojected to longitude/latitude format before use. These coverages, in vector (i.e. polygon) format, were turned into raster (i.e. pixel-based) format at 0.05 degrees resolution for use in the analyses.

Table 8.1. The 31 tsetse (*Glossina*) species and subspecies based on Ford and Katondo (1977) and Jordan (1993). Abbreviations are those used in later tables. Those with common numbers in the last two columns indicate no distinction in Ford and Katondo (1977) and Jordan (1993). No prediction was possible for *Glossina frezili*.

Group	Species	Abbreviation	Source	Ford	Jordan
fusca	*G. brevipalpis*	gbrevi	Newstead, 1910	5	15
	G. fuscipleuris	gfscpl	Austen, 1911	6	7
	G. fusca fusca	gffusc	Walker, 1849	**1**	1
	G. fusca congolensis	gfcong	Newstead & Evans, 1921	**1**	5
	G. haningtoni	ghanin	Newstead & Evans, 1922	10	6
	G. longipennis	glngpe	Corti, 1895	3	14
	G. medicorum	gmedic	Austen, 1911	7	4
	G. nashi	gnashi	Potts, 1955	12	8
	G. nigrofusca nigrofusca	gnnigr	Newstead, 1910	**4**	2
	G. nigrofusca hopkinsi	gnhopk	van Emden, 1944	**4**	13
	G. severini	gsever	Newstead, 1913	8	11
	G. schwetzi	gschwe	Newstead & Evans, 1921	9	9
	G. tabaniformis	gtaban	Westwood, 1850	2	3
	G. vanhoofi	gvanho	Henrad, 1952	11	12
	G. frezili		Goutex, 1987		10
palpalis	*G. caliginea*	gcalig	Austen, 1911	17	20
	G. fuscipes fuscipes	gffsci	Newstead, 1910	14	22
	G. fuscipes martinii	gfmart	Zumpt, 1935	15	24
	G. fuscipes quanzensis	gfquan	Pires, 1948	16	23
	G. palpalis palpalis	gppalp	Rob.-Desvoidy, 1830	**13**	18
	G. palpalis gambiensis	gpgamb	Vanderplank, 1949	**13**	16
	G. pallicera pallicera	gpplic	Bigot, 1891	18	19
	G. pallicera newsteadi	gpllic	Austen, 1929	19	21
	G. tachinoides	gtachi	Westwood, 1850	20	17
morsitans	*G. austeni*	gauste	Newstead, 1912	24	28
	G. longipalpis	glngpa	Wiedemann, 1830	21	26
	G. morsitans morsitans	gmmors	Westwood, 1850	**22**	30
	G. morsitans submorsitans	gmsubm	Newstead, 1910	**22**	25
	G. morsitans centralis	gmcent	Machado, 1970	**22**	27
	G. pallidipes	gpalli	Austen, 1903	23	26
	G. swynnertoni	gswynn	Austen, 1923	25	29

Satellite data

NOAA-AVHRR data at 8 km spatial resolution were obtained from NASA's Pathfinder program (James and Kalluri, 1994) for the years 1982–1994 inclusive. AVHRR channel 3 (MIR) and NDVI data were used directly and an LST data layer was produced from AVHRR channels 4 and 5 (Price, 1984). Finally a series of 8 km CCD images derived from Meteosat was obtained from the FAO ARTEMIS program, Rome, for the period 1989–1998 inclusive. All imagery was temporal Fourier processed (see next section), taking care to exclude both high and low extreme values in each data series (when monthly values for any pixel departed from the initial Fourier fit by more than a specified value, data for these months were interpolated from 'valid' data before and after this month, and the Fourier transform reapplied). Finally all Fourier imagery was reprojected to longitude/latitude format at a resolution of 0.05 degrees. The mean, amplitudes and phases of the annual, biannual and triannual components, the maximum, minimum and variance of each variable were available for selection in the analyses.

Data processing

Temporal Fourier analysis

Temporal Fourier analysis describes variations through time of satellite signals as the sum of a series of sine curves with different frequencies and amplitudes. It is applicable to regularly collected data such as maximum value composite monthly AVHRR data, $\{x_t\}$, collected over 1 or more years (for simplicity, temporal Fourier analysis should only be applied to entire years of data, not to partial years).

The Fourier series representation of $\{x_t\}$ is found from the following:

$$x_t = a_0 + \sum_{p=1}^{N/2-1} [a_p \cos(2\pi pt/N) + b_p \sin(2\pi pt/N)] + a_{N/2} \cos \pi t, \; (t = 1,2,\ldots N) \tag{8.1}$$

with coefficients $\{a_p, b_p\}$ defined as follows:

$$\left.\begin{aligned} a_0 &= \bar{x} \\ a_{N/2} &= \Sigma(-1)^t x_t/N \\ a_p &= 2[\Sigma x_t \cos(2\pi pt/N)]/N \\ b_p &= 2[\Sigma x_t \sin(2\pi pt/N)]/N \end{aligned}\right\} \; p = 1,\ldots N/(2-1) \tag{8.2}$$

Despite the rather daunting appearance of Equation 8.1 its interpretation is relatively straightforward. For any particular value of p in the summation term Σ, the terms in the square bracket define a single sine curve with a period of N/p time units (frequency = p/N). This is because the sum of a cosine and a sine curve with the same argument ($2\pi pt/N$ in Equation 8.1) is another sine curve of the same period but with a different amplitude and displaced in time by an amount dependent on the relative contributions of the cosine and sine curves to the total. These relative contributions are determined by the coefficients a_p and b_p, respectively, which therefore fix the amplitude and timing of the peak(s) of the combined curve within the interval from $t = 1$ to N. Equation 8.2 simply describes how to estimate these important coefficients from the sample data, and also suggests that there are $(N/2)$ pairs of coefficients of this sort ($a_{N/2}$ can be regarded as the final 'pair' of coefficients, since in this final term $\sin \pi t$ is always zero, and $b_{N/2}$ is therefore also zero), implying that there are also $(N/2)$ different sine curves (each with a different period of oscillation) in the description of x_t in equation 1. Each of these curves is called a harmonic, so there are $N/2$ harmonics overall.

$$\begin{aligned} &R_p = \text{the amplitude of the } p\text{th harmonic} = \sqrt{(a_p^2 + b_p^2)} \\ &\phi_p = \text{the phase of the } p\text{th harmonic} = \tan^{-1}(-b_p/a_p) \end{aligned} \tag{8.3}$$

and R_p and ϕ_p thus uniquely define the amplitude and position of the pth harmonic. Further details of time series analysis may be found in Chatfield (1980) and Diggle (1990).

The inverse Fourier transform is a way of reconstructing the original signal from its (forward) Fourier transform. Since the calculation of the forward transform (Equation 8.2) uses the same expressions (sine and cosine curves) as the inverse transform (Equation 8.1), a single algorithm may be used, with care, for both forward and inverse Fourier transforms of data sets.

Temporal Fourier analysis has a number of useful characteristics. The sum of all of the harmonics exactly describes the original time series. This means that the harmonics are orthogonal to (i.e. uncorrelated with) each other. The variance of each harmonic (for a sine curve this is simply the square of its amplitude) therefore contributes additively to the total variance and each harmonic may be examined in turn to determine its contribution to overall variance. Harmonics of low amplitude may be dropped from reconstructions of any signal from its Fourier transform, thereby achieving efficient data ordination (the reduction of a data set without severe loss of information). Similarly the omission of high-frequency harmonics often achieves noise reduction, a useful operation with time series of remotely sensed data. In work on tsetse and other vectors the harmonics with periods of 12, 6 and 4 months are generally the most useful descriptors of habitat variability and these are here called the annual, biannual and triannual Fourier components. Figure 8.1 illustrates these three important Fourier components of the LST and NDVI time series of satellite data from a single point in northern Côte d'Ivoire, West Africa, and shows how their sum provides a smoothed description of the raw data. Figure 8.1 shows how Fourier analysis captures the different shapes of the NDVI and LST cycles and how it can quantify the time delay between the peak vegetation activity and peak temperatures each year.

Plate 4 shows examples of wet and dry season pictures from two sites in West Africa, one in the forest zone and one in the savannah zone, and the NDVI satellite signals and derived Fourier variables for both sites. The forest site shows relatively few seasonal changes (photographs) and little satellite signal variation around the mean value, but signs of stronger biannual than annual cycles (probably due to the annual advance and retreat of rainfall associated with movement of the Inter-tropical Convergence Zone). The savannah site, *c.* 600 km north of the forest site, shows obvious seasonal changes and an NDVI signal that is much more variable and dominated by the annual Fourier component. Plate 5 shows a series of images of Africa with the mean, annual amplitude and phase of the NDVI signal shown separately in the red, blue and green channels of the computer screen, and then together, as they are normally displayed.

Analytical methods

In its simplest form, discriminant analysis assumes both multivariate normality and a common within-group covariance of the variables for all points defining vector or disease presence and absence. Covariances are estimated from the training set. Means of multivariate distributions are referred to as centroids and are defined by mathematical vectors $\{\bar{\mathbf{x}}_v\}$ where v is the number of dimensions (= variables) (here we follow the usual convention that heavy type indicates a row or column vector, or a matrix, and the bar above a variable indicates the mean). The Mahalanobis distance, D^2, is the distance between two multivariate distribution centroids, or between a sample point and a centroid, and is defined as follows:

$$D^2_{12} = (\bar{\mathbf{x}}_1 - \bar{\mathbf{x}}_2)' \mathbf{C}^{-1}_w (\bar{\mathbf{x}}_1 - \bar{\mathbf{x}}_2) = \mathbf{d}' \mathbf{C}^{-1}_w \mathbf{d} \qquad (8.4)$$

where the subscripts now refer to groups 1 (e.g. for vector absence) and 2 (e.g. for vector presence), $\mathbf{d} = (\bar{\mathbf{x}}_1 - \bar{\mathbf{x}}_2)$ and $\mathbf{C}^{-1}_w$ is the inverse of the within-groups covariance (dispersion) matrix (Green, 1978) (the single quotation, ′, indicates the transpose of a row or column vector). Thus D^2 is the Mahalanobis distance between the sample centroids adjusted for their common covariance. Equation 8.4 may be used in a number of ways. Firstly, it may be used to assign new data points to one or other category (of presence or absence) by examining the value of D^2 between each point and each of the training-set defined centroids. The point is then assigned to the group for which D^2 is a minimum. Secondly, the equation may be used to calculate the probability with which each data point belongs to each of the training set groups. This involves defining the position of the point within each multivariate distribution around each centroid (most easily

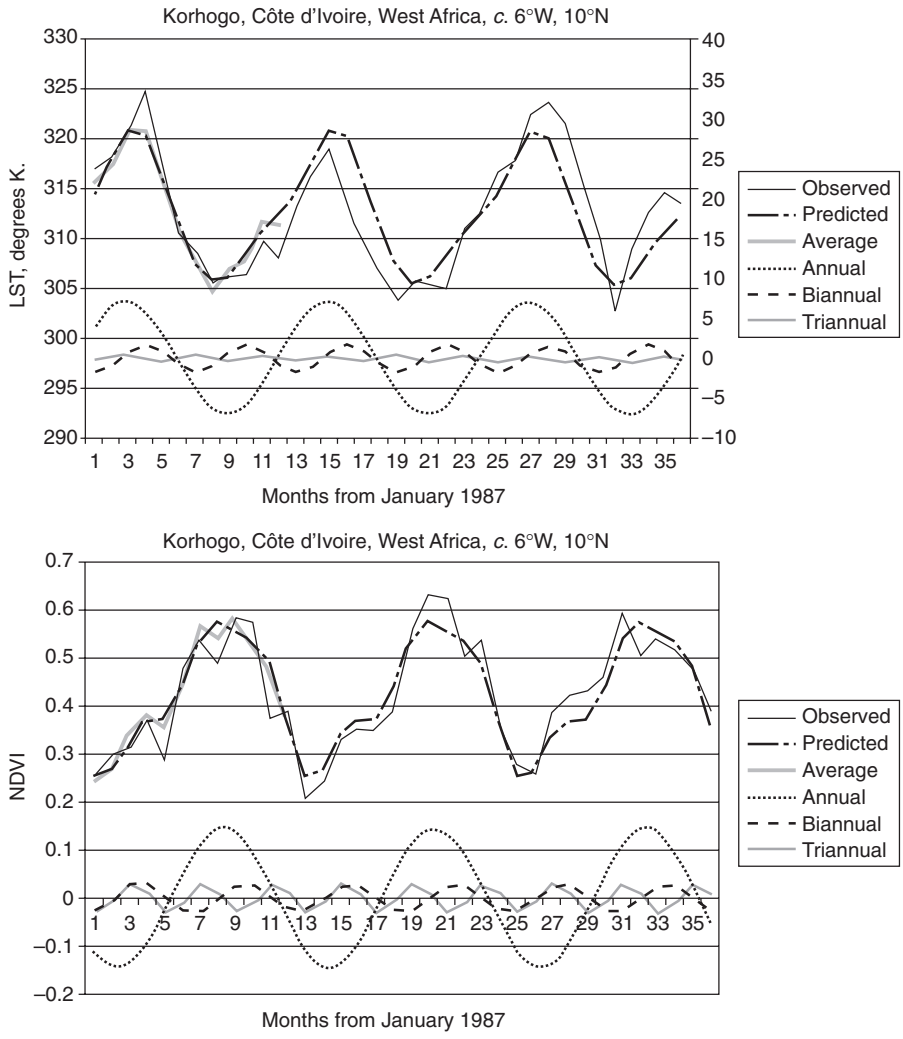

Fig. 8.1. Temporal Fourier analysis of satellite data. Land surface temperature (LST) and Normalized Difference Vegetation Index (NDVI) from NOAA satellites for a 3-year period at a site near Korhogo, northern Côte d'Ivoire (observed lines), the average signal over this time (average lines shown in the first year only) and their temporal Fourier descriptions (predicted lines) in terms of the annual, biannual and triannual cycles (the annual, biannual and triannual curves that sum to give the predicted lines). The predicted lines successfully describe the average cycles of LST and NDVI variation throughout the year. The annual to triannual cycles are shown as variations around their mean values (right-hand scale for LST).

achieved by calculating D^2 which is distributed as χ^2 with (v-1) d.f., where v, as before, is the number of variables defining each centroid). In general these measures are normalized by dividing each by the sum of all measures (i.e. the sum of the probabilities across all classes in the training set) to give posterior probabilities, defined as follows:

$$P(1|\mathrm{x}) = \frac{p_1 e^{-D^2_1/2}}{\sum_{g=1}^{2} p_g e^{-D^2_g/2}}$$

and

$$P(2|\mathrm{x}) = \frac{p_2 e^{-D^2_2/2}}{\sum_{g=1}^{2} p_g e^{-D^2_g/2}} \qquad (8.5)$$

where P(1|x) is the posterior probability that observation **x** belongs to group 1 and P(2|x) the posterior probability that it belongs to group 2 (Green, 1978) (the exponential terms in Equation 8.5 are those of the multivariate normal distributions defining groups 1 and 2; all other terms of the multivariate distributions are the same in numerator and denominator and therefore cancel out) (Tatsuoka, 1971). In Equation 8.5, p_1 and p_2 are the prior probabilities of belonging to the same two groups, respectively, defined as the probabilities with which any observation might belong to either group given prior knowledge or experience of the situation. In the absence of any prior experience it is usual to assume equal prior probability of belonging to any of the groups; in the simple case of two-group discrimination, therefore, $p_1 = p_2 = 0.5$. Great care should be taken with the normalization step of Equation 8.5 since it assumes that observation **x** must come from one or other of the classes defined in the training set data. This emphasizes the importance of carefully selecting the training set to be representative of all possible presence and absence sites, not just some of them. In general it is advisable to produce, along with the output image of predicted probabilities, a second image of the Mahalanobis distance to the nearest cluster in the training set, i.e. the cluster to which each pixel is assigned. This image can then be examined to find areas where the Mahalanobis distances are very large and therefore where predictions are likely to be inaccurate.

As indicated earlier, Equations 8.4 and 8.5 should be modified when the assumption of common covariances is obviously invalid. Not only may areas of presence and absence differ in their environmental characteristics, but different parts of a species range may also show more subtle differences, requiring separate multivariate descriptions of their climatic conditions. Separate cluster analysis of the environmental variables defining presence and absence was employed here using the *k-means cluster* algorithm of the SPSS statistical package (© SPSS Inc., Chicago, Illinois). Each cluster (either for presence or absence) was then treated as a separate multivariate normal distribution, with its own covariance characteristics, and the posterior probabilities were calculated by summing across all distributions. In the case of two groups only (one for presence and one for absence), Equation 8.5 is then modified as follows:

$$P(1|\mathbf{x}) = \frac{P_1|\mathbf{C}_1|^{-1/2} e^{-D^2_1/2}}{\sum_{g=1}^{2} p_g|\mathbf{C}_g|^{-1/2} e^{-D^2_g/2}}$$

and (8.6)

$$P(2|\mathbf{x}) = \frac{P_2|\mathbf{C}_2|^{-1/2} e^{-D^2_2/2}}{\sum_{g=1}^{2} p_g|\mathbf{C}_g|^{-1/2} e^{-D^2_g/2}}$$

where $|\mathbf{C}_1|$ and $|\mathbf{C}_2|$ are the determinants of the covariance matrices for groups $g = 1$ and 2, respectively. The Mahalanobis distances in Equation 8.6, calculated from Equation 8.4, are evaluated using the separate within-group covariance matrices $\mathbf{C}_1$ and $\mathbf{C}_2$ (Tatsuoka, 1971). When there is more than a single class of presence or absence data (e.g. multiple clusters) the summation in the denominators of Equation 8.6 covers the entire set of $g > 2$ groups and there are as many posterior probability equations as there are groups. With unequal covariance matrices the discriminant axis (strictly speaking a plane) that separates the two groups in multivariate space is no longer linear, and Equation 8.6 then effectively defines the maximum likelihood solution to the problem (Swain, 1978).

Clustering was usually beneficial in the analyses described here and increased the fit of the discriminant analysis models, but it should never be allowed to produce clusters with fewer than a minimum number of data points, since this results in badly defined covariance matrices (which sometimes cannot be inverted) and inaccurate predictions. A single faulty covariance matrix can affect all outputs of discriminant analysis models.

There is no obvious rule about the use of expected or observed prior probabilities in Equations 8.5 or 8.6. Use of observed (generally training set) prior probabilities shifts the equiprobability contours towards the smaller groups, resulting in a larger proportion of assignments to the classes with larger group sizes. This shift generally increased predictive accuracy in the present case.

Further details of multivariate analysis may be found in several useful texts (Tatsuoka, 1971; Green, 1978; Krzanowski and Marriott, 1995; Legendre and Legendre, 1998).

The analysis proceeded as follows. The tsetse distribution maps (Ford and Katondo, 1977a) were obtained in digital form from the International Laboratory for Research on Animal Diseases (ILRAD, now the International Livestock Research Institute, ILRI) and were reprojected from the Miller Oblated Spheroid projection to geographical (i.e. latitude/longitude) coordinates using inverse projection formulae in Snyder's manual of map projections (Snyder, 1987). They were then turned into raster images of the same extent and resolution as the satellite data, using the IDRISI GIS package (© The IDRISI Project, Clark Labs, Worcester, Massachusetts). A regular sampling grid was then set up, with sample points every 5 pixels (i.e. every one-quarter of one degree, equivalent to a distance of about 20–25 km at the equator), sampled every fifth row (with alternate rows offset by 2 pixels) within the area of 20°N to 20°S, and every 10 pixels and rows (offset by 5 pixels) outside this area within Africa. A land–water mask image ensured that sample points falling into the sea or inland lakes were excluded from the sample. This sample grid, involving a total of over 29,000 points, was used to select presence/absence sites from the raster images of each tsetse species, and was also used to sample the corresponding satellite imagery. A single database was constructed for all tsetse species.

Before discriminant analysis of the distribution of each species, the data for that species were subjected to the *k-means cluster* algorithm of the SPSS program. SPSS scripts were written to produce one to eight clusters each for areas of presence and absence. In order to overcome the potentially serious problem of data 'outliers' (points with extreme values in one or more satellite channels), clustering occurred in two steps. After the first step, the standard deviation of each data point from its assigned mean for each variable was calculated and points falling more than six standard deviations away from any mean were excluded from the analysis, which then reclustered the remaining data again and calculated means (i.e. centroids) and covariances for the new clusters. This information was then available for custom programs that carried out the discriminant analysis. As indicated above, there is a variety of criteria by which variables might be selected during analysis. In the analyses here, the kappa statistic was used to select no more than ten predictor variables in a step-wise inclusion fashion, to describe the distribution of each tsetse species. At each step, each variable was examined in turn for its ability to maximize κ, which was calculated *after* combining all predictions of absence and all of presence into a 2×2 table, regardless of the numbers of clusters of absence or presence actually used. This thus highlights the technique's ability to distinguish presence and absence only, rather than its ability correctly to assign observations to one of a number of clusters representing either absence or presence. In each run of the model the number of presence and absence clusters was varied (with up to eight each of presence and absence), and final selection of the number of clusters to use was based on maximizing the κ value achieved across all combinations of cluster numbers. Other accuracy statistics calculated included the overall percentage correct, the percentage of false positives (i.e. false predictions of presence) and false negatives (false predictions of absence), sensitivity (the accuracy of describing presences only) and specificity (the accuracy of describing absences only), though none of these is as good a measure of overall accuracy as is the kappa statistic.

The variables selected for the best model for each species/subspecies were then given a ranking score (1 for the first, 10 for the tenth, and 11 for all those not used in each model), and the average score for each variable was calculated from all 30 species' models. The variable with the lowest average score (DEM) was therefore the variable with the highest average ranking across all species, and was arguably the 'most' important in determining the distribution of tsetse in Africa. The top ten variables of this sort were used to generate a matrix of average multivariate distances between species (i.e. by applying Equation 8.4 and the

species-specific inverse covariance matrix for each pair of species in turn, averaging the two resultant Mahalanobis distances). This matrix thus represents the average pair-wise differences in the habitats of all tsetse species in Africa, in terms of the environmental variables that are most important in determining tsetse distributions. The matrix was put through the hierarchical cluster analysis of SPSS (using the average linkage option) to generate a dendrogram of environmental cluster linkages between the species: on this dendrogram, species that are linked at smaller distances occur in more similar habitats than those linked at larger distances. If tsetse evolution has been directed or channelled by the abiotic environment, it might be expected that hierarchical clustering using environmental data will group species according to their taxonomic relationships.

Results

A description of the variables used in the analyses is given in Table 8.2. Most of these variables were derived from temporal Fourier processing of the individual satellite data layers, such as LST or NDVI, but a few were composites, such as the phase difference between thermal and NDVI Fourier variables. The ten variables selected to describe each species are given in Table 8.3, together with the accuracies of these descriptions and the numbers of clusters of presence and absence that were chosen to give these results. Table 8.3 shows that, in terms of the overall figure (% correct), all models exceeded 95% accuracy. Kappa, however, is much more variable, ranging from 0.37 for *G. austeni* to 0.92 for *G. haningtoni*. Overall, and using Landis' criteria for categorizing accuracy (Landis and Koch,

Table 8.2. Satellite and other variables used in the analysis of tsetse distributions.

Variable	Description
DEM	Elevation, hundreds of metres
pa03a0ll	AVHRR Channel 3, middle infrared, mean value, °C
pa03p1ll	AVHRR Channel 3, phase of the annual cycle, Decimal months
pa03a1ll	AVHRR Channel 3, amplitude of the annual cycle, °C
pa03p2ll	AVHRR Channel 3, phase of the biannual cycle, Decimal months
pa03a2ll	AVHRR Channel 3, amplitude of the biannual cycle, °C
pa03p3ll	AVHRR Channel 3, phase of the triannual cycle, Decimal months
pa03a3ll	AVHRR Channel 3, amplitude of the triannual cycle, °C
pa03mxll	AVHRR Channel 3, maximum of Fourier fitted function (annual+biannual+triannual), °C
pa03mnll	AVHRR Channel 3, minimum of Fourier fitted function (annual+biannual+triannual), °C
pa03vrll	AVHRR Channel 3, variance of the raw data,°C^2
pa07a0ll–pa07vrll	AVHRR derived Land Surface Temperature (LST), description otherwise as for channel 3, °C etc.
pa14a0ll–pa14vrll	AVHRR derived Normalized Difference Vegetation Index (NDVI), description otherwise as for Channel 3, no units, etc.
pa20a0ll–pa20vrll	AVHRR derived Vapour Pressure Deficit (VPD), description otherwise as for Channel 3, mbar, etc.
F025a0ll–F025vrll	Meteosat Cold Cloud Duration (CCD), description otherwise as for Channel 3, hours per month, etc.
pndcdmr	Ratio of NDVI/CCD means, per h
pndthmr	Ratio of NDVI/AVHRR Channel 4 means, per °K
patnthnd	Arctan(AVHRR Channel 4 /NDVI), degrees
pndthar	Ratio(pa14a1ll/pa07a1ll), per °C
pndcdp1	pa14p1ll-F025p1ll, Decimal months
pndthp1	pa14p1ll-pa07p1ll, Decimal months

Table 8.3. List of the 30 tsetse species and subspecies for which predictions were made and their abbreviation, with (a) the numbers of absence and presence clusters in the best model for each, and model details, and (b) the top ten variables selected. Explanations of the variable codes are provided in Table 8.2.
(a)

Group	Species	Abbreviation	Absence	Presence	Kappa	Correct (%)	False +ve%	False −ve%	Sensitivity	Specificity
fusca	*G. brevipalis*	gbrevi	6	6	0.52	99.07	0.67	0.26	0.67	0.99
	G. fuscipleuris	gfscpl	4	2	0.57	98.38	1.45	0.17	0.87	0.99
	G. fusca fusca	gffusc	6	2	0.87	99.31	0.55	0.14	0.95	0.99
	G. fusca congolensis	gfcong	5	2	0.82	97.59	1.6	0.81	0.88	0.98
	G. haningtoni	ghanin	7	1	0.92	99.56	0.22	0.22	0.93	1.00
	G. longipennis	glngpe	6	2	0.48	99.53	0.36	0.11	0.66	1.00
	G. medicorum	gmedic	5	2	0.87	99.46	0.46	0.08	0.96	1.00
	G. nashi	gnashi	5	1	0.59	99.90	0.06	0.04	0.66	1.00
	G. nigrofusca nigrofusca	gnnigr	6	2	0.44	98.99	0.88	0.13	0.75	0.99
	G. nigrofusca hopkinsi	gnhopk	3	2	0.87	99.97	0.02	0.01	0.91	1.00
	G. severini	gsever	5	2	0.69	99.87	0.08	0.05	0.75	1.00
	G. schwetzi	gschwe	3	2	0.79	99.38	0.58	0.04	0.96	0.99
	G. tabaniformis	gtaban	6	2	0.90	98.06	1.12	0.82	0.92	0.99
	G. vanhoofi	gvanho	5	1	0.62	99.96	0.03	0.01	0.75	1.00
palpalis	*G. caliginea*	gcalig	4	1	0.65	99.64	0.29	0.07	0.83	1.00
	G. fuscipes fuscipes	gffsci	6	2	0.90	97.96	1.33	0.71	0.94	0.99
	G. fuscipes martinii	gfmart	5	1	0.75	99.18	0.73	0.09	0.93	0.99
	G. fuscipes quanzensis	gfquan	6	2	0.84	98.59	1.2	0.22	0.95	0.99
	G. palpalis palpalis	gppalp	6	2	0.80	97.71	1.7	0.6	0.89	0.98
	G. palpalis gambiensis	gpgamb	5	2	0.79	98.85	0.99	0.16	0.94	0.99
	G. pallicera pallicera	gpplic	6	2	0.85	99.31	0.62	0.07	0.97	0.99
	G. pallicera newsteadi	gpllic	5	1	0.43	97.68	1.91	0.41	0.69	0.98
	G. tachinoides	gtachi	7	1	0.84	97.84	1.41	0.75	0.90	0.99
morsitans	*G. austeni*	gauste	2	2	0.37	99.79	0.16	0.05	0.53	1.00
	G. longipalpis	glngpa	7	2	0.74	98.20	1.33	0.47	0.85	0.99
	G. morsitans morsitans	gmmors	3	1	0.76	98.27	1.54	0.19	0.94	0.98
	G. morsitans submorsitans	gmsubm	5	2	0.75	95.54	3.44	1.02	0.89	0.96
	G. morsitans centralis	gmcent	7	1	0.64	97.07	2.24	0.69	0.80	0.98
	G. pallidipes	gpalli	6	2	0.45	96.85	2.40	0.75	0.65	0.98
	G. swynnertoni	gswynn	4	2	0.62	99.78	0.19	0.03	0.85	1.00

(b)

Group	Species	Abbreviation	Var 1	Var 2	Var 3	Var 4	Var 5	Var 6	Var 7	Var 8	Var 9	Var 10
fusca	*G. brevipalpis*	gbrevi	pa14a3ll	pa03a0ll	pa07a3ll	pndcdmr	pa07a0ll	pa07p1ll	pa14a0ll	F025a1ll	pa14p3ll	F025p3ll
	G. fuscipleuris	gfscpl	F025a0ll	pndcdp1	pa03p2ll	pa14p2ll	F025p2ll	pa14p3ll	F025mxll	F025a2ll	pa14a0ll	pa14a3ll
	G. fusca fusca	gffusc	pa20p1ll	F025a0ll	pa03vrll	pa03a0ll	F025a2ll	pa07a2ll	F025p3ll	F025a3ll	pa20p3ll	pa14a0ll
	G. fusca congolensis	gfcong	F025a0ll	F025p1ll	pa07vrll	F025a2ll	F025p3ll	F025a1ll	F025p2ll	F025a3ll	pa14a2ll	pa07p2ll
	G. haningtoni	ghanin	pa20mxll	pa20p1ll	pa03a1ll	pa03vrll	F025p2ll	F025a0ll	F025a2ll	F025vrll	pndthar	pa14vrll
	G. longipennis	glngpe	pa14a3ll	F025a2ll	F025p1ll	pa07a3ll	pa03mnll	pa20a1ll	pa03vrll	pa20p3ll	pa14vrll	pndthmr
	G. medicorum	gmedic	pa20p1ll	F025a0ll	F025a2ll	pa14p3ll	F025a3ll	F025mxll	pa03p3ll	pa14a0ll	pa07mnll	pa20a3ll
	G. nashi	gnashi	F025a2ll	F025p1ll	pa20mnll	pa07p1ll	pa03p2ll	pa03p3ll	pa20a3ll	pa14a2ll	pa03a0ll	pa07a2ll
	G. nigrofusca nigrofusca	gnnigr	F025a2ll	F025p1ll	F025a0ll	F025a3ll	pa07mnll	pa14a3ll	pa03mnll	F025p3ll	F025p2ll	pa14mnll
	G. nigrofusca hopkinsi	gnhopk	DEM	pa03a0ll	F025a1ll	pndcdmr	pa14a2ll	F025p1ll	F025a2ll	pndthp1	pa07mnll	pa07a3ll
	G. severini	gsever	F025a0ll	pndcdp1	pa03a0ll	pndthar	pa14p3ll	pndcdmr	pa07p3ll	pndthp1	pa20a1ll	pa03mxll
	G. schwetzi	gschwe	F025a3ll	F025p1ll	F025mxll	F025a2ll	DEM	F025a1ll	pa14a3ll	pa14p3ll	pa07p3ll	pa03a2ll
	G. tabaniformis	gtaban	pa03mxll	DEM	pa07p2ll	pa14a2ll	pa14a1ll	pa20p1ll	F025p2ll	pndcdp1	F025a1ll	F025vrll
	G. vanhoofi	gvanho	F025a0ll	F025a3ll	F025a2ll	F025p3ll	pa03p3ll	pa20mnll	pa20a3ll	pa07a1ll	pa14mxll	pa03a0ll
palpalis	*G. caliginea*	gcalig	DEM	F025a2ll	pndcdmr	pa03a2ll	F025p1ll	F025a3ll	patnthnd	F025vrll	pa14a2ll	pndthmr
	G. fuscipes fuscipes	gffsci	pa14mnll	F025a0ll	F025vrll	pa20a0ll	DEM	F025p2ll	F025mxll	pndthmr	F025a2ll	pa14p1ll
	G. fuscipes martinii	gfmart	DEM	F025p1ll	F025a0ll	F025p3ll	pndthar	pa14vrll	pndcdp1	F025p2ll	pa07a3ll	F025a3ll
	G. fuscipes quanzensis	gfquan	pndthar	F025a1ll	pa14a2ll	F025a0ll	F025p3ll	pa03p2ll	F025vrll	F025mxll	pa14a1ll	pa03a3ll
	G. palpalis palpalis	gppalp	F025a2ll	pa14a2ll	pa07mnll	DEM	pa14mxll	pndcdmr	F025a3ll	F025p3ll	pndthar	F025mxll
	G. palpalis gambiensis	gpgamb	DEM	pa20p1ll	pa20mxll	F025a3ll	pa03a0ll	F025vrll	pa07p3ll	pa03a2ll	pa07a0ll	pa14a3ll
	G. pallicera pallicera	gpplic	pa20p1ll	F025a0ll	F025a2ll	F025p3ll	pa03mnll	pa20a1ll	F025a1ll	pa03a2ll	pa14a3ll	pa20p3ll
	G. pallicera newsteadi	gpllic	DEM	pa20a1ll	pndcdmr	F025mxll	F025a3ll	pa03p2ll	pa03p1ll	pndcdp1	pa14vrll	pa03p3ll
	G. tachinoides	gtachi	pa03a1ll	pa14mxll	pa07vrll	F025vrll	F025a3ll	pa03a3ll	pa03a0ll	pa07a0ll	DEM	F025p2ll
morsitans	*G. austeni*	gauste	DEM	pa03a0ll	F025mxll	pa07a0ll	pndthmr	pa20a3ll	pa07a3ll	pa03a3ll	pa07vrll	F025p3ll
	G. longipalpis	glngpa	pa14p1ll	DEM	pa20mxll	F025mxll	pa14mnll	F025a3ll	F025p2ll	pa03p3ll	F025a0ll	pa20a2ll
	G. morsitans morsitans	gmmors	pa03p1ll	DEM	F025p2ll	F025mxll	pa03a0ll	pa07mxll	pndcdmr	pa07vrll	pa14a3ll	pa20a2ll
	G. morsitans submorsitans	gmsubm	pa03a1ll	pa20mnll	pa03p2ll	pa14vrll	patnthnd	F025a3ll	pa03a3ll	F025p3ll	F025a2ll	pa20a2ll
	G. morsitans centralis	gmcent	pa14p1ll	pndthp1	pa14vrll	pa03mnll	F025a1ll	F025vrll	pa07p3ll	pa03p3ll	F025mxll	pa14a0ll
	G. pallidipes	gpalli	pa20p2ll	pa03a3ll	pndcdmr	F025a1ll	DEM	pa14p1ll	F025a3ll	pndcdp1	patnthnd	pa03vrll
	G. swynnertoni	gswynn	pndthmr	pndthar	F025mxll	F025p1ll	pa20a0ll	pa03a0ll	pa14mnll	F025a3ll	DEM	pndthp1

1977), only one species, *G. austeni*, is poorly described by satellite data ($\kappa < 0.4$); 13 species are well described ($0.4 < \kappa < 0.75$) and the remaining 16 species are excellently described by satellite data ($\kappa > 0.75$).

The predictive maps for all tsetse species are shown in Figs 8.2–8.4. Because of the limitations of black-and-white printing, the probability scale of the output imagery has been reduced to two categories: below (in white) and above (in grey) a level of 0.5. Thus the grey areas in Figs 8.2–8.4 represent areas that are environmentally similar to areas from which each species has been recorded in the past. In order to show as much detail as possible, the maps are drawn on a scale appropriate to show both the entire mapped distribution of each species and the entire area predicted to be suitable; areas outside the frame of each figure, therefore, not only contain no record of the presence of each species but are also predicted to be unsuitable for that species. As explained above, such maps should not be taken as predicted distribution maps, since species in general do not occupy all of the areas that are climatically suitable for them, but a smaller or larger subset of such areas. Species cannot, however, inhabit areas that are climatically unsuitable for them and so false negative predictions (i.e. incorrect predictions of absence) are a better guide to the adequacy or otherwise of this modelling approach. Table 8.3 shows that false positive predictions (mean 0.985%, range 0.02–3.44%) always outnumber false negative predictions (mean 0.306%, range 0.01–1.02%), all of which, with the single exception of *G. m. submorsitans*, are < 1%.

The best models for each species tended to have more clusters for absence (mean, mode and median of 5.2, 6 and 5, respectively) than for presence (1.7, 2 and 2, respectively) (Table 8.3), indicating that, for any particular tsetse species, the environmental variability in areas of absence is much greater than in areas of presence. This is not surprising, given the great preponderance of absence observations (drawn from throughout Africa) in the data sets for all species, but may also reflect the fact that each tsetse species has evolved in a particular and relatively restricted set of environmental conditions.

There were significant correlations between each of the measures of accuracy and the relative frequency (which in this case also means the absolute frequency, since the total sample sizes were about the same) of presence observations in the data set. Thus κ and sensitivity were positively correlated with the percentage (or number) of presence observations in the total sample ($r = 0.548$, $P < 0.01$ and $r = 0.444$, $P < 0.05$, respectively), whilst both the percentage of all observations correctly identified and the specificity were inversely correlated with the same measures of relative (or absolute) frequency ($r = -0.678$, $P < 0.001$ and $r = -0.617$, $P < 0.001$, respectively). Both the percentage of false positives and the percentage of false negative predictions increased with the percentage (or number) of presence observations ($r = 0.586$, $P < 0.01$ and $r = 0.838$, $P < 0.001$, respectively), the former two to three times faster than the latter (unconstrained regression slopes of 0.160 and 0.086, respectively; slopes, constrained to pass through the origin, of 0.251 and 0.097, respectively). It is likely that many of these effects are due to the patchy distribution of tsetse within Africa, and the fact that narrow-spread species are likely to have more 'edges' per unit area occupied than are widespread species. Edges, of course, represent places where the distribution of any species ends, and capturing edges with satellite descriptors (and hence providing an accurate fit to the species' distribution) will therefore be proportionally more difficult for the narrow-spread or patchy species.

Perhaps not unexpectedly, the average fit to the savannah-dwelling *morsitans* group species distributions was marginally worse than the average fit to the forest-dwelling *fusca* group species distributions (mean κ of 0.618 vs. 0.710; mean overall percentage correct of 97.93% vs. 99.22%; mean false positives of 1.61% vs. 0.58%; mean false negatives of 0.46% vs. 0.21%; mean sensitivity of 0.786 vs. 0.828 and mean specificity of 0.983 vs. 0.994, respectively). Values for the *palpalis* group species are intermediate for all except mean κ and mean sensitivity, both of which are higher (mean $\kappa = 0.761$, mean sensitivity = 0.988). Some of these

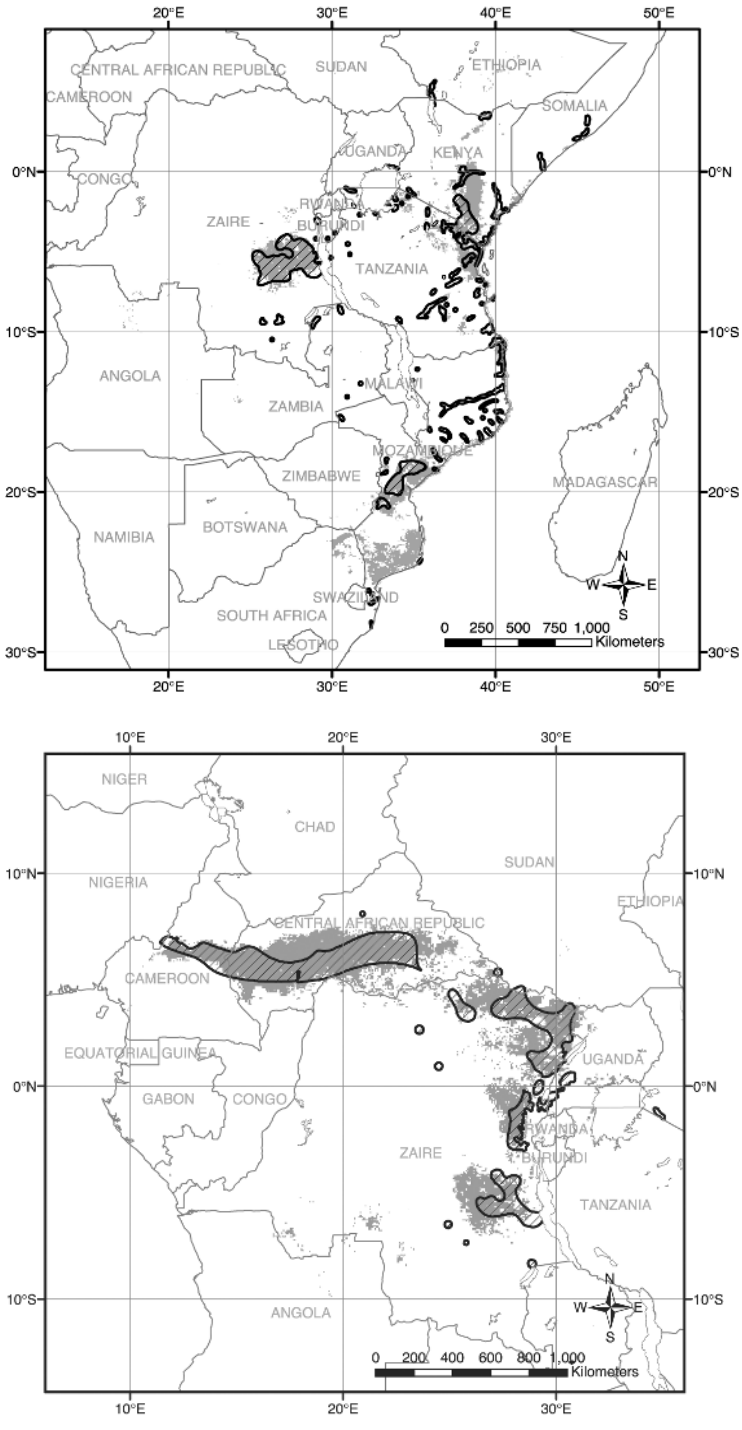

Fig. 8.2.(a) *fusca* group. *G. brevipalpis, G. fuscipleuris.*

Figs 8.2–8.4. Predicted areas of suitability for tsetse in Africa. Predicted areas are based upon the similarity of environmental conditions (as detected by satellites) to those at sample sites within the historical distribution of each species (cross-hatched, from Ford and Katondo, 1977a).

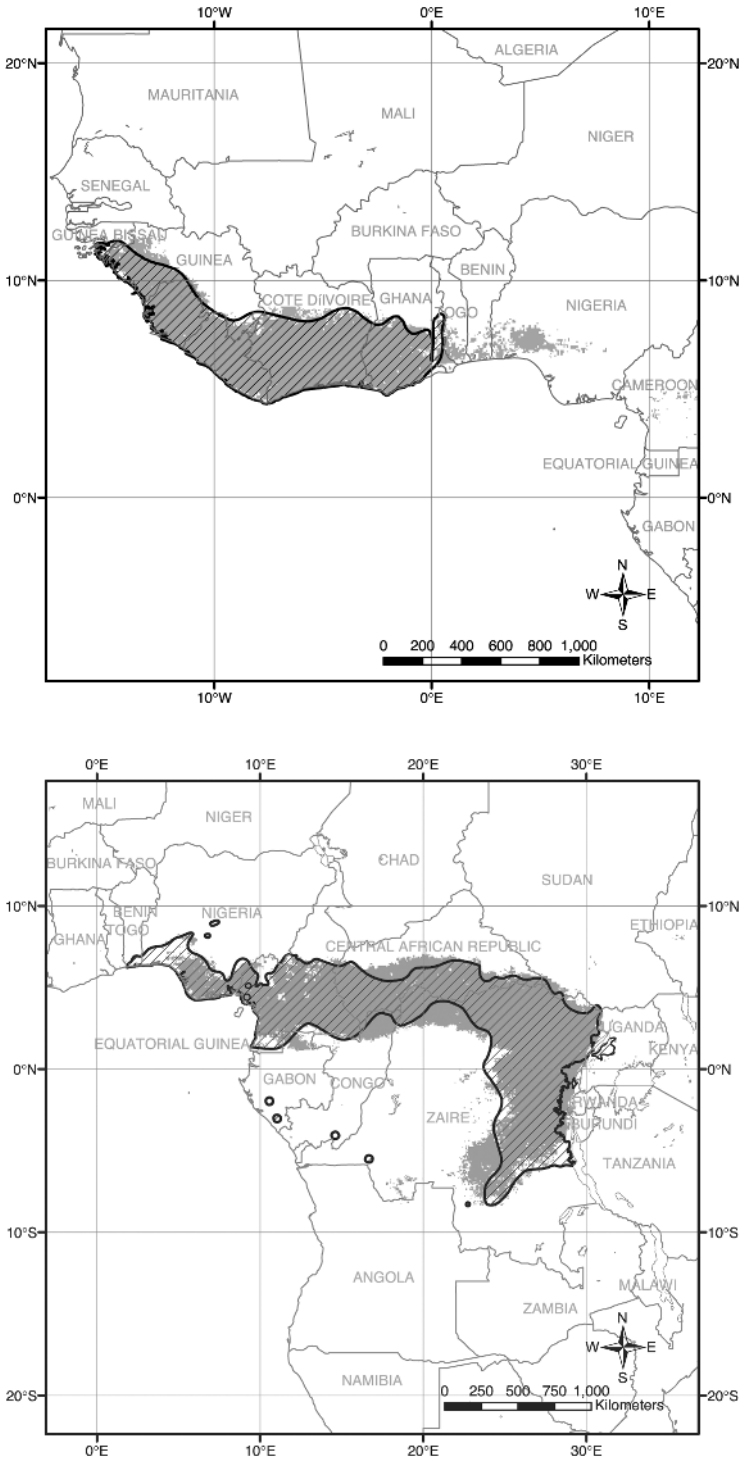

Fig. 8.2.(b) *fusca* group (*continued*). *G. fusca fusca, G.f. congolensis.*

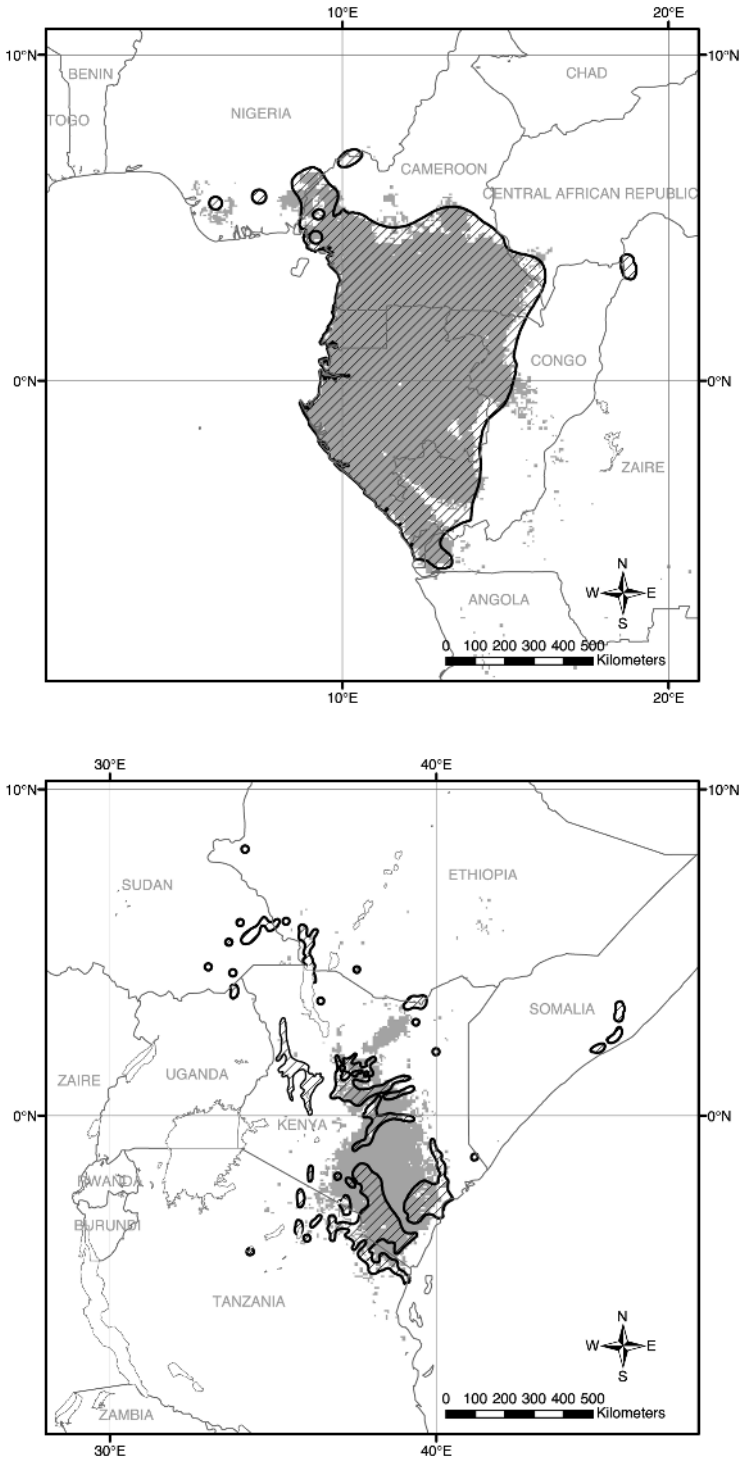

Fig. 8.2.(c) *fusca* group (*continued*). *G. haningtoni, G. longipennis.*

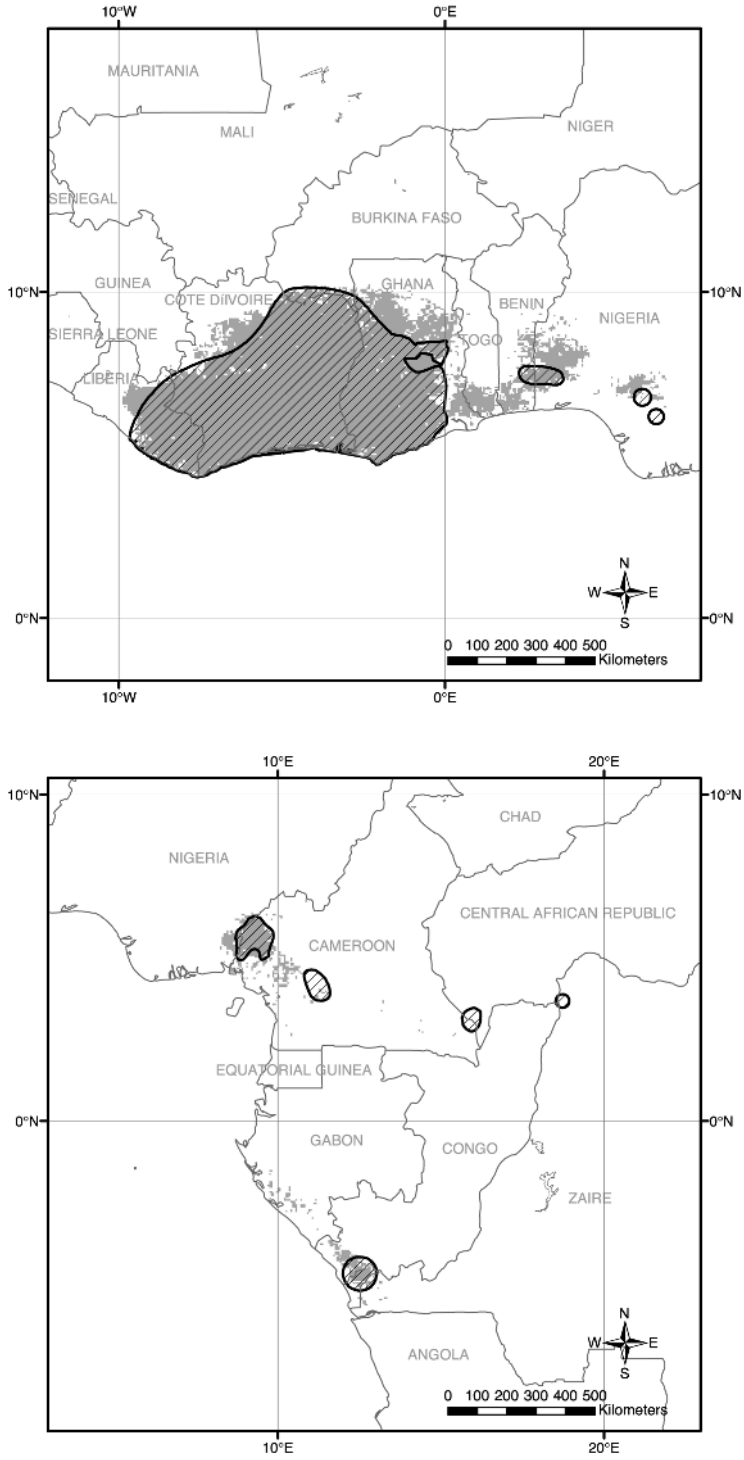

Fig. 8.2.(d) *fusca* group (*continued*). *G. medicorum, G. nashi.*

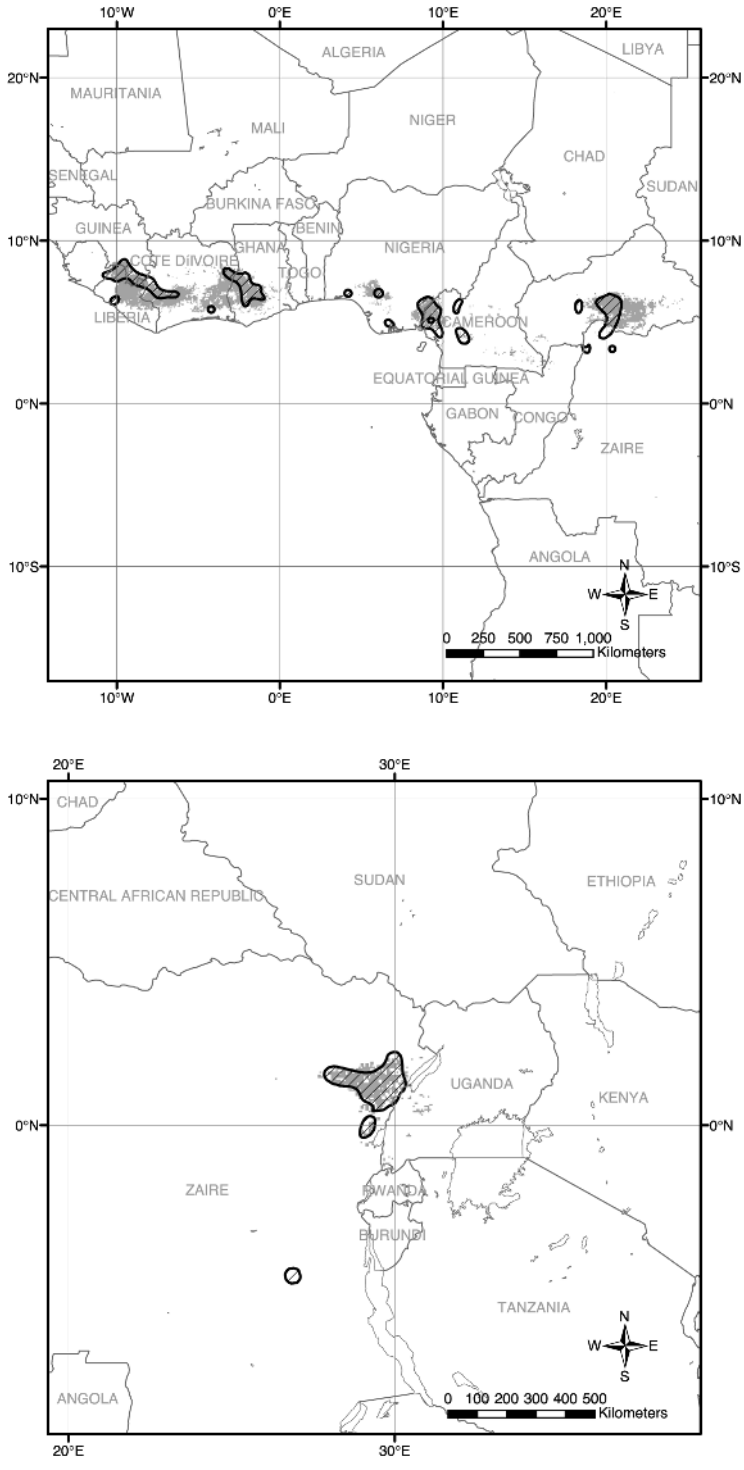

Fig. 8.2.(e) *fusca* group (*continued*). *G. nigrofusca nigrofusca, G. n. hopkinsi.*

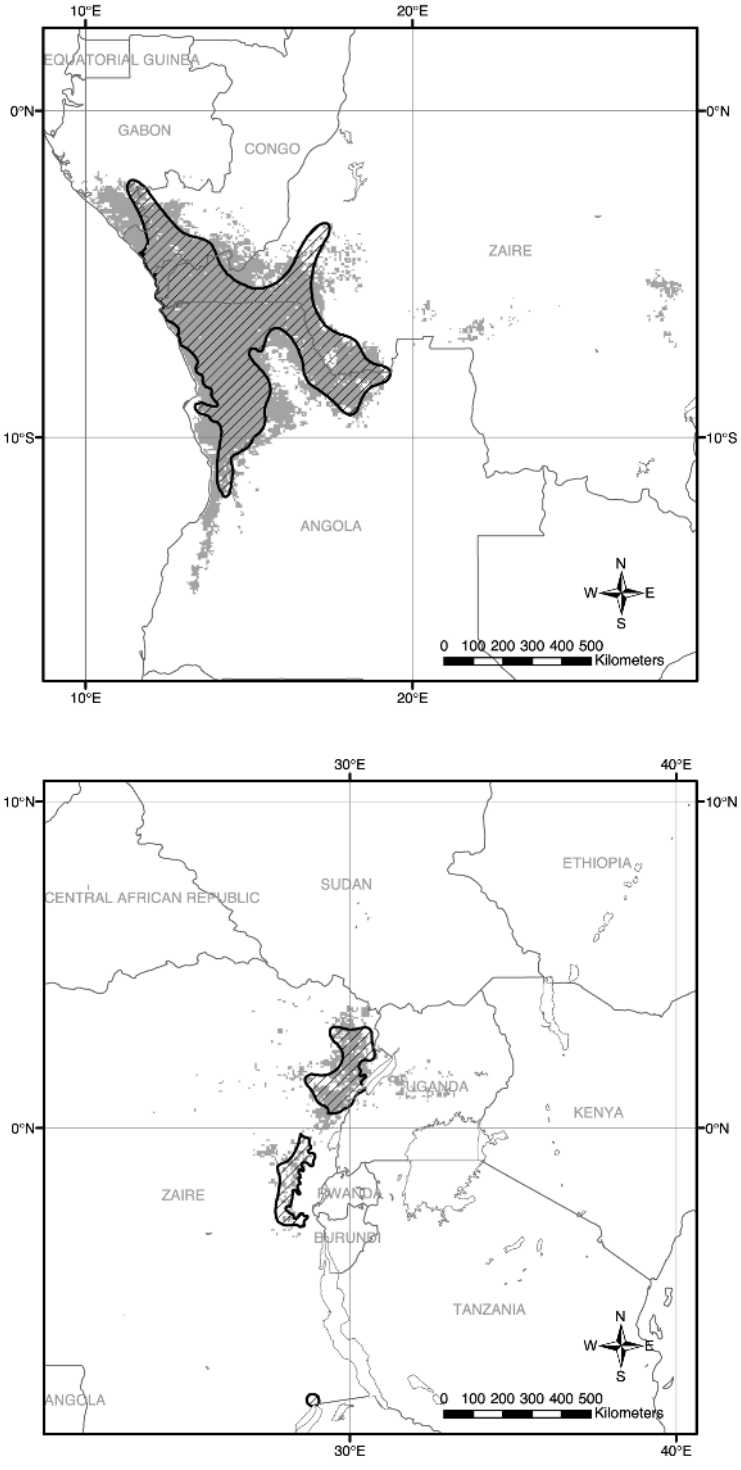

Fig. 8.2.(f) *fusca* group (*continued*). *G. schwetzi, G. severini.*

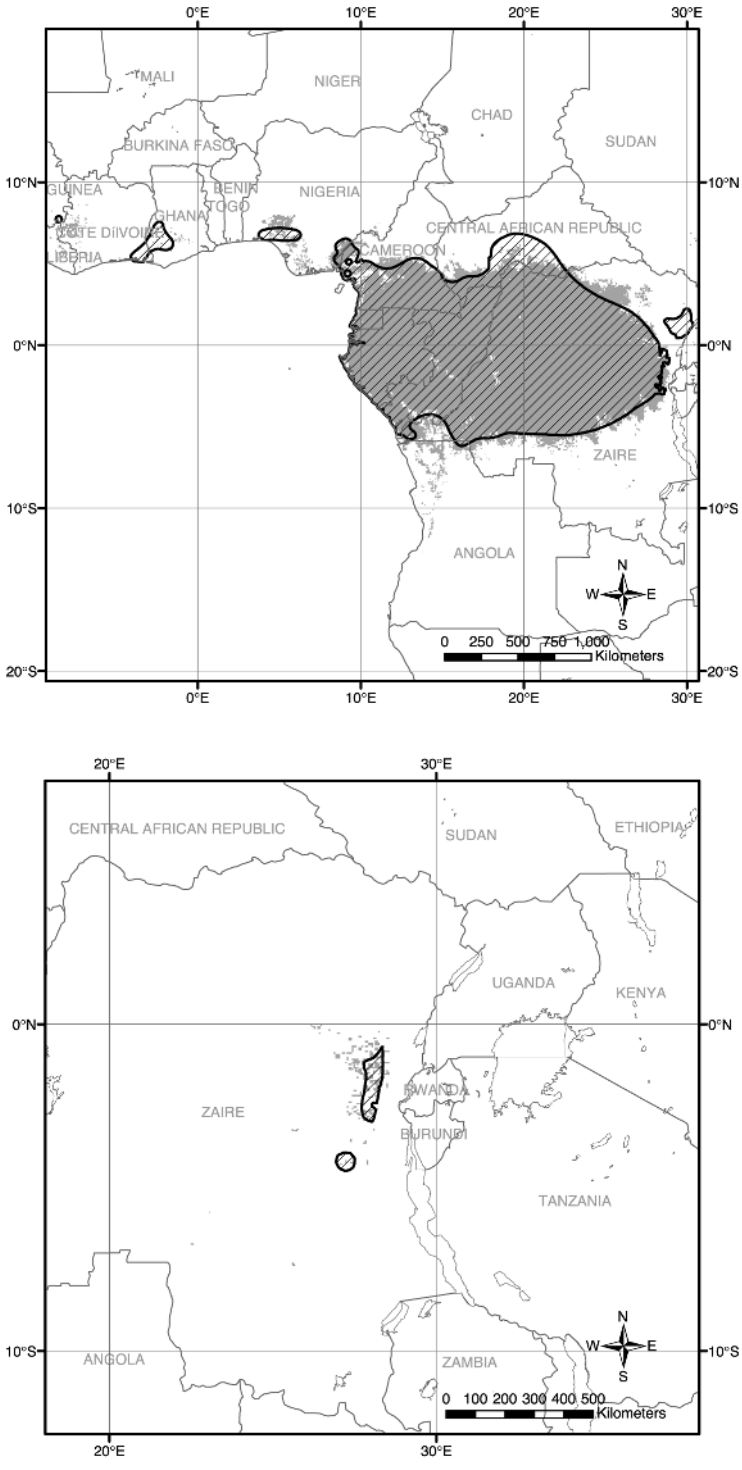

Fig. 8.2.(g) *fusca* group (*continued*). *G. tabaniformis, G. vanhoofi.*

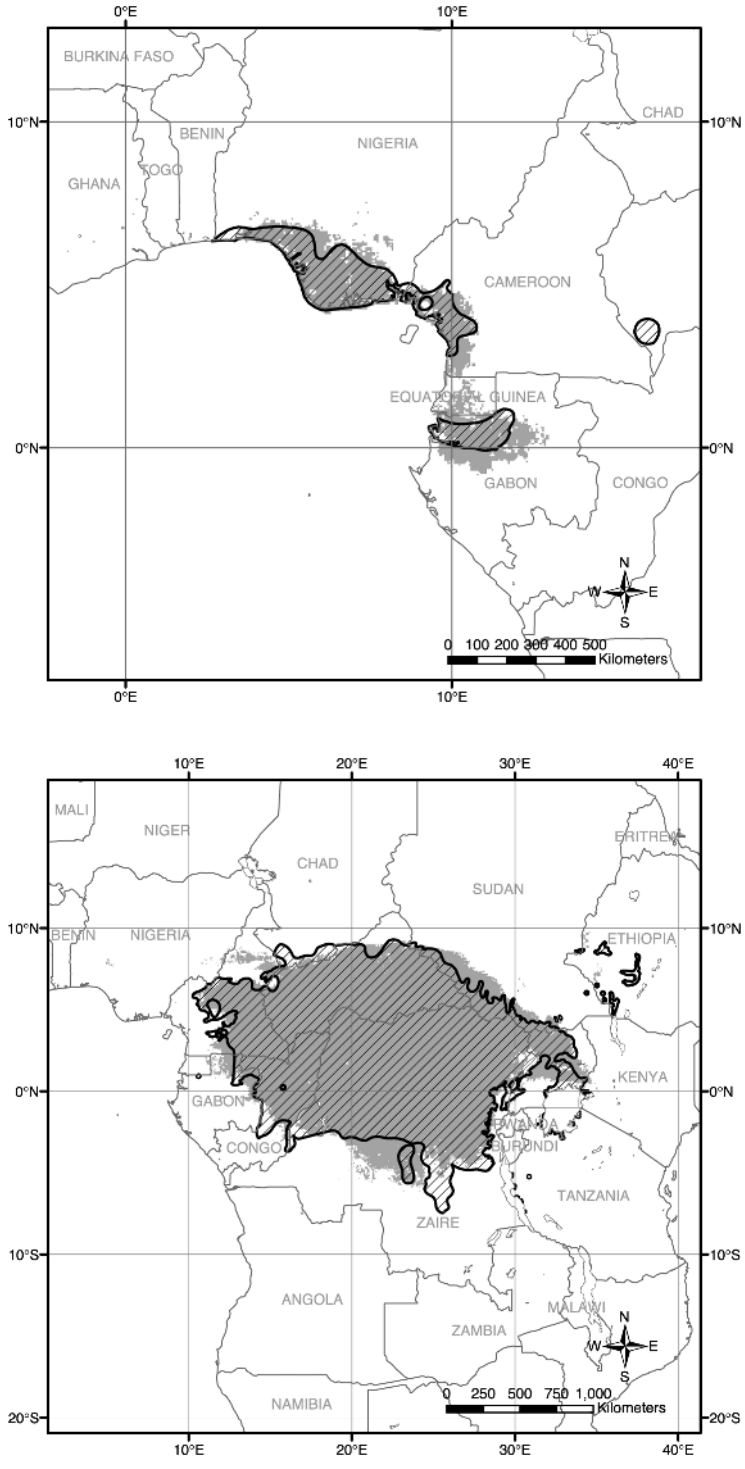

Fig. 8.3.(a) *palpalis* group. *G. caligenea, G. fuscipes fuscipes.*

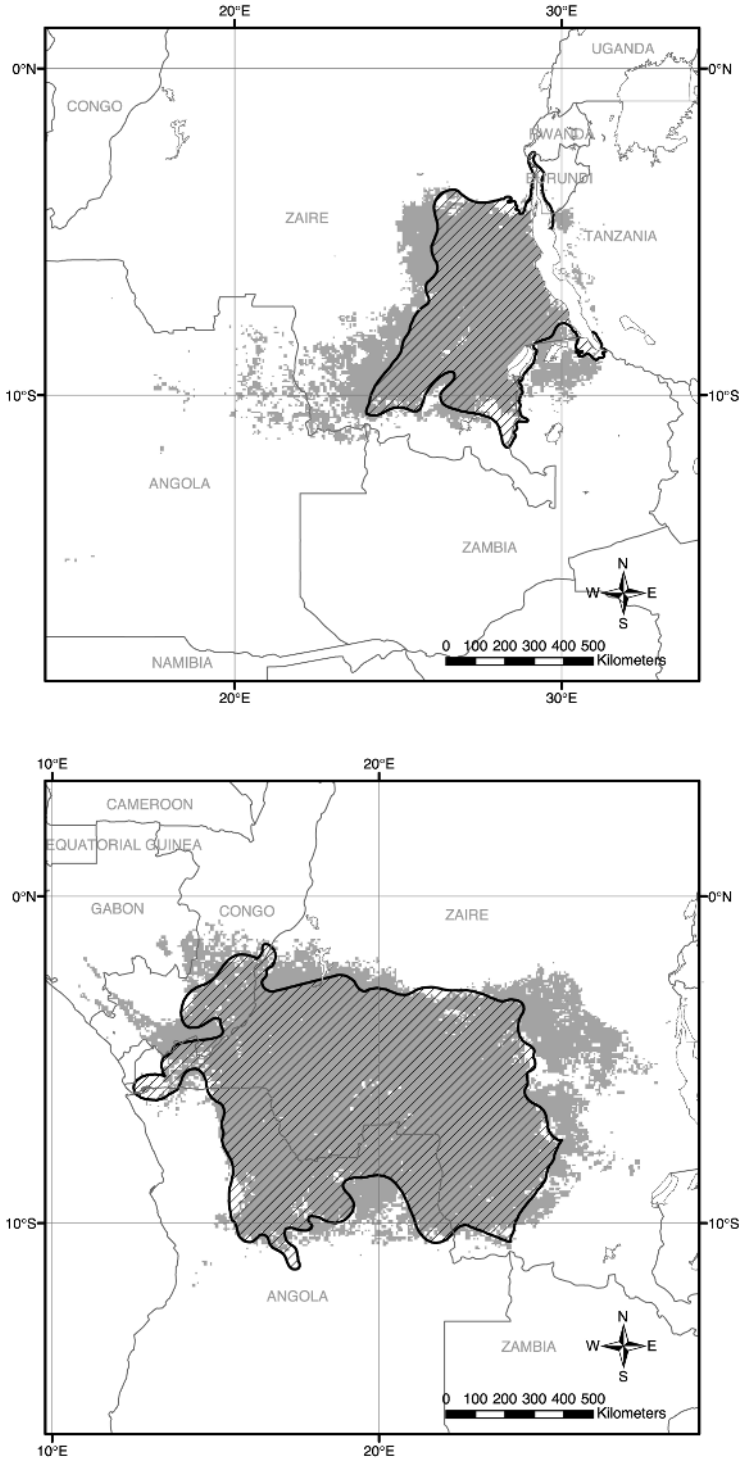

Fig. 8.3.(b) *palpalis* group (*continued*). *G. f. martini, G. f. quanzensis.*

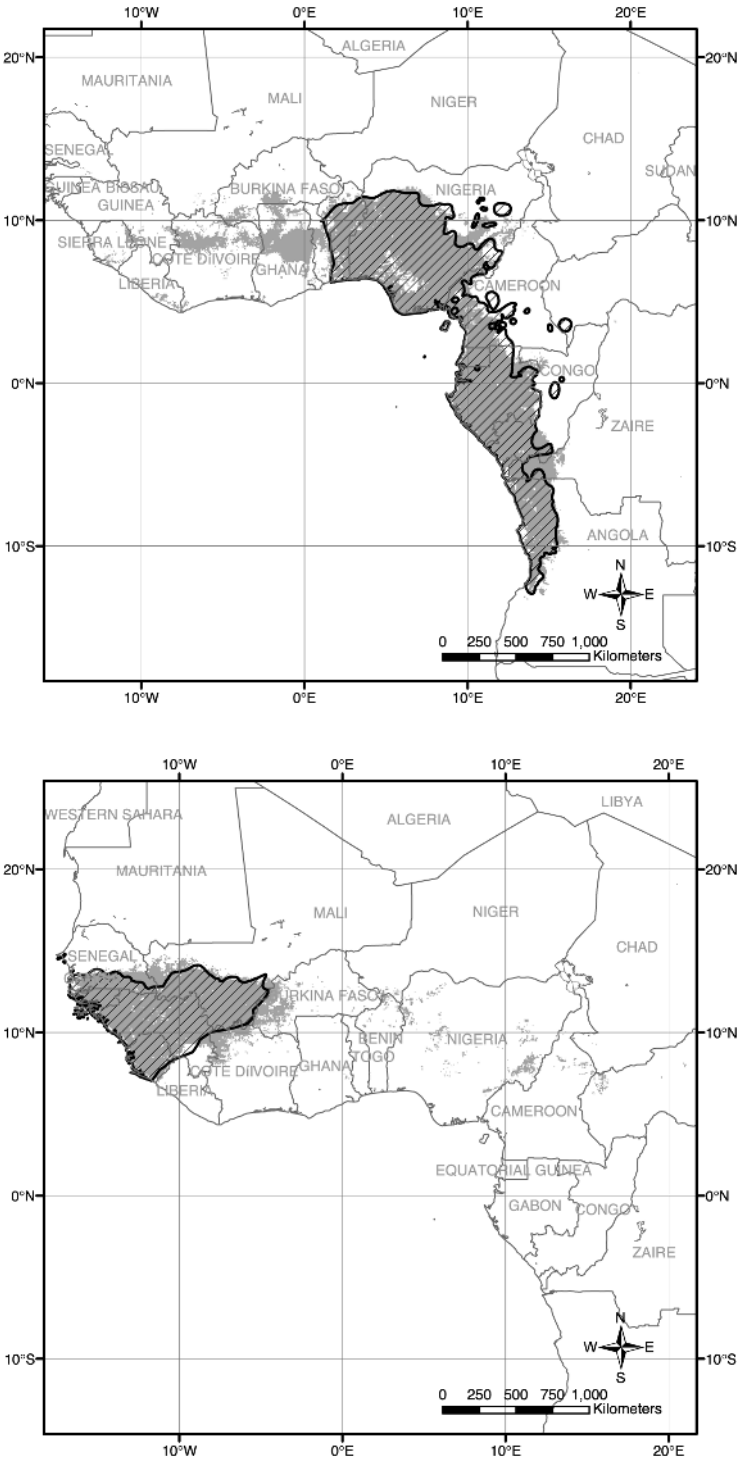

Fig. 8.3.(c) *palpalis* group (*continued*). *G. palpalis palpalis, G.p. gambiensis.*

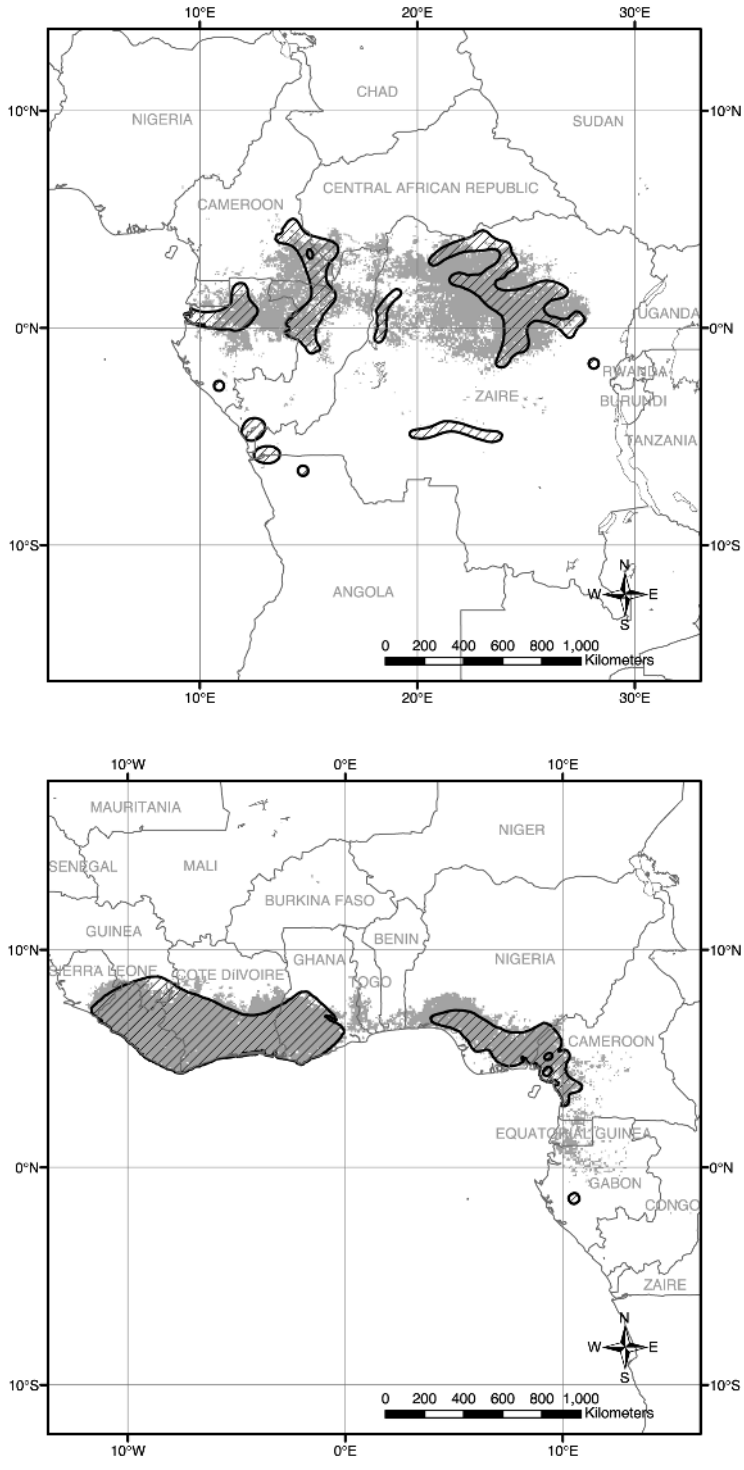

Fig. 8.3.(d) *palpalis* group (*continued*). *G. pallicera newsteadi, G. p. pallicera.*

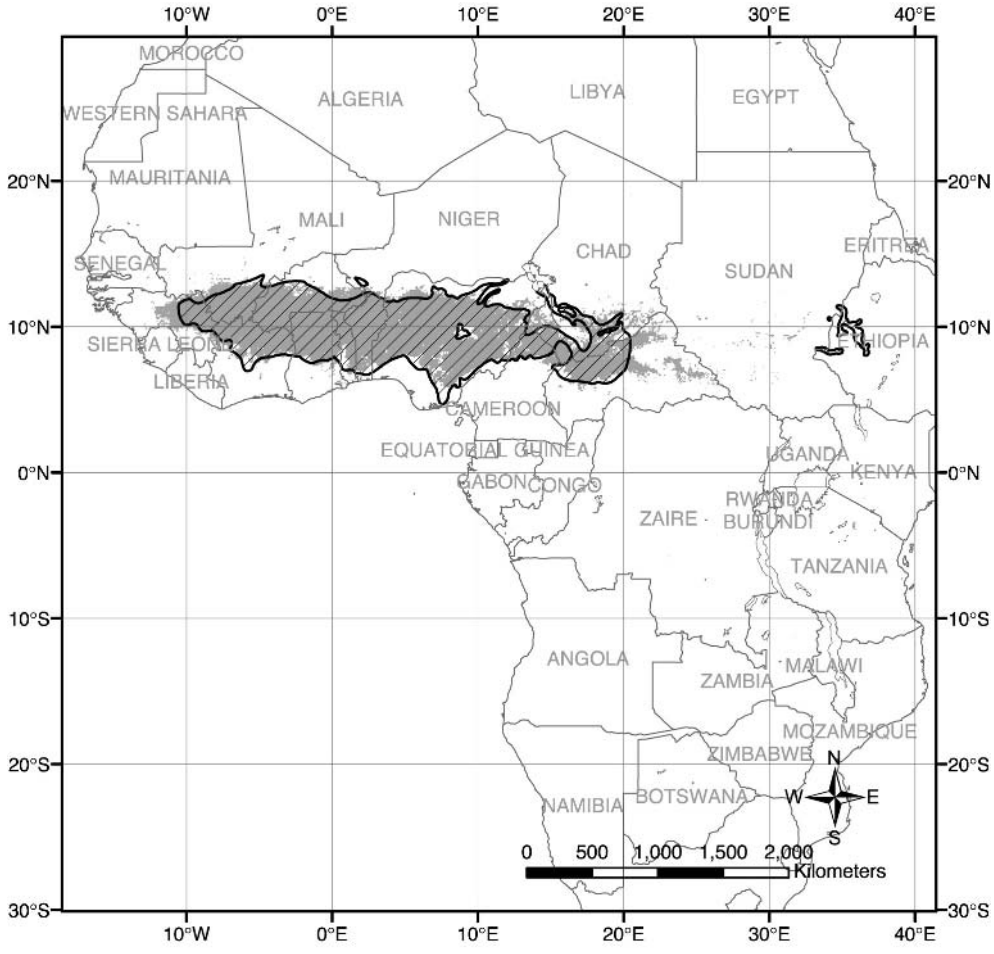

Fig. 8.3.(e) *palpalis* group (*continued*). *G. tachinoides.*

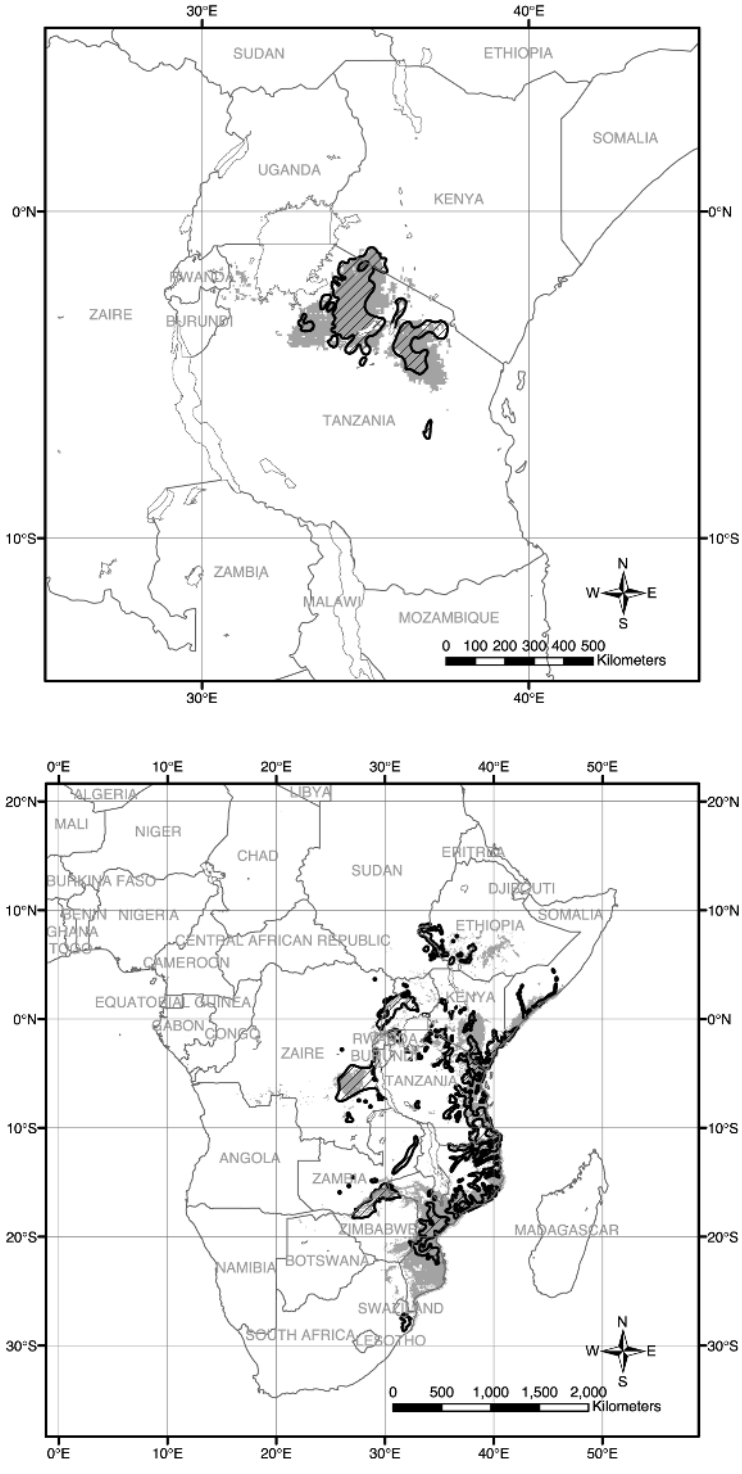

Fig. 8.4.(a) *morsitans* group. *G. swynnertoni, G. pallidipes.*

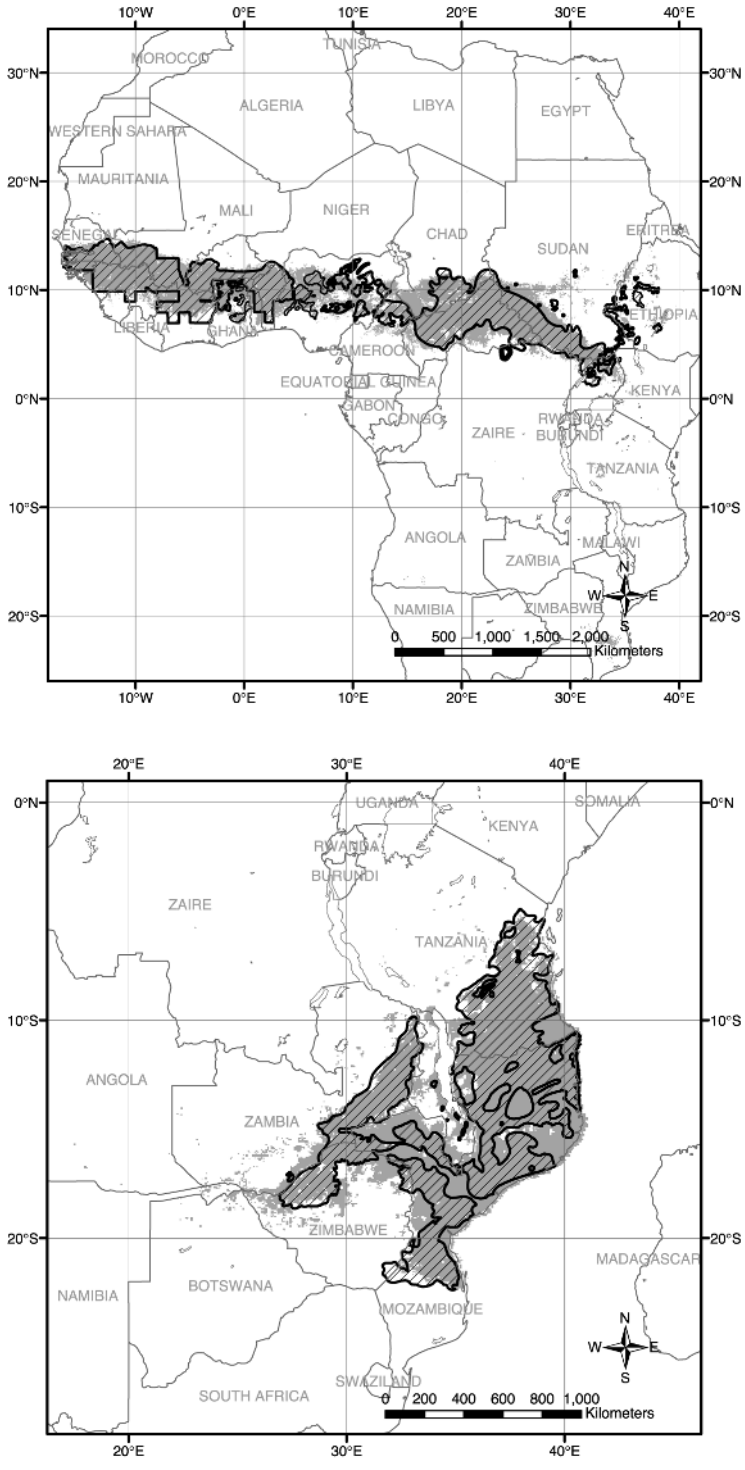

Fig. 8.4.(b) *morsitans* group (*continued*). *G. morsitans submorsitans, G. m. morsitans.*

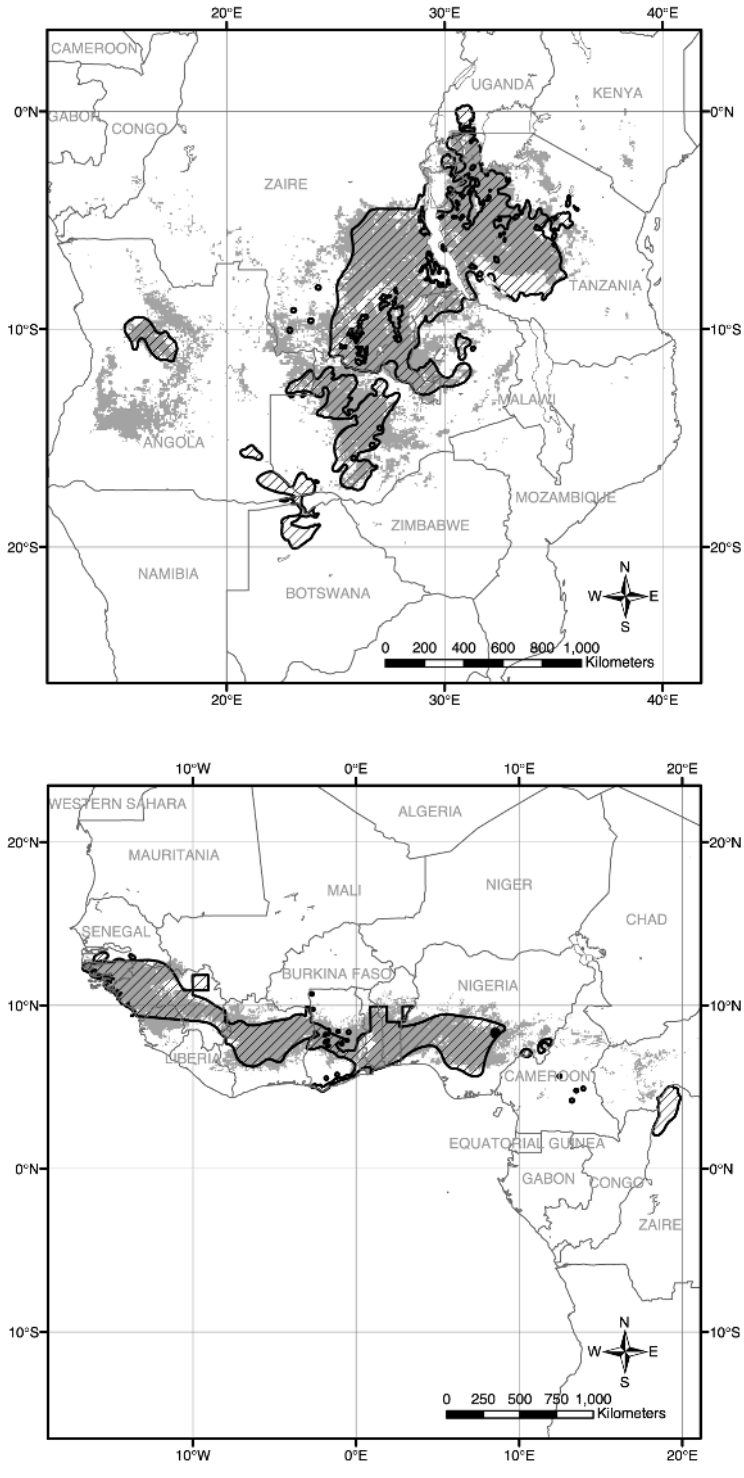

Fig. 8.4.(c) *morsitans* group (*continued*). *G. m. centralis, G. longipalpis.*

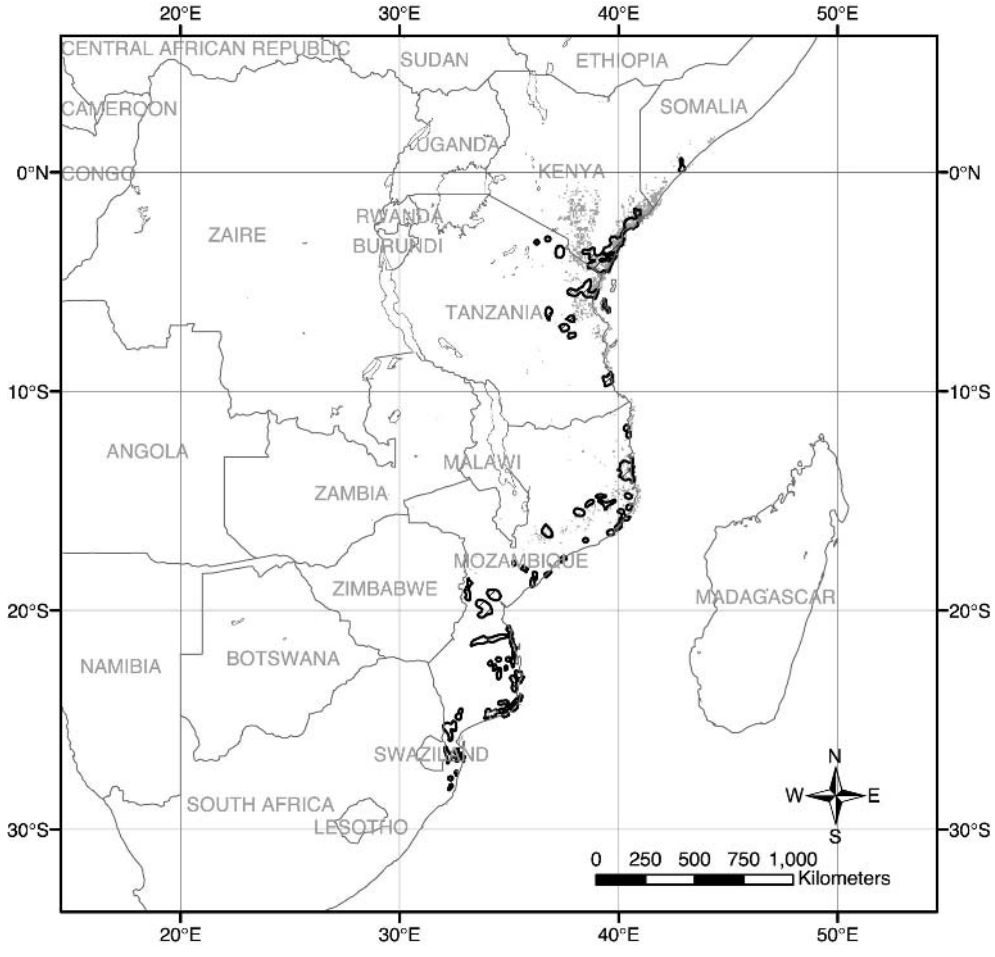

Fig. 8.4.(d) *morsitans* group (*continued*). *G. austeni*.

differences may arise from the different total areas occupied by the three species groups, coupled with the area effects outlined in the previous paragraph. The mean percentages of sample pixels occupied by any one species of the *fusca*, *palpalis* or *morsitans* groups are 2.04%, 3.83% and 2.91%, respectively; thus, per species, the *palpalis* group is on average the most widespread. The poorer mean κ value, and the poorer sensitivity, for *morsitans* group species compared with the *fusca* group species (despite the expectation of larger κ and sensitivity values, given the larger area occupied by an average *morsitans* species) therefore confirms the suggestion that the mapped distributions of these savannah-dwelling species are rather more difficult to describe using satellite data than are those of species of the *fusca* group.

Table 8.4 shows the percentage occurrence of the different types of satellite data in the top five predictor variables for each species group. By definition, the top five variables make the greatest contribution to describing the distribution of each species. Table 8.4 shows that the greatest difference between the species groups is in terms of the CCD imagery, a surrogate for rainfall. Both the *fusca* and *palpalis* group species have a higher percentage of CCD variables in their top five predictors (45% and 43%, respectively) than do *morsitans* group species (22%). This probably reflects the greater requirements of the first two species groups for well-vegetated or forest habitats that obviously depend upon rain.

The dendrogram of the linkage between the environments of the different tsetse species, based on the top ten environmental variables for all species, is given in Fig. 8.5. This shows certain similarities with both the taxonomic relationship between the species and their geographical distribution. The *fusca* group species are concentrated in the upper half of the dendrogram, whilst all the *morsitans* group species are restricted to the lower half. The arrangement of each group appears to reflect their geographical distributions, since the *fusca* group species near the top of the dendrogram occur in Central Africa (near to the putative centre of evolution of all tsetse species; Ford, 1971), whilst those lower down occur in West or Central/West Africa (*G. f. fusca, G. medicorum, G. haningtoni, G. n. nigrofusca*), Central/East Africa (*G. n. hopkinsi, G. severini, G. vanhoofi*) or East Africa (*G. brevipalpis, G. longipennis*). Similarly the *morsitans* group species in the lower half of the dendrogram occur mostly in East Africa; with *G. m. submorsitans* and *G. longipalpis* the 'outliers', with distributions mostly or exclusively in West Africa. The linkage of these two species to the other *morsitans* species occurs at the largest rescaled distance.

Discussion and Conclusions

Satellites appear able to describe with remarkable statistical accuracy the distribution of virtually all tsetse species in Africa. The fixed sampling grid used in the present study means that all species data sets were dominated by absence observations, with the result that more absence than presence clusters were required in the best-fitting models for each. In order to avoid the models selecting variables that just described these absence clusters, the data were aggregated into a single absence and a single pres-

Table 8.4. Percentage occurrence of the different types of satellite and other data (see Table 8.2) in the top five variables selected to describe the distributions of species in the three tsetse groups. Variables composed of differences or ratios (see Table 8.2) are counted once to each of the contributory variables.

Group	DEM	Channel 3	LST	NDVI	VPD	CCD	Sum
fusca	4.0	16.0	10.7	17.3	6.7	45.3	100
palpalis	12.2	8.2	8.2	18.4	10.2	42.9	100
morsitans	9.8	17.1	14.6	26.8	9.8	22.0	100

LST, Land Surface Temperature; NDVI, Normalized Difference Vegetation Index; VPD, Vapour Pressure Deficit; CCD, Cold Cloud Duration.

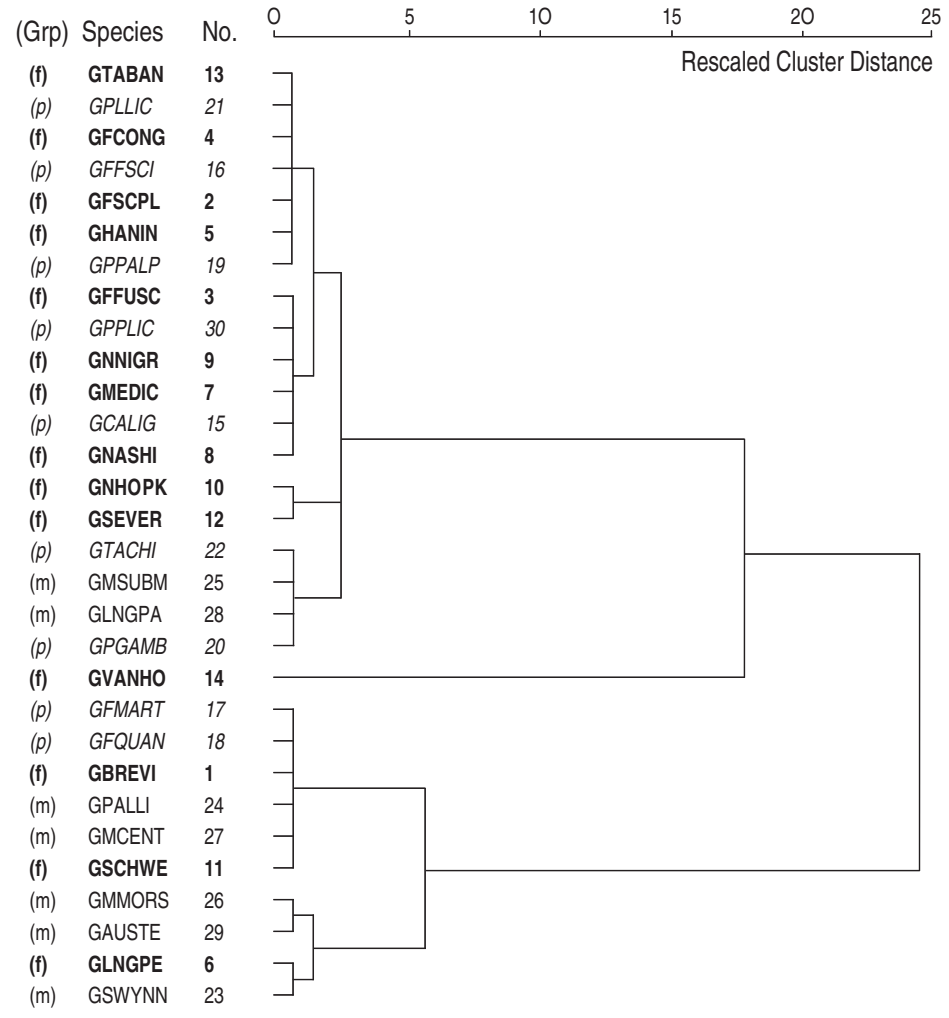

Fig. 8.5. Average linkage dendrogram for environmental distances between tsetse species. Dendrogram of average between-species distances (linearly rescaled to a maximum of 25) based on the top ten environmental variables used to describe the distributions of all 30 species/subspecies of tsetse in Africa. The *fusca, palpalis* and *morsitans* group species are identified by bold, italic or normal text respectively. (The numbers are for database identification only).

ence class before calculating the κ accuracy statistic that formed the basis for variable selection. Thus clustering produces distributions that are individually more likely to be multivariate normal, thereby conforming to the assumptions of the maximum likelihood methods used, whilst aggregating the results before calculating the accuracy metric concentrates on the ability of the method to distinguish fly-presence from fly-absence sites.

Of the various measures of model accuracy used here, the κ statistic appears to reflect best the actual fit of each model to the mapped distributions of each species. For example, taking the first ten species of the *fusca* group (Table 8.3), κ ranges between 0.44 (for *G. n. nigrofusca*) and 0.92 (for *G. haningtoni*), whilst the overall percentage correct figures range only from 97.59% (for *G. f. congolensis*) to 99.97 (for *G. n. hopkinsi*).

The κ values appear to reflect well the visual impression given by the predicted distributions shown in Figs 8.2–8.4 for these species.

It is not at all clear whether the residual errors in the predictions in Figs 8.2–8.4 are because the initial maps or the modelling approach itself is in error. In all likelihood there are errors in both, and it thus becomes essential that there should be a process of model validation, where model predictions are tested with targeted field sampling in key areas of Africa.

The dendrogram of habitat relationships between tsetse species (Fig. 8.5) appears to be a relatively novel way of using satellite data to investigate interspecies relationships. In the present instance the dendrogram appears to borrow more from the geographical arrangement of tsetse in Africa than from the evolutionary history of the group, though the two are obviously related. The very close linkage between *G. austeni* and *G. m. morsitans* in Fig. 8.5 (linked at the shortest rescaled distance) allows us to revisit Bursell's opinion in the Introduction, that *G. austeni* is a species that has 'reverted' to the moist habitats characteristic of the more primitive tsetse species. In fact the environment of this species, as detected by satellites, is relatively typical of the other members of the *morsitans* group and *G. austeni* is, at least in these terms, not at all special. Dendrograms of the sort shown in Fig. 8.5 are also potentially useful when examining the transfer of zoonotic diseases between a variety of potential vector species in a group such as the tsetse. It is obviously more likely that such transfers take place between species inhabiting the same environments (i.e. linked at shorter rather than longer distances) than between species inhabiting different ones. Dendrograms may also help us to identify crucial bridging vectors that carry diseases over into new habitats, and therefore host groups.

Tsetse remain taxonomically relatively straightforward, without any recognition to date of the species complexes that occur in many other vector groups (e.g. mosquitoes, blackflies). There are, nevertheless, signs of within-species differences that have both environmental and genetic correlates. For example, regional differences in the genetics and behaviour of *G. pallidipes* have been recognized for some time (Langley *et al.*, 1984) and population analyses have suggested that this species of tsetse also shows a different tolerance to atmospheric dryness in the Lambwe Valley of Kenya (where conditions are near to ideal for it) and in Somalia (Rogers, 1990). A challenge for future research is to investigate whether the environmental variable clusters of a single species, range map in any way on to genetic differences between flies from the different areas. This is the within-species equivalent of the between-species dendrogram shown in Fig. 8.5.

Several problems remain for local, regional and continental mapping of tsetse distributions using satellite data, the most important being the ultimate degree of accuracy that such an approach can provide. Tsetse control personnel want a map that is 100% reliable, but this degree of accuracy is in practice only essential when tsetse eradication is the ultimate aim. Even ground surveys fail to detect some tsetse species (e.g. *G. austeni* is difficult to sample in the field) and therefore cannot be 100% reliable; and no method is particularly efficient at detecting the presence of virtually all tsetse species at very low densities. The prohibitive costs of total eradication and the difficulty of maintaining this situation over time have caused many to think more realistically of fly control. Control, rather than eradication, requires the identification of tsetse 'hotspots' which must be targeted by control measures, and of ecological corridors along which flies might move to reinvade controlled areas. Each of these tasks should be more easy to accomplish using satellite data and carefully prepared predictive risk maps of the sort described here. It would seem ideal to combine this activity with that of model validation, stressed above.

Finally, new generations of satellites (Terra, Aqua and MSG – see Introduction) promise more data channels at better spatial and spectral resolution than previous generations of remote-sensing platforms. The challenge to the research community is to identify which channels and combinations

of channels are better able to identify the unique habitat fingerprints that define the distribution of tsetse, and other important vectors, in Africa and elsewhere. We do not suggest that these methods can ever totally replace traditional tsetse survey methods, firmly based on field sampling; they should be used rather like a cell biologist uses a microscope – to make a difficult job easier.

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9 Triatominae: Systematics, Morphology and Population Biology

Jean-Pierre Dujardin and Chris J. Schofield

Introduction

The Triatominae form a well-characterized subfamily of the Reduviidae (Hemiptera, Heteroptera), defined on the basis of their blood-sucking habits and features associated with their haematophagous diet (Usinger, 1939; Lent and Wygodzinsky, 1979). All other reduviids are predators on invertebrates, which is seen as the primitive state. Many predatory reduviids will probe and occasionally feed from vertebrates. Conversely, many Triatominae will feed from invertebrates, especially during their younger developmental stages. It is generally assumed, therefore, that the Triatominae have arisen from reduviid predators, probably through stages of facultative haematophagy in vertebrate nests, and then to the obligate haematophagy shown by most of them (Schofield, 1988). The evolutionary process in these bugs then continues as a process of adaptation to the most stable habitats, particularly human dwellings (Schofield *et al*., 1999). Those that have fully adapted to live in human dwellings feed on humans and domestic animals, are generally dispersed by humans, and can become important as vectors of *Trypanosoma cruzi* – causative agent of Chagas disease or American trypanosomiasis.

Chagas disease, as a human infection, is widespread in Latin America from Mexico to southern Argentina, and is ranked by the World Bank (1993) as the most serious parasitic disease of the Americas in terms of its social and economic impact. The human infection is difficult to treat, and control relies largely on elimination of the domestic triatomine vectors. Since 1991, the estimated infection prevalence of 16–18 million people has declined to about 11 million as a result of extensive control campaigns directed against the domestic vectors (Schmunis, 1999). However, *T. cruzi* remains widespread as a zoonotic infection of small mammals and marsupials, transmitted by silvatic Triatominae over a region extending from the Great Lakes of North America to southern Patagonia. All species of Triatominae seem capable of transmitting *T. cruzi*, but a distinction is made between three groups: (i) domestic vectors that colonize human dwellings and so transmit the parasite to humans; (ii) silvatic species that may be in the process of adaptation to human dwellings (candidate vectors); and (iii) silvatic species that remain closely associated with wild mammals (potential vectors). A further distinction can be made between domiciliary vectors that have colonized the house from local silvatic foci, and domestic vectors that have colonized the

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house by being brought in from other domestic colonies (Dujardin *et al*., 2000). This distinction has operational importance because domestic populations that have no local silvatic foci may be candidates for local eradication, whereas domiciliary populations will require continual surveillance in order to impede recolonization from local silvatic sources. Several species of Triatominae are associated with birds' nests or reptiles but these are less frequently infected because birds and reptiles are refractory to infection with *T. cruzi*.

Of the 138 species of Triatominae currently recognized (Table 9.1), research has concentrated on those of greatest epidemiological significance as domestic vectors of *T. cruzi*, especially *Triatoma infestans, Triatoma brasiliensis* and *Panstrongylus megistus* from the Southern Cone countries, and *Rhodnius prolixus* and *Triatoma dimidiata* from the Andean Pact countries and parts of Central America. The domestic vector species are generally easy to rear in the laboratory, and have provided excellent models for fundamental studies of insect physiology as well as studies related to their control. In recent years, they have also been used for studies of population genetics and basic evolutionary processes, largely linked to their process of adaptation to human dwellings. This chapter discusses the systematics, morphology and population biology of Triatominae, as a prelude to discussion of the epidemiology and control of Chagas disease (Chapters 13 and 31).

Table 9.1. Tribes and Genera of the Triatominae (Hemiptera: Reduviidae) (for an updated species list, consult the ECLAT website: http://ECLAT.fcien.edu.uy/).

Tribe	Genus	No. of species
Alberprosenіini	*Alberprosenia*	2
Bolboderini	*Belminus*	6
	Bolbodera	1
	Microtriatoma	2
	Parabelminus	2
Cavernicolini	*Cavernicola*	2
	Torrealbaia	1
Rhodniini	*Psammolestes*	3
	Rhodnius	16
Triatomini	*Dipetalogaster*	1[a]
	Eratyrus	2
	Linshcosteus	6
	Hermanlentia	1
	Panstrongylus	14[b]
	Paratriatoma	1
	Mepraia	2[c]
	Triatoma	76[d]
Total		138[b]

[a]The type species, *Dipetalogaster maxima*, was originally described as *D. maximus*, but the spelling should be corrected under Article 30 of the International Code for Zoological Nomenclature, since nouns ending in *-gaster* are feminine.

[b]These totals can be reduced by 1 if synonomy of *P. herreri* with *P. lignarius* is accepted.

[c]Some authors place *T. spinolai* in a separate genus, *Mepraia*, following Lent *et al*. (1994). However, acceptance of this taxon leaves unresolved the position of *T. breyeri* and *T. eratyrusiformis*, which have been classed within the *spinolai* complex (Lent and Wygodzinsky, 1979; Schofield, 1988). Pending clarification, we retain in the text the previous generic designation of Lent and Wygodzinsky (1979).

[d]Some authors have proposed revalidation of the genus *Meccus* for species of the *T. phyllosoma* group, and of *Nesotriatoma* for species of the *T. flavida* group. We consider these suggestions to be unsupportable, given the close relationship between *T. flavida* and the *phyllosoma* group (see Table 9.3).

Systematics and Distribution

The Hemiptera or 'true bugs' form the largest of the exopterygote orders of insects, with over 80,000 described species. They are characterized by having piercing-and-sucking mouthparts, and the majority feed on plant fluids. Hemiptera are currently classified into four suborders, with the Coleorrhyncha, Auchenorryhncha and Sternorryhncha (previously grouped together as the Homoptera) all being plant feeders, and the Heteroptera comprising plant feeders, predators and blood-suckers. Only the Heteroptera have a gula, or neck, which brings the rostrum forwards and allows it greater mobility, facilitating the adaptation from plant feeding to feeding on invertebrates. Good descriptions of the heteropteran families, with keys to subfamilies, are given by Schuh and Slater (1995).

The Reduviidae form a large family of heteropteran Hemiptera, with well over 6000 described species distributed throughout the world. With the exception of the Triatominae, all Reduviidae are predators on insects and other invertebrates, though facultative or opportunistic haematophagy is seen in many of the predatory subfamilies and in the closely related Phymatidae, which are often considered a subfamily of the Reduviidae. At the time of writing, there seems no universal agreement on the classification of the subfamilies of the Reduviidae. The most recent catalogue (Maldonado Capriles, 1990) recognizes 25 of the 32 subfamilies that have been proposed, comprising at least 6230 species in 912 genera. However, Schuh and Slater (1995) have reduced several of these subfamilies to tribal status, recognizing only 23 subfamilies.

The haematophagous Reduviidae were originally listed in the subfamily Reduviinae (formerly Acanthaspidinae). They were formally differentiated as the tribe Triatomini by R. Jeannel in 1919 and finally raised to subfamily level by Usinger (1939). (The generic name *Triatoma* was given by F.L. Laporte in 1832 based on the erroneous idea that these bugs had three-segmented antennae. On seeing fresh specimens with complete four-segmented antennae, Laporte revised this name to *Conorhinus* but, under the International Code for Zoological Nomenclature, *Triatoma* has priority.) The predatory Reduviidae are often known as 'assassin bugs', reflecting their predaceous habits. The Triatominae are sometimes referred to in english as 'kissing bugs', which seems to derive from domestic species feeding on the face of sleeping people (the face is generally the most exposed part of a sleeping person). In Latin America, triatomine bugs have a range of local names in Spanish, Portuguese and Amerindian languages. Table 9.2 summarizes some of the more common vernacular names, with approximate translations.

The 138 described species of Triatominae are currently grouped into 17 genera forming five tribes (Table 9.1). Of these, the Alberproseniini, Bolboderini, Cavernicolini and Rhodniini are customarily considered to be monophyletic (Lent and Wygodzinsky, 1979) with the Triatomini clearly polyphyletic. All are of New World origin, except for the Indian genus *Linshcosteus* which is currently grouped within the Triatomini but may merit separate tribal status to reflect its apparently independent origin (Carcavallo *et al*., 2000; Dujardin *et al*., 2000). A brief description of tribes and genera is given below. For more detailed descriptions see Lent and Wygodzinsky (1979), Lent *et al*. (1994) and Dujardin *et al*. (2000).

Tribe Alberproseniini Martinez & Carcavallo, 1977

The tribe Alberproseniini contains one genus, *Alberprosenia*, with two species from Brazil (*A. malheiroi*) and northeast Venezuela (*A. goyovargasi*). They are small Triatominae, with adults just 5 mm long, known only from a few collections under the bark of dead trees. *A. goyovargasi* nymphs have been reared to adult by feeding on human blood but these bugs appear to have no epidemiological significance. Morphometric analysis suggests that *Alberprosenia* tends to form an external group with the Cavernicolini and Bolboderini, distant from the other Triatominae (Dujardin *et al*., 2000).

Table 9.2. Examples of common vernacular names for Triatominae.[a]

Country	Names
USA	Kissing bug, cone-nose bug (note that 'assassin bug' refers to predatory Reduviidae)
Mexico	Chinche besucona (Spanish – 'kissing bug') Pik (Nahuatl – refers to sound made when flying bug strikes wall) Chinche hocicona (Spanish – 'cone-nosed bug')
Central America	Chinche besucona
Cuba	Sangrejuela (Spanish – 'leech')
Colombia	Pito (Spanish – whistle or horn, but probably also refers to extended mouthparts of the bug)
Venezuela	Chipo (colloquial Spanish – 'bug')
Ecuador	Chinchorro (Quechua – 'large bug')
Peru	Chirimacha (Quechua – 'bug that dislikes the cold' or 'drunk with the cold')
Paraguay	Chichá guazu (Guarani – 'big bug') Itchajuponja (Ayoreo – 'sucking bug') Sham bui tá (Aché – 'insect that does harm by its dejections', mainly used for blister beetles)
Bolivia	Vinchuca (Quechua – 'bug that lets itself fall', e,g. from roof, to reach sleeping people)
Chile	Vinchuca
Argentina	Vinchuca
Uruguay	Vinchuca
Brazil	Barbeiro (Portuguese – 'barber' or 'shaving bug') Chupão (Portuguese – 'big sucking bug') Bicudo (Portuguese – 'beaked bug') Furão (Portuguese – 'big piercing bug')

[a]Note that Triatominae may also be described by general terms such as 'hitas' (Spanish, which can also refer to ticks, mites, bedbugs etc.), 'percevejas' (Portuguese) or 'chinches de cama' (Spanish), which are more usually applied to Cimicid bedbugs.

Tribe Bolboderini Usinger, 1944

The Bolboderini comprise four genera (*Bolbodera, Belminus, Parabelminus* and *Microtriatoma*) with 11 species. All are small, with adults generally less than 12 mm long, collected from a variety of silvatic habitats including rodent nests, bromeliads and palm-tree crowns. The monotypic genus *Bolbodera* has only been collected from Cuba, but the other species have been collected from sites in southern Mexico and in Central and South America as far south as the state of Paraná in southern Brazil. It seems likely that current collections of these species are under-represented, and intermediate populations may be expected, especially in forest areas.

Species of *Belminus* and *Microtriatoma* have generally been encountered in association with other reduviids, including species of *Rhodnius*, and are suspected of having predaceous as well as blood-sucking habits. *B. herreri* has been laboratory reared by allowing it to take blood from engorged nymphs of *Rhodnius* – a process known as cleptohaematophagy (literally, stealing the bloodmeal) (Sandoval *et al*., 2000a). *M. trinidadensis* has occasionally been collected in peridomestic habitats, for example in chicken coops in Bolivia associated with *Rhodnius stali* (De la Riva *et al*., 2001).

Tribe Cavernicolini Usinger, 1944

The Cavernicolini comprise two genera (*Cavernicola* and *Torrealbaia*) with three species. They are small, with adults rarely more than 13 mm in length. *C. pilosa* seems the most widely distributed, from Panama to central Brazil, and is invariably associated with bats. *C. lenti* has been collected from hollow trees in the Amazon forest, also associated with bats. The biology of *T. martinezi* is unknown as the species is known only from the holotype.

Tribe Rhodniini Pinto, 1926

The Rhodniini comprise two genera: *Rhodnius* with 16 species and *Psammolestes* with three. They are small to medium-sized Triatominae, with adults generally within the range 12–25 mm long. All are generally of arboreal habit. The three species of *Psammolestes* are all associated with the woven-stick nests of furnariid birds (particularly *Annubius annubis* and *Phacelodomus rufifrons*) and feed primarily on these birds or on rodents that may occupy vacated nests. Their biology is poorly known since they are difficult to rear in the laboratory, but although they have been found naturally infected with *T. cruzi* they do not associate with humans and are considered to be without epidemiological significance.

Species of *Rhodnius,* however, are generally very easy to rear in the laboratory and provide excellent models for studies of basic insect physiology. They are readily distinguished from other Triatominae by the apical insertion of their antennae and distinct callosities behind the eyes (this latter character being a tribal trait). They are mainly associated with mature palm trees, living between the leaf axils and feeding on the wide range of vertebrates that may be found there. Exceptions include *R. pictipes* of the Amazonian forest, which occupies a wider range of ecotopes, and *R. domesticus* of the coastal Atlantic forest, which has almost invariably been found between the leaf axils of epiphytic bromeliads. The rare *R. paraensis* has only been recorded from arboreal rodent nests in a restricted area of the Amazon forest.

Several species of *Rhodnius* are important domestic vectors of Chagas disease. *R. prolixus* is particularly important in Venezuela, Colombia and parts of Central America, and may have derived from silvatic populations of the closely related *R. robustus*. In northwest Colombia and Panama, *R. pallescens* is known from palm trees and peridomestic habitats, but is considered the most important vector in Panama. *R. ecuadoriensis* is known from palm trees and peridomestic habitats in Ecuador but seems to be exclusively domestic in arid regions of northern Peru, where it is an important local vector. Several other species of *Rhodnius* have been recorded flying into human dwellings at night, presumably attracted by lights, and some seem able to form small domestic colonies, including *R. neglectus* in parts of central Brazil and *R. stali* in eastern Bolivia (Matias *et al.*, 2002).

Tribe Triatomini Jeannel, 1919

As currently defined, the tribe Triatomini includes seven genera in the Americas, together with the atypical genus *Linshcosteus* recorded only from the Indian subcontinent. A group of seven closely related species of *Triatoma* that occurs in parts of South-East Asia and southern China appears to have derived from *T. rubrofasciata* exported from the Americas in association with rats on sailing ships (Patterson *et al.*, 2001). Apart from records of *T. rubrofasciata* in port areas, no Triatominae have been recorded from Africa.

Genus Dipetalogaster

This is represented by a single species, *D. maxima*, known only from rocky outcrops at the southern tip of Baja California, Mexico. *D. maxima* is the largest species of Triatominae, with adults that can reach up to 45 mm in length. It seems a highly opportunistic feeder, sometimes feeding during the day on resting vertebrates. Occasionally it enters houses.

Genus Eratyrus

This is represented by two species, *E. cuspidatus* and *E. mucronatus*, characterized by conspicuous tubercles or spines on the anterior lobe of the pronotum. *E. cuspidatus* occurs in Central America and South America west of the Andes, while *E. mucronatus* occurs in South America east of the Andes. Both species have a range of silvatic habits, often associated with hollow trees and palm-tree crowns. Both have been reported in peridomestic habitats, and *E. mucronatus* has been found colonizing houses in parts of Bolivia.

Genus Hermanlentia

This is represented by a single species, *H. matsunoi*, known only from bat caves in the Peruvian Andes. *H. matsunoi* is a large species, up to 40 mm long, characterized by an unusually long head.

Genus Mepraia

This is a somewhat controversial genus, revalidated by Lent *et al*. (1994) for the atypical species previously known as *Triatoma spinolai*. This species occurs only in the arid central and northern regions of Chile, and is the only triatomine species to show conspicuous alary polymorphism. Females are invariably wingless, while males may be wingless or have normal or enlarged wings (Schofield *et al*., 1998). Populations from the northern Atacama desert region are generally entirely wingless and these populations have recently been ascribed to a separate species, *M. gajardoi*. Unfortunately, the revalidation of *Mepraia* by Lent *et al*. (1994) did not consider two other species previously grouped by Lent and Wygodzinsky (1979) within the 'spinolai complex'. These two species, *T. eratyrusiformis* and *T. breyeri* from central and northeastern Argentina, do not show alary polymorphism but do have morphological, morphometric and genetic similarities with *spinolai*, as well as similar ecology. *T. eratyrusiformis* also shows a similar karyotype to *spinolai*, with multiple X chromosomes (Panzera *et al*., 1998). The karyotype of *T. breyeri* is not yet known. All these species are of silvatic habit, generally associated with small mammals amongst rockpiles. None is of epidemiological significance.

Genus Panstrongylus

The genus *Panstrongylus* comprises 14 species (or 13 species, if synonomy of *P. herreri* with *P. lignarius* is accepted) distributed throughout Latin America (except Chile). They are generally large and robust species, with adults up to 40 mm long, characterized by a relatively short head with antennae inserted immediately in front of the eyes. Most are associated with terrestrial habitats, often in rockpiles or burrows such as those made by armadillos. Forest species such as *P. geniculatus*, *P. lignarius* and *P. rufotuberculatus* can also be found in hollow trees and palm-tree crowns. *P. megistus* is an important domestic vector of Chagas disease in Brazil. It was one of the first vectors to be incriminated by Carlos Chagas and was the principal domestic vector in central Brazil until being progressively replaced by *Triatoma infestans* since the 1930s. Today it remains widespread in Brazil, but mainly in silvatic and peridomestic habitats – particularly in the more humid coastal regions. *P. rufotuberculatus* and *P. herreri* are of localized epidemiological significance in parts of Bolivia and Peru, respectively, and *P. geniculatus* has recently been reported invading houses in parts of the Amazon region of Brazil and Venezuela.

Genus Paratriatoma

This is represented by a single species, *P. hirsuta*, characterized by many short bristles over the body surface. *P. hirsuta* is a chromatically variable species, associated with nests of packrats (*Neotoma* spp.) throughout the southwestern USA and northern states of Mexico.

Genus Triatoma

The genus *Triatoma* is by far the most numerous of triatomine genera, with 75 currently named species and numerous subspecies and morphological variants, many of which are important domestic vectors of Chagas disease. Adults of most species are around 25 mm long, but within the genus the adult size ranges from 10 to 40 mm. Within the genus, a series of specific groupings has been proposed (Lent and Wygodzinsky, 1979) which we have revised on the basis of recent morphometric analyses (Table 9.3). Morphometric analysis based on head characters suggests two main clades within the genus *Triatoma*, corresponding to those of North and Central America and those of South America. The difference between these two groupings is also supported by comparison of 18S ribosomal DNA sequences (Marcilla *et al*., 2001), suggesting that the ancestral forms of the two groups of *Triatoma* were separated prior to the closing of the isthmus of Panama about 3 million years ago.

Table 9.3. Probable species groups and complexes within the genus *Triatoma*. Each of the complexes indicated is currently believed to be monophyletic. However, available genetic and morphometric data suggest that the *rubrofasciata* and *phyllosoma* groups may also have a common ancestor, whereas the *dispar* complex may have evolved independently from other species of the *infestans* group.

Group	Complex	Subcomplex	Species
Rubrofasciata (mainly N. American and Old World)	protracta		*barberi*, *incrassata*, *neotomae*, *nitida*, *peninsularis*, *protracta*, *sinaloensis*
	rubrofasciata	rubrofasciata	*amicitiae*, *bouvieri*, *cavernicola*, *leopoldi*, *migrans*, *pugasi*, *rubrofasciata*, *sinica*
		lecticularia	*gerstaeckeri*, *indictiva*, *lecticularia*, *recurva*, *rubida*, *sanguisuga*
Phyllosoma (mainly Mesoamerican and Caribbean)	flavida		*bruneri*, *flavida*, *obscura*
	phyllosoma		*bassolsae*, *bolivari*, *brailovskyi*, *dimidiata*, *gomeznunezi*, *hegneri*, *longipennis*, *mazzottii*, *mexicana*, *pallidipennis*, *phyllosoma*, *picturata*, *ryckmani*
Infestans (mainly South American)	dispar[a]		*carrioni*, *dispar*, *nigromaculata*, *venosa*
	infestans	brasiliensis	*brasiliensis*, *melanocephala*, *petrochiae*, *wygodzinskyi*
		infestans	*delpontei*, *infestans*, *melanosoma*, *platensis*
		maculata	*maculata*, *pseudomaculata*,
		oliveirai	*baratai*, *deaneorum*[b], *guazu*, *jurbergi*, *klugi*, *matogrossensis*, *oliveirai*, *williami*
		rubrovaria	*arthurneivai*, *carcavalloi*, *circummaculata*, *costalimai*, *limai*, *rubrovaria*
		sordida	*garciabesi*, *guasayana*, *patagonica*, *sordida*
		Others	*lenti*, *tibiamaculata*[c], *vitticeps*
	spinolai	(*Mepraia*)	*breyeri*, *eratyrusiformis*, *spinolai*, *gajardoi*

[a]There is preliminary genetic evidence to suggest that the *dispar* complex may have evolved independently from other species placed here in the *infestans* group.
[b]= *T. deanei*
[c]*T. tibiamaculata* is chromosomically similar to members of the *brasiliensis* subcomplex, except for having multiple X chromosomes.

In the USA, species of *Triatoma* can be found in silvatic habitats (most frequently associated with packrats) almost as far north as the Canadian border. In the southern states, species such as *T. protracta*, *T. lecticularia*, *T. gerstaeckeri* and *T. sanguisuga* sometimes invade peridomestic habitats such as dog kennels, and occasionally transmit *T. cruzi* to domestic mammals. Five cases of vector-borne transmission to humans have also been recorded from the USA, in southern California, New Mexico and Texas.

In Latin America, species of *Triatoma* can be found in most regions except the high Andes (above 3500 m above sea level) and the southernmost parts of Chile and Argentina. They occupy a great range of silvatic habitats, including rockpiles, burrows, caves, birds' nests, fallen trees and bromeliad epiphytes. Many species invade peridomestic and domestic habitats, and include species of greatest importance as vectors of Chagas disease. Most significant is *T. infestans*, a species thought to have originated in central Bolivia and to have been transported in association with human migrations to Argentina, Chile, Brazil, Paraguay, Uruguay and southern Peru (Schofield, 1988; Dujardin *et al*., 1998a). By 1991, *T. infestans* was considered responsible for well over half of all cases of Chagas disease and became the principal target of vector control operations throughout the Southern Cone region. As a result of the Southern Cone initiative, it has now been

eliminated from most of Brazil, Uruguay and Chile and also from extensive areas of Argentina, Paraguay and southern Bolivia (see Chapter 31).

Other important vector species include *T. brasiliensis* in northeastern Brazil, *T. dimidiata* in Central America, Colombia and Ecuador, and various species of the *phyllosoma* complex in Mexico. *T. maculata* and *T. pseudomaculata* are common peridomestic species in Venezuela and northeast Brazil, respectively, and *T. sordida* is common in peridomestic habitats in central Brazil and the *chaco* region of Paraguay, southeastern Bolivia and northwestern Argentina.

Genus Linshcosteus

The genus *Linshcosteus* includes six species, known only from the Indian subcontinent, that clearly differ from other Triatominae by their relatively broader body and shorter proboscis. These species tend to occur in isolated rockpile habitats, probably feeding on rupicoline rodents, bats and lizards, and they probably represent isolated morphological variants from a common ancestor. They are without epidemiological importance, though they have been found infected with trypanosomes resembling the rat parasite, *Trypanosoma conorhini*. From their geographical, morphological and morphometric differences from other Triatomini, they probably merit separate tribal status (Carcavallo *et al*., 2000; Dujardin *et al*., 2000).

Morphology and Development

The Hemiptera are exopterygote insects (this name derives from the fact that the wings develop outside the body, instead of within a pupal case). Their life cycle proceeds from eggs through various nymphal stages to the adults. In the case of Triatominae and other Reduviidae there are five nymphal stages (see Plate 6). These differ from the adults primarily by their lack of fully developed wings or genitalia, although they generally occupy the same habitat and feed on the same hosts as the adults. For Triatominae, this means that all five nymphal stages, and both sexes of adult, feed on vertebrate blood and are capable of becoming infected by and transmitting *T. cruzi*.

Adults

The main anatomical features of adult Triatominae are shown in Plate 7. They can generally be distinguished from other reduviids by the straight, slender, three-segmented proboscis adpressed to the underside of the cone-shaped head, which reaches the prosternal stridulatory sulcus in all genera except *Cavernicola* and *Linshcosteus*. By contrast, the proboscis of predatory reduviids is often curved, and usually more heavily chitinized. However, many predatory Reduviidae – especially of the subfamily Reduviinae – can appear very similar to Triatominae.

Most adult Triatominae are dark grey, brown or black, but many have characteristically coloured markings (e.g. red, brown, yellow, orange or pink) along the connexivum, often with similar coloured markings on the legs and thorax. Sexes are readily distinguished from the dorsal view, with the males having a smoothly rounded tip to the abdomen compared with the pointed or trilobed tip of the females. Males are often smaller than females, but this is not always so.

Adults and nymphs have a pair of compound eyes that are generally of the same colour as the rest of the body but may fade to grey after death. Eye colour is genetically determined, and white- and red-eye forms have been recorded in some species; red-eye is an autosomal recessive character (Pires *et al*., 2002). Adults and nymphs also have a pair of laterally inserted, four-segmented filiform antennae and the position of insertion can be used to distinguish important genera: in *Rhodnius* the antennae are inserted almost at the front of head; in *Panstrongylus* they are just in front of the eyes; and in *Triatoma* they are intermediate in position. Adults also have a pair of ocelli just behind the eyes but nymphs do not.

The adult thorax has a prominant pronotal shield, generally heavily chitinized and

occasionally bearing spines or tubercles. The metathoracic segment contains two pairs of scent glands. The dorsal Brindley's glands appear to be unique to the Reduviidae. They secrete isobutyric acid in all species so far examined (together with some minor components), which is corrosive and may act both as a defensive secretion and possibly also as an alarm pheromone. The ventral metasternal glands open to the metacoxal cavities. They are common to most Heteroptera, with various functions. In Triatominae, their function is unknown and the secretion has been characterized only in *D. maxima*, as 3-methyl-2-hexanone (Rossiter and Staddon, 1983).

Important taxonomic features of Triatominae include colour and chromatic patterns, and features of the head capsule, wings and male genitalia. Measures of the head capsule and wings are also used in quantitative morphometric analysis (Dujardin *et al*., 2000). Keys to genera and most species are given by Lent and Wygodzinsky (1979).

In the laboratory, adult bugs may live for several months – in some cases more than a year. In natural populations, however, adult lifespan is generally reduced. In the case of *T. infestans*, for example, which is one of the most extensively studied species, adults can live for up to 18 months in the laboratory but average only 3 months in natural conditions (Gorla and Schofield, 1989). During this time, they will typically feed every 4–9 days if hosts are available but can survive for several weeks without feeding.

Eggs and nymphs

Eggs of Triatominae may be laid singly or in clumps, loose or adhered to the substrate, depending on the species. Most are pearly-white or pinkish when laid, gradually becoming darker as the embryo develops inside. The ovoid eggs have a distinct surface architecture, which has taxonomic value in some groups of species, and they have a distinct operculum or cap that opens on maturity to allow the first instar nymph to emerge. In general, species with arboreal habits tend to adhere their eggs to the substrate, often in small groups. For *Psammolestes* (and also many predatory reduviids) the egg clusters seem to provide some protection against hymenopteran egg parasitoids, since it is generally only the outer eggs of the cluster that become parasitized. In silvatic and domestic species of Triatominae, around 10–40% of eggs tend to become parasitized by Hymenoptera such as *Telenomus fariai* and other species of Scelionidae and Encyrtidae. However, the egg stage of Triatominae generally carries < 1% of the reproductive capacity of each generation (meaning that destruction of all eggs of a specified age would only reduce the subsequent number of females by 1%) and so egg parasitism has no significant role in population regulation (Rabinovich, 1972; Gorla and Schofield, 1985, 1989) (see next section).

Egg laying generally begins a few days after emergence of the female and may continue throughout the life of the female. Unmated females may lay a few infertile eggs. Mated females appear able to store sperm for a long period, but there is evidence of sperm depletion over time and so multiple mating can increase the total number of fertile eggs laid (Pires *et al*., 2002). Eggs are generally laid a few at a time, during the bug's circadian period of activity. The number of eggs laid depends primarily on the quantity of blood ingested by the female, giving a linear correlation between blood intake and number of eggs laid. In the case of *T. infestans,* for example, each egg corresponds to a blood intake of about 20–25 mg. Under natural conditions in Argentina, *T. infestans* females lay up to 160 eggs per month during the warmer summer months, but fewer than 60 per month during the winter (when they feed less often), giving an average total egg output of about 250 eggs per female (Gorla and Schofield, 1985, 1989). The time of egg hatching depends primarily on temperature, with the hatching of most species occurring after 14–28 days at 24–28°C. Higher temperatures accelerate hatching but can also increase egg mortality. Low temperatures can inhibit hatching for several weeks and also increase mortality (Gorla and Schofield, 1985, 1989).

As with the adults, nymphs of Triatominae can usually be distinguished from those of other Hemiptera by their straight proboscis adpressed to the underside of the cone-shaped horizontal head. Unlike most predatory Reduviidae, nymphs of Triatominae do not have abdominal scent glands. Keys to nymphs of many species of Triatominae are given by Lent and Wygodzinsky (1979), but nymphs collected from the wild may be 'camouflaged' by the adhesion of fine dust over their body surface. Note that it is always preferable to rear nymphs to adults in order to confirm their identification.

Newly emerged nymphs are soft and pinkish, but the cuticle soon hardens and darkens and within 2–3 days the nymph is ready for a bloodmeal. At least one full bloodmeal, or several smaller meals, is required for moulting to each successive nymphal stage, though nymphs of many species can survive for several months without feeding if no host is available. Successive nymphal stages differ from each other by minor morphological details, but they can be readily distinguished by the size of head capsule and thickness of the legs. Overall body size is not a good guide to nymphal stage, because a well-fed nymph may appear larger than an unfed nymph of the succeeding stage. Wingpads are clearly visible on fifth-stage nymphs and can usually be discerned on fourth-stage nymphs. Future males and females can be determined by examination of the posterior sternites of fourth- and fifth-stage nymphs (Gillett, 1935).

The development time from egg to adult varies with species but is typically 4–12 months. Smaller species such as *R. prolixus* may complete egg to adult development in about 4 months, and some larger species such as *T. dimidiata* and *D. maxima* may take more than 12 months, but most species seem capable of two generations per year under good conditions of temperature and host availability. Feeding is inhibited and development is slowed by low temperatures; development is faster at higher temperatures, but mortality also increases. Most species thrive at temperatures of 24–28°C and atmospheric humidity of 30–80% relative humidity. Development generally halts at temperatures below 16°C, while temperatures above 40°C are usually lethal.

Feeding and defecation behaviour

The role of triatomine bugs as vectors of *T. cruzi* is crucially dependent on their feeding and defecation behaviour. *T. cruzi* is a 'posterior station' parasite. It is ingested as bloodstream trypomastigotes when the bug feeds on an infected mammal. The parasite develops and multiplies in the hindgut of the bug, with production of infective forms in the rectum that can be subsequently passed out with the bug's excreta. *T. cruzi* does not survive passage through the bug haemolymph, and so does not develop in any other part of the bug and is not transmitted by the bite. By contrast, the related parasite *T. rangeli* is able to transit the haemolymph and can develop in the salivary glands of some triatomine species (almost invariably species of *Rhodnius*), so that *T. rangeli* is primarily transmitted by the bite. (Note, however, that *T. rangeli* appears to be non-pathogenic in humans).

Some species of Triatominae defecate long after feeding and so tend to be poor vectors: an example is *T. protracta,* whose specific name derives from the 'protracted' time between feeding and defecation. Several other species defecate while feeding, while still in contact with the host, in which case parasites in the faecal droplet may enter the host through abraded skin, or sometimes by the bite wound, with local development producing a small lesion known as a 'chagoma' at the portal of entry. Most frequently, however, inadvertent touching of the faecal droplet can allow the contaminants to be passed to eye, nose or mouth, when the parasites can pass rapidly across the mucosa. Unilateral swelling around the ocular cavity, which is thought to result from parasite passage across the ocular mucosa, is known as 'Romaña's sign' and is strongly diagnostic for the acute stage of human infection (unilateral ocular swelling can also be caused by the toxic action of the

bug's saliva but in this case has a short duration). *T. cruzi* can readily transit the oral mucosa and there are several recorded cases of human infection due to ingesting food or drink contaminated with infected bug faeces. It is believed that this is the primitive route of transmission, so that in silvatic situations it seems most likely that reservoir hosts such as opossums or rodents acquire the infection by eating infected bugs or licking the hair coat that has been contaminated with bug faeces. In domestic situations, the probability that a bug will defecate while in contact with the host is inversely density dependent – meaning that the likelihood of parasite transmission per infected bug is greatest at low bug densities (Kirk and Schofield, 1987). This apparent paradox results because the probability of a bug taking a replete meal is reduced at high bug densities, with a concurrent delay in its likelihood of defecation (see next section).

Nymphs and adults of Triatominae feed from the same range of available hosts. In general they appear to have little host specificity, but instead will probe and feed from whatever vertebrate hosts are available. Warm moist carbon dioxide seems to act as an alerting stimulus, but the primary mechanism of orientation to the host seems to involve radiant heat. No specific kairomones are currently known, though the antennae of Triatominae bear a range of chemoreceptors that can respond to fatty acid odours (Catalá, 1997). Most hungry bugs will probe a warm surface and sample the underlying fluid. Receptors in the food channel of the proboscis respond to engorgement stimuli in the form of ATP and its analogues (Friend and Smith, 1982). The bug then injects saliva with powerful antihaemostatic properties (Ribeiro *et al.*, 1998) and feeding commences. Bug saliva is complex, with various pathways to inhibit platelet aggregation, coagulation, and mast-cell release. As a result, the bite of the more domesticated species tends to be fairly imperceptible to humans, though the bite of some silvatic species can be very painful. Bites of *P. geniculatus* on humans and pigs, for example, can leave lesions that resemble cutaneous leishmaniasis, and at least one case of death has been recorded due to anaphylactic shock following the bite of *T. rubrofasciata* (Ryckman and Bentley, 1979).

Most species of Triatominae require up to 20 min for complete engorgement but readily break off from feeding if disturbed. The amount of blood taken by nymphs increases with each stage, with the fifth-instar nymphs sometimes taking 8–10 times their unfed weight in blood. In the laboratory, a fifth-instar nymph of *D. maxima* has been recorded taking nearly 4.5 g of blood in a single meal. Adults typically take 3–5 times their body weight of blood at each meal if allowed to engorge. Thus during adult life, a female *T. infestans* will ingest about 10 g of blood, while larger species such as *P. megistus* may take twice this amount. From the amount of blood ingested by nymphs and adult bugs, it is deduced that domestic infestations of Triatominae can make a significant contribution to chronic iron-deficiency anaemia. Calculations based on domestic infestations of *R. prolixus* in Venezuela, and of *T. infestans* in Brazil, suggest that each person in a typically infested house is losing an average of about 2.5 g of blood/day due to the feeding bugs.

During or after feeding, the bugs begin to defecate, voiding excess water from the current bloodmeal, together with uric acid (their nitrogenous waste product) and haem from the undigested part of the previous bloodmeal. Bug faeces on the walls of infested houses often appear as characteristic white and black streaks due to the uric acid and haem, respectively. The faecal deposits also contain a volatile pheromone of complex action, which may signal the presence of a host to unfed nymphs but can also mark refuges where fed nymphs and adults can hide (Lorenzo and Lazzari, 1996). The identity of this pheromone is not known but may be ammonia (Taneja and Guerin, 1997).

Population Dynamics

Triatominae have a longer life cycle and lower rate of reproduction than most other insects of medical importance. They are often described as *K*-strategists, adapted for

efficient exploitation of the stable habitats offered by vertebrate nests or human dwellings. This is in contrast to *r*-strategists, such as mosquitoes, that are better adapted to more unstable changeable conditions. Highly domestic species such as *T. infestans* and *R. prolixus* can reach high densities in human dwellings, maintaining population numbers that are similar from year to year, though there may be seasonal changes in numbers and population age structure. The 'record' is currently held by *R. prolixus* in Colombia, where over 11,400 bugs were collected from a single rural house (Sandoval *et al*., 2000b).

The process of density regulation in domestic populations of Triatominae seems to involve three main mechanisms mediated by density-dependent access to bloodmeals and modulated by ambient temperatures. As bug density increases, relative to a fixed availability of hosts, then the likelihood of host disturbance also increases. This means that each bug becomes more likely to break off from feeding before it has become fully engorged. As a result, its nutritional status declines, with three main consequences. In the case of females, reduced blood intake results in reduced egg production; in the case of nymphs, reduced blood intake results in slower development. Both these consequences act to reduce the rate of recruitment from one developmental stage to the next, so that the overall bug density tends to decline. The third consequence is that reduced blood intake for the adults can promote dispersive flight. This seems to affect primarily male bugs, and has been described as a form of 'pseudo-apoptosis' whereby males disperse to leave more resources available for their offspring (see next section). In the reverse sense, reductions in bug density mean that each bug now has greater access to a bloodmeal, so that development and egg production can increase. By this means, the bug population is maintained at more or less constant levels in terms of the overall blood demand. However, the age structure of the bug population (and hence total numbers of individual bugs) can vary within this system, since, for example, the blood demand of younger nymphs is less than that of older nymphs and adults, and so seasons of peak adult emergence and egg laying may be characterized by a higher proportion of recently emerged younger nymphs.

There are several epidemiological consequences of this system of population regulation. Firstly, the overall likelihood of transmission of *T. cruzi* to humans will depend on the relative proportion of older nymphs and adults in the population, because these have a higher probability of already having taken an infected bloodmeal. As a result, there is a degree of correlation between the frequency of acute cases (i.e. recently transmitted) of Chagas disease and the greater proportion of older nymphs and adult bugs present during the warmer summer months. Secondly, since density regulation depends almost entirely on density-dependent access to bloodmeals, reductions in bug density – for example, by a control intervention that is only partially effective – will tend to be compensated for by rapid development of the remaining bug population back to its original levels. Moreover, attempts to reduce bug population density by reducing the number of hiding spaces available will tend to be ineffective, since bug population density is primarily regulated by bloodmeal resources rather than by spatial resources or predators. Finally, since the likelihood of transmission depends on defecation while still in contact with the host, which is in turn dependent on the size of bloodmeal ingested, then the highest risk of transmission of *T. cruzi* occurs while the bug population is growing towards its maximum density, i.e. when bugs are able to engorge fully, without density-dependent competition for bloodmeals. This means that a partial reduction in bug density – for example, due to an ineffective control intervention – may be counterproductive in terms of increasing the transmission risk.

The system of density regulation of domestic Triatominae also has important genetic consequences. When the bug population reaches maximum levels, its rate of population increase declines to unity, i.e. the population is neither increasing nor decreasing. On average, therefore, each adult female at this point is giving rise to only one

adult daughter. However, the adult females retain the capacity to lay about 200 eggs during adult life, of which around 100 would be future females. This means that at this point in population development there is intense intersibling competition, resulting in the failure of about 99% of offspring to reach maturity. This implies very strong selection pressure favouring those individuals that are most energetically efficient in utilization of available resources. The result is a trend amongst domestic Triatominae towards genetic and physiological simplification, and reduced genetic variability (Schofield *et al*., 1999). Genetic variability of each domestic population is also reduced by the original founder effect, since each new population generally results from only a single founding female, and also by the low immigration rate from other populations (see later). In consequence, bug populations in a single house or discrete village community tend to be relatively monomorphic, though genetic drift in isolation from other populations often means that bugs from each locality may conform to a locale-specific genotype (Dujardin *et al*., 1998a). These genetic characteristics strongly favour insecticidal control of domestic Triatominae, because each bug in the target populations can be expected to respond in the same way to a given control intervention, and the reduced genetic variability offers a low likelihood of selecting for different attributes such as insecticide resistance (see Chapter 31).

Dispersal

Active dispersal

Triatomine bugs can disperse by two mechanisms: actively by walking or adult flight, and passively by being carried by an appropriate vertebrate. When bugs are disturbed from their refuges they may attempt to walk away, or they may become immobile – 'feigning death'. In general, however, walking by nymphs and adults is for short-range movements, to and from refuges, or from a refuge in search of a bloodmeal, and usually follows a circadian rhythm. For the majority of species associated with diurnally active hosts, the main period of locomotory activity is nocturnal, beginning shortly after dusk (Lorenzo and Lazzari, 1996). Species that feed on nocturnally active lizards and some species of desertic environments (such as *D. maxima* in Baja California, or *T. spinolai* in the arid north of Chile) are often active during the day. Their locomotor activity seems to depend more on host presence, and such bugs have been seen to scurry over rocks to approach a seated observer (e.g. Marsden *et al*., 1979).

Active flight by adult bugs is generally associated with bug populations in unstable habitats with poor access to a blood source. Thus adult flight may be triggered by ecological changes leading to host migration or death, so that bugs become hungry and are prompted to disperse. Bugs collected flying to light sources are invariably starved, and field and laboratory studies show that flight is generally associated with low nutritional status, often combined with above-average temperatures (Lehane *et al*., 1992). Many flights are trivial, covering distances of only a few metres and generally in a downward path, but some bugs are able to make sustained flights covering distances of several hundred metres. A model of dispersive flight by *T. infestans*, solved with field data from Brazil and Argentina, showed that the average effective flight range for this species was around 200 m (Schofield and Matthews, 1985) but natural flights of up to 1 km have been recorded (Schweigmann *et al*., 1988). Studies of forced flight in the laboratory show that some bugs can beat their wings for an hour or more, achieving forward speeds of around 1–1.5 m/s.

For most species, flight can be initiated equally by males and females, though it is often a higher proportion of males that flies. The reasons for this are unclear, especially since the likelihood of finding a suitable new habitat would be very low and dispersing male bugs would be unable to colonize a new habitat. For this reason, male dispersal has been described as 'pseudo-apoptosis' whereby males are effectively committing suicide in order to leave more resources available for their offspring (Schofield *et al*.,

1999). Recent studies have revealed polymorphism in the flight muscle enzymes of fliers and non-fliers. Bugs that fly have a much more active form of the enzyme alpha-GPD, and the proportion of bugs with this active form of the enzyme seems to vary within species between populations.

A wide range of triatomine species has been recorded flying to house lights at night, including, for example, *P. megistus, P. rufotuberculatus, P. lutzi, T. dimidiata, T. pallidipennis, T. vitticeps* and *R. pictipes*. Many have also been taken at light traps, though most come to rest some metres before the light source itself. However, several species show no obvious tendency to be attracted to a light source and it is not at all clear what would be the normal orientation signal for flying bugs. Field experiments on flying male *T. infestans* in the salt flats of central Argentina showed no deviation from a randomly directed dispersal pattern (Schofield *et al*., 1992) but *R. brethesi* has been reported flying to attack people in the Amazon region of Brazil (Coura *et al*., 1994). It may be that some bugs disperse randomly, while others use heat (e.g. infrared radiation) and/or chemical signals for flight orientation.

Passive dispersal

In general, it seems that silvatic species have a greater tendency to disperse by adult flight and that this tendency becomes progressively reduced amongst populations adapted to live in more stable habitats – especially those that live in domestic habitats. For example, simulation modelling solved with field data from Brazil shows that observed patterns of house colonization by *T. infestans* can be best explained by passive dispersal of bugs into a locality, followed by active and passive dispersal from one house to another within that area (Jedwab, 1987). This also accords with genetic studies indicating that bugs within a village tend to form a panmictic unit, with genetic drift allowing differentiation between bugs from neighbouring villages (Dujardin *et al*., 1998a). Prior to current control campaigns (see Chapter 31) *T. infestans* was commonly found amongst the clothes and belongings of travellers from endemic areas – even under horses' saddles and within the seats of long-distance trains and buses. Moreover, historical reconstruction suggests that the geographical spread of *T. infestans* from its presumed origin in central Bolivia has been associated with documented human migrations (Schofield, 1988), and the spread of *R. prolixus* into Central America seems to have been entirely due to accidental human carriage (Dujardin *et al*., 1998b; Schofield and Dujardin, 1999). Similarly, the spread of *T. rubrofasciata* can be attributed to passive transport on sailing ships (Patterson *et al*., 2001) and there is evidence from genetic and morphometric studies that *T. dimidiata* was similarly carried from Mexico to Ecuador (Abad-Franch *et al*., 2001; Marcilla *et al*., 2001; Schofield, 2002).

Amongst some silvatic species and peridomestic populations, there is strong anecdotal and circumstantial evidence for passive dispersal associated with the vertebrate hosts. For example, eggs of *Psammolestes tertius* have been found adhered to the feathers of its furnariid bird hosts; nymphs of *T. dimidiata* in Honduras have been found amongst the feathers of domestic chickens, and young nymphs of *T. sordida* have been reported amongst the feathers of sparrows in Brazil (Forattini *et al*., 1971). Dispersal of coastal populations of *T. spinolai* in Chile has been suggested to occur in association with marine birds or by way of young nymphs within the pelage of sea-lions – which has been shown to be at least experimentally feasible (Schofield *et al*., 1998).

In evolutionary terms, therefore, the capacity for adult flight may be regarded as the primitive state amongst silvatic Triatominae, becoming progressively reduced with adaptation to more stable habitats offering a more predictable food supply. The trend amongst Triatominae seems to be towards specialization and habitat stability, leading to greater dependency on the vertebrate host for passive dispersal. In extreme cases, such as populations of *T. spinolai* in the Atacama desert of Chile, where survival away from the host becomes most unlikely, the bug populations have lost their wings entirely (Schofield *et al*., 1998).

Similarly, in long-established laboratory colonies where food is regularly offerred to the bugs, complete atrophy of the adult flight muscles is common.

Evolution and Population Genetics

Current theory considers the Triatominae to represent a polyphyletic assemblage of Reduviidae that have adapted towards particular habitats offering shelter from climatic extremes together with a more abundant supply of proteinaceous food. In primitive terms, an adaptive process from a free-living predatory form to a nest-dwelling haematophagous form can be envisaged and it can be supposed that this has happened several times within the Reduviidae to give rise to the various tribes, genera and species groups of Triatominae. Such a trend has been associated with various morphological, biochemical and reproductive changes but can also be envisaged as a typical evolution of demographic strategy (*sensu* Rabinovich, 1974) from *r*-strategists (free-living predators adapted to relatively unstable conditions of habitat and food supply) to *K*-strategists (nest-dwelling predators exploiting a more stable habitat and food supply). Similar adaptations are thought to have occurred in other Hemipteran families – for example, amongst the predatory Anthocoridae to give rise to the haematophagous Cimicidae and Polyctenidae, and also amongst the predatory Lygaeidae to give rise to the Cleradini, of which several species seem to be at least facultatively haematophagous.

Within the Triatominae there are several lines of evidence to suggest that this adaptive process has been relatively recent (Schofield, 2000). The absence of autochthonous species from Africa dates them after continental separation, and they could not have followed our proposed evolutionary route before the advent of nest-building mammals and birds in the neotropics, which already places them in the post-Cretaceous period. Their frequent association with rodents would suggest that they evolved well after the mid-Tertiary period and, from their relative lack of divergence from the basic reduviid form, we see no reason to suggest that they would have evolved even prior to the Quaternary period. Apart from characters directly associated with their blood-sucking habit, such as mouthparts, digestive system and sensilla patterns, their body form is little changed from that of their putative reduviid ancestors. Several are known to feed facultatively from invertebrates and the bite of many Triatominae remains painful to the vertebrate host (cf. Ryckman and Bentley, 1979), which recalls the painful bite of most predatory reduviids. Also, like all obligate bloodsuckers, Triatominae require intestinal symbionts to provide essential vitamins that are lacking in their blood diet, but while other obligate bloodsuckers carefully store their symbionts either intracellularly or in a specialized part of the fat-body (the mycetome) the symbiotic fauna of Triatominae is less consistent and lives haphazardly in the gut lumen.

Comparative genetic studies are also lending support to the idea that Triatominae are a relatively recent evolutionary development, and have also shown evidence of polyphyletic evolution (eg. Monteiro *et al*., 2000; Marcilla *et al*., 2001). This is most striking in comparisons between the Triatomini and Rhodniini, which appear to have little in common other than a general reduviid form and haematophagous habit. They are well separated however, by morphology and morphometric characters, and isoenzymes (Chávez *et al*., 1999; Dujardin *et al*., 1999a) and also by cuticular hydrocarbon comparisons (Juárez *et al*., 2001), mitochondrial, nuclear and ribosomal DNA sequences (Monteiro *et al*., 2000; Marcilla *et al*., 2001) and salivary proteins (Pereira *et al*., 1996; Ribeiro *et al*., 1998; Soares *et al*., 1998). It seems likely, therefore, that the bloodsucking habit has arisen several times within the Reduviidae to give the various tribes and genera of Triatominae that are currently recognized, but it is also likely that the ancestral forms of the different bloodsucking groups were related to different predatory subfamilies. A striking feature of the distribution of triatomine species groups is that each tends to form a geographically discrete grouping, with the occasional anomalies being readily explained by passive

carriage by vertebrate hosts (Schofield, 1988). This suggests that each of the recognized species groups (Table 9.3) has arisen from a specific ancestral form that has subsequently radiated. A discussion of apparent radiative adaptation in the Rhodniini is given by Schofield and Dujardin (1999).

These ideas of the evolution of Triatominae are summarized in Fig. 9.1, where the adaptive process leads ultimately to the development of domestic populations that assume particular significance as vectors of human Chagas disease. For the bugs, this process has important genetic consequences that favour current attempts to eliminate domestic populations. In general, each domestic population is founded by a single female, either entering by active flight or brought to the house by passive carriage associated with a vertebrate host. The female feeds and lays eggs, and the emerging nymphs may, or may not, succeed in feeding and completing their development. There are thus two scenarios in the initial entry of a bug into a human home: successful or unsuccessful colonization. Both can be epidemiologically significant, because even unsuccessful colonization can involve point transmission of *T. cruzi*. Such cases have occurred in some urban areas, where adventitious female bugs occasionally fly into apartments, feed, transmit the parasite and then die.

In cases of successful colonization there is a genetic founder effect, in the sense that the original female carries only a fraction of the available gene pool of the species. The offspring are all genetically related and, if the house is relatively isolated and receives no further immigrant bugs, the resulting population will remain genetically restricted. Then, as the bug population increases towards its maximum level, inter-sibling competition becomes an important additional factor acting to reduce genetic variability of the domestic bugs (see earlier). This sequence of founder effect, genetic drift and inter-sibling competion, can then be repeated as domestic bugs are carried from one house to another (Fig. 9.2).

The best studied example of this process concerns *T. infestans*, which is considered the most highly domestic of all species of Triatominae, and responsible for most transmission of human Chagas disease in the Southern Cone countries of Latin America. Species homogeneity of various domestic and silvatic populations of *T. infestans* from Bolivia, Brazil, Peru and Uruguay showed that the species was structured into small units between which very little gene flow seemed to occur, and that geographical distances rather than selective factors could account for most of the heterogeneity between populations (Dujardin *et al*.,

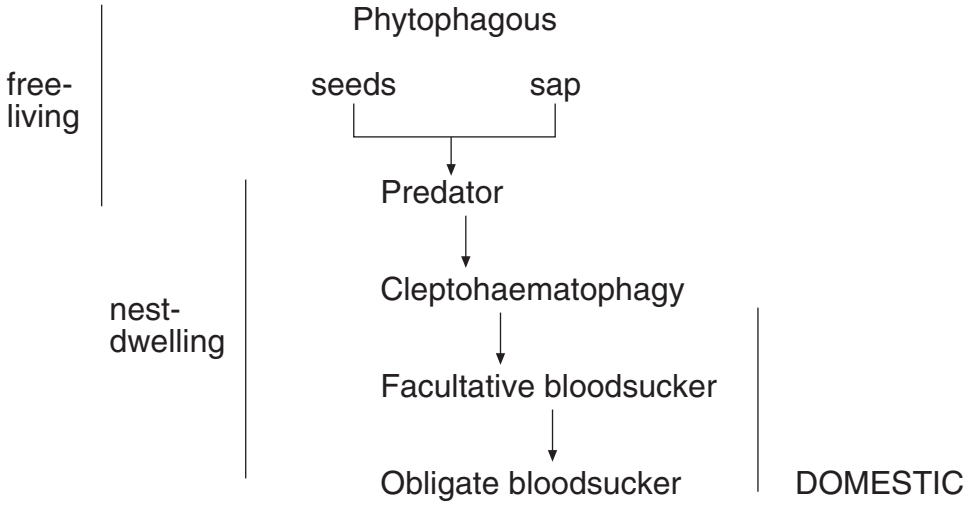

Fig. 9.1. Summary of the presumed evolutionary stages towards bloodsucking in Triatominae.

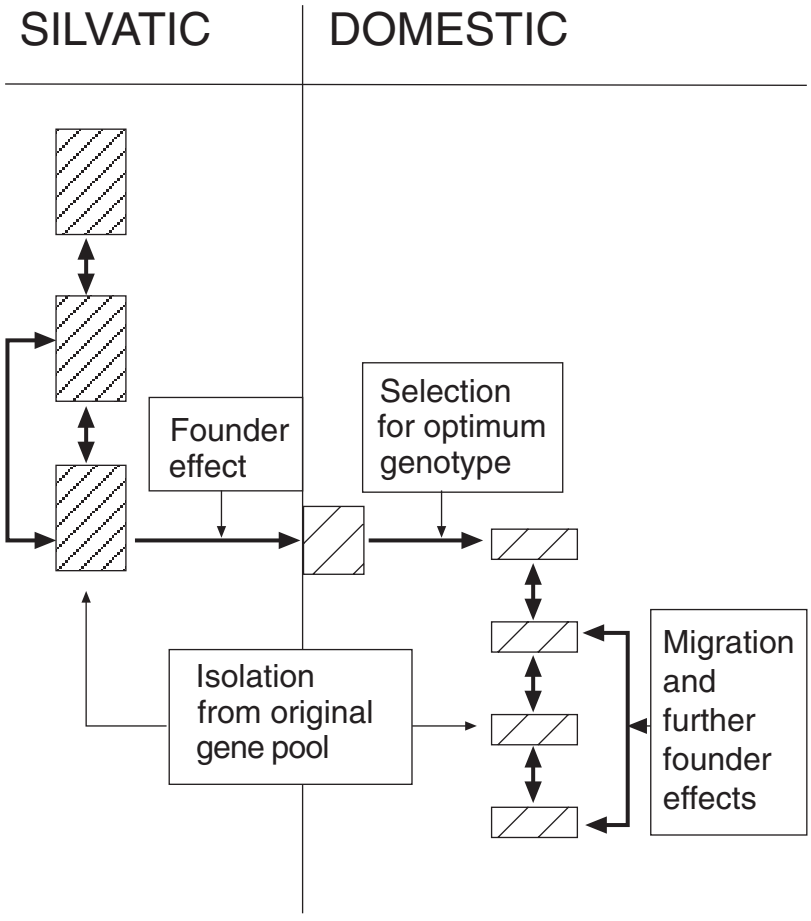

Fig. 9.2. Diagrammatic representation of the genetic processes involved in domestication of Triatominae. From the original gene pool of the silvatic population, a founder effect occurs when the first silvatic female enters and lays eggs in the domestic habitat (i.e. this female can only carry a fraction of the genetic material represented by the original population). If the resulting domestic population remains isolated from the original gene pool, genetic drift can also be expected. Further genetic simplification then occurs through intersibling competition as the domestic population increases towards its maximum levels, leading to selection for the most energetically efficient individuals. The process may be repeated as individuals from each domestic population are transported to colonize further domestic habitats (see text) (modified from Schofield *et al.*, 1999).

1998a). Different electrophoretic techniques diverged in their estimates of heterozygosity but agreed on the relatively low variability of *T. infestans* compared with silvatic species. This, as well as the very low genetic distances among natural populations of the Southern Cone countries, suggested a recent spread of the domestic populations of *T. infestans* (Dujardin *et al.*, 1998a).

Genetic analysis of these data also supported the notion of a unique ancestral population of *T. infestans* (Dujardin *et al.*, 1998a). This hypothesis was investigated assuming that the population dispersal of domestic *T. infestans* had produced random dispersal of different genes – in which case the initial population is expected to present gene frequencies similar to those found in the whole sample. On this basis, Bolivia was incriminated as the geographical origin of the domestic populations of *T. infestans*. This accorded with the existence of silvatic

colonies of *T. infestans* known only from the Cochabamba and Sucre regions of Bolivia, as well as with historical records suggesting that *T. infestans* had entered Peru, Uruguay and Brazil at around the turn of the 20th century (Schofield, 1988). Thus, the hypothesis of a recent and rapid spread of *T. infestans* from one geographical source could be supported by population genetic studies and by biogeographical and historical criteria. It was also supported by the existence of geographical clines from the supposed origin in Bolivia to more peripheral areas. From Bolivia to Uruguay, for example, cytological analyses showed a decrease of chromosomal C-banding, as well as a striking decrease of DNA content in the gonads of male *T. infestans* (Panzera *et al*., 1998). Between the same countries, head morphometrics showed a consistent cline of decreasing size (Dujardin *et al*., 1998b).

Similar features were found in *Rhodnius prolixus*, a highly adapted domestic species and principal vector of Chagas disease in Venezuela, Colombia and parts of Central America. Isoenzyme surveys of natural populations of this species were consistent with low genetic variability, while RAPD and morphometric comparisons suggested that this species in Central America could represent a recent and genetically limited subset of the original South American populations, probably those of Venezuela (Dujardin *et al*., 1998b). This was in accord with historical records suggesting that *R. prolixus* in Central America was derived from an accidental escape of laboratory-bred bugs in 1913, which were subsequently transported in association with people visiting different rural areas (Schofield and Dujardin, 1999). In this way, *R. prolixus* appears to have been dispersed as a domestic species in much the same way that domestic *T. infestans* was dispersed through the Southern Cone countries during the last 100 years (Schofield, 1988). A similar sequence of events – of a domestic species being carried by people to other countries – is now supported for domestic *T. dimidiata* populations carried along pre-Columbian maritime routes from Mexico to Ecuador (Abad-Franch *et al*., 2001; Schofield, 2002).

In epidemiological terms, the picture of Triatominae that emerges involves a series of species adapting to stable habitats, which, for some, means domestication (i.e. adaptation to the most stable habitat offered by human habitations). Once domesticated, the genetic consequences of founder effects, drift, inbreeding and strong intersibling competition lead to progressive genetic simplification, often with evidence of developmental instability such as increased fluctuating asymmetry or unilateral morphological monstrosities (Dujardin *et al*., 1999b), a decrease in body size (Dujardin *et al*., 1998b,c) and reduced sexual dimorphism (Dujardin *et al*., 1999c). The domesticated populations also become increasingly reliant on their hosts – people and domestic animals – for dispersal, but by this means they can spread to other domestic habitats in areas beyond the natural ecological limits of the original species. These features – close association with humans and wide geographical dispersal – on the one hand make these populations the most significant as vectors of human Chagas disease, but on the other hand the genetic consequences make such populations highly vulnerable to well-proven control interventions (see Chapter 31). Indeed, where domestic populations have been imported as exclusively domesticated entities, as seems to be the case for *T. infestans* in most of the Southern Cone, *R. prolixus* in Central America and central Colombia and *T. dimidiata* in Ecuador, then such populations can be proposed as candidates for complete eradication. Perhaps of greater concern, therefore, are those species that are currently in the process of domestication, but which still maintain close genetic links with their original silvatic populations. For these, we have yet to understand fully the underlying ecology of the transition, and we have yet to develop satisfactory practical approaches for inhibiting the process.

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PART 3.
EPIDEMIOLOGY AND DIAGNOSIS

10 Diagnosis of Human African Trypanosomiasis

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Introduction

Diagnosis of human African trypanosomiasis (HAT) or sleeping sickness can be defined as the demonstration of infection by *Trypanosoma brucei gambiense* (*T. b. gambiense*) or *rhodesiense* (*T. b. rhodesiense*). Evidence of infection may be indirect (clinical and biological features, serological and molecular test results) or direct (demonstration of the parasite). For reasons related to costs and risks associated with treatment, the demonstration of the parasite is mandatory for primary diagnosis. Yet primary diagnosis is not sufficient for deciding on treatment. Since not all drugs pass the blood–brain barrier, it should first be determined whether the parasite has reached the central nervous system. This 'stage determination' may be accomplished by direct (parasite detection) or indirect diagnostic techniques. Finally, after treatment, both direct and indirect diagnostic techniques should repetitively be applied to assess the disappearance of the parasite. It is recommended (but almost impossible) to follow up the patient during 2 years after treatment.

Diagnosis of the infection is important not only for the patient but also for the community. Undiagnosed, patients will remain untreated and will die sooner or later. In the meantime, they act as a reservoir on which teneral tsetse flies will infect themselves to spread the infection. In addition, due to the debilitating character of the disease, patients become a burden to their families. As the disease progresses from first to second stage, treatment becomes more expensive and the risk of severe side effects and sequellae increases.

As long as vaccination or prophylaxis against the infection and effective vector control are lacking, diagnosis will remain one of the cornerstones in the control of the disease, hence the importance of correct application of the different techniques currently available. Since sleeping sickness occurs in rural environments in sub-Saharan Africa, it is clear that only those diagnostic techniques that combine cost effectiveness, rapidity, simplicity and diagnostic performance will be applied within sleeping sickness control activities. Nevertheless, techniques not readily applicable for control activities may be very useful for research purposes, such as studies on epidemiology, on vaccination and alternative treatment, on the animal reservoir and on transmission dynamics.

Clinical Diagnosis

Clinical suspicion might be considered as indirect evidence of trypanosome infection. However, due to the aspecific, variable and

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inconstant character of clinical symptoms and signs, suspects should be confirmed by parasite detection. Only for stage determination and treatment failure cases may clinical signs, indicating central nervous system involvement, be sufficient to decide on the treatment protocol or on retreatment. A comprehensive review of clinical manifestations of sleeping sickness can be found in Dumas and Girard (Dumas and Girard, 1978).

Clinical signs associated with the initial and haemolymphatic stage

The first or haemolymphatic stage corresponds to the invasion of lymph, blood and other tissues by the trypanosomes. The reaction of the reticuloendothelial system to the presence of the parasite is characterized by a great variety of clinical manifestations. The variability and inconstancy of these signs is illustrated by the fact that many patients infected by *T. b. gambiense* are unaware of infection, since they feel in reasonable health for weeks, months or even years. In *T. b. rhodesiense* infections, outward signs of well-advanced disease may occur within 2 months.

Chancre

At the site of inoculation, signs of a local inflammation may appear: itching, erythema, swelling, pain and local heat. The first sign of infection occurs after 5–15 days: the trypanosomal chancre. It expands within a few days from a red skin patch with a little point in the centre (erythematous macula) to a hard and painful round pruritic nodule (erythematous pseudo furuncle) up to 3 cm in diameter. The chancre disappears without leaving a trace within 2–3 weeks. It is more commonly observed in *T. b. rhodesiense* than in *T. b. gambiense* infections and more often in Europeans, but only rarely in Africans.

Fever and general malaise

From 1 to 3 weeks after the infective bite, an initial fever develops in response to the invasion of the bloodstream by the trypanosomes. The fever (often high) lasts for a maximum of 1 week and can be accompanied by headache and general malaise. The first febrile attack is followed by bouts of fever, separated by prolonging remissions as the disease progresses.

Adenopathy

Accompanying the immune stimulation, the enlargement of glands of the posterior cervical and supraclavicular groups is one of the cardinal signs of *T. b. gambiense* infection. Sometimes enlarged neck glands are easily visible (Winterbottom's sign) but usually the neck has to be palpated to discover them. They are the size of a bean or larger, typically feel rubbery and move freely, are painful at the onset and can persist over weeks or months. Later, they tend to shrink. In *T. b. rhodesiense* infections, neck glands are often not enlarged.

Skin rash and pruritus

From 6 to 8 weeks after onset, scattered large erythematous circinate patches 7–10 cm in diameter develop mainly on trunk and shoulders. They often fade and reappear and are invisible on dark skin. They are not tender and do not itch and are therefore not commonly a presenting symptom. The skin rash is too inconstant and aspecific to be of diagnostic value. Pruritus is often associated with skin rash and is observed in almost half of the cases. It may be discrete in the beginning but persists throughout the disease.

Local oedema

Another inconstant characteristic of trypanosome infection is the partly oedematous swelling of lower eyelids and the puffy swollen appearance of the face, which gives some patients an expression of dullness or sadness. The eyes may look alert or stare, contrasting with the stillness of face. Many patients seem unaffected in early phase but this symptom progressively becomes more characteristic. Later, local oedema develops on other body parts, e.g. peripheral oedema on the legs associated with anaemia.

Cardiovascular disturbances

Cardiac involvement is one of the earliest signs suggestive of trypanosomiasis infection and virtually a common feature of *T. b. rhodesiense* and *gambiense* infections. Thorough examination is required, since there is rarely any overt symptomatology. Physical examination can reveal anomalies of rhythm, soft cardiac murmur and hypotension. Thoracic radiography can show cardiomegaly due to pericardial effusion and dilatation of cardiac cavities. Electrocardiogram tracings may have marked abnormalities.

Endocrine dysfunction

Endocrine dysfunction is marked by permanent feeling of cold and by disturbance of vegetative and sexual functions. In women, sterility, amenorrhoea and abortion is observed. Amenorrhoea may appear soon after onset but is common later on. Abortion is often associated with uterine hypoplasia secondary to defective hormonal secretions. Premature birth, stillbirth and perinatal death are frequent. In men, impotence and in later stages excessive development of mammary glands (gynaecomastia) occurs.

Neurological disorders

Neurological disorders may appear very early, indicating the precocity of meningeal and encephalitic involvement. Behavioural disturbances may occur but often remain unrecognized. Headache, debility and weakness are too common in other diseases to be of diagnostic value. Less frequent but almost diagnostic is a peculiar deep hyperaesthesia (Kerandel's sign), a sensation of pain when soft tissues are compressed. Pain is not immediate but occurs after 1–2 s; it rapidly becomes severe and out of proportion to the force causing it, lasts some seconds, fades and disappears within a few minutes.

Clinical signs associated with the meningoencephalitic stage

Signs and symptoms described for the haemolymphatic stage persist or may even worsen but, as the disease progresses, signs of nervous system injury become more obvious. Manifestations depend on which part of the central nervous system is affected. Neurological changes are most extensive in *T. b. gambiense* infection with its duration of years and its characteristic sleep-pattern disturbance. In *T. b. rhodesiense* infections with duration of only 6–9 months, the marked neurological symptoms are not seen, though there may be some drowsiness, tremors and unsteadiness preceding the terminal coma.

Disturbances of consciousness and sleep

Daytime somnolence can be considered as most characteristic of all signs. At any moment, awakening succeeds periods of sleep with variable periodicity, according to the gravity of the disease. Slowing down of mental functions with diminution of attention, total indifference and episodes of somnolence prevail. A state of apathy overcomes the patient and may accompany narcoleptic states, which occur unexpectedly. Together with disorders of awakening, a loss of muscle tone and drooping of the eyelids are seen. Drowsiness gradually becomes more pronounced until sleep is continuous. Finally somnolence deepens to coma.

Disorders of tonus, motility and abnormal movements

These signs vary in distribution, intensity and onset and reveal lesions of the diencephalon and the superior mesencephalon. Tonus disturbances include a hypertonicity (of extrapyramidal origin), which is variable in time and place, or a hypotonicity (of cerebellar origin, due to sensitivity troubles). In association with the disturbed awakening it may sometimes produce the apathic appearance of patients. A great diversity of abnormal movements due to muscle instability is observed. Trembling of hands and fingers and choreiform, athetoid or oscillatory movements of the arms, head, neck or trunk are variable in topography, intensity and rhythm. Exceptionally, real paralysis occurs. Perioral and cheiro-oral reflexes of the cere-

bral trunk are almost a constant. Also in *T. b. rhodesiense* sleeping sickness, tremor of hands and tongue, unsteadiness and walking difficulties may appear from the second or third month on.

Mental changes

In *T. b. gambiense* infections, temporary mental confusion and psychiatric problems may occur. These include emotional lability, indifference, aggression, asocial behaviour, stereotypic behaviour, impulsive actions, fugue states, manic episodes, melancholia, delirium and/or dementia. Even in the early phase subtle changes can be present, whereas psychiatric problems may dominate. Dementia only develops at terminal stages of the disease. In *T. b. rhodesiense* patients, mental slowness, dullness and temporary delirium, mania, confusional or hallucinatory states may be observed.

Parasitological Diagnosis

Definite diagnosis, i.e. direct evidence for trypanosome infection, is obtained by microscopic examination of lymph, blood or cerebrospinal fluid from the putative host. Unfortunately, the demonstration of the parasite still relies on often poorly sensitive yet laborious techniques that have been used for many decades. In practice, therefore, parasitological examination is generally limited to clinical or serological suspects. Failure to demonstrate parasites, however, does not necessarily exclude infection. Due to the sometimes low parasite load, particularly in *T. b. gambiense* infections, trypanosomes can be difficult to detect. Concentration or cultivation techniques, often combined with optimized visualization systems, can improve the sensitivity of parasitological diagnosis. For efficient parasitological examination of samples, it is important to keep the time between sampling and examination as short as possible to avoid lysis of the trypanosomes in the sample. Trypanosomes are rapidly killed by direct sunlight but can survive longer when the sample is kept cool in a dark place. Since parasite detection is mandatory for definite diagnosis, special attention should be paid to the quality of reagents and materials and to proper maintenance of the equipment, in particular the microscope. In general, a 20 × 10 or 40 × 10 magnification is used for screening the sample preparation under the microscope. Few authors have studied the comparative sensitivity of several tests in a more or less standardized way but, in general, the detection limit of a test depends on the amount of sample screened (WHO, 1998).

The following is an exhaustive list of trypanosome detection techniques, of which only some are actually applied for diagnosis of sleeping sickness. More details on some of the techniques can be found in Van Meirvenne (1999).

Chancre aspirate

Trypanosomes can be detected in the chancre 2 days earlier than in the blood. The chancre is punctured and the juice obtained is microscopically examined as a fresh, fixed or Giemsa-stained preparation. This method is seldom applied, since most of the patients are detected much later after infection when the chancre has already disappeared.

Lymph node aspirate

If enlarged lymph nodes are present, they are punctured and the fresh aspirate is microscopically examined. Due to its simplicity and low cost, this technique remains widely applied, particularly in *T. b. gambiense* infections. Sensitivity varies from 40 to 80% and seems to depend on the parasite strain, on the occurrence of other diseases causing lymphadenopathy and on the disease stage (more common in the early stage).

Blood

Wet blood film

About 5–10 µl of finger-prick blood are applied on a slide and examined microscopically under a coverslip. If present, try-

panosomes are mainly revealed by their moving between the red blood cells. Although the detection limit is only 10,000 tryps/ml and the sensitivity is therefore low, this technique is still in use most probably because of its low cost, its simplicity and its immediate result.

Thick blood film

A small drop (20 µl) of finger-prick blood is spread over an area of 1 cm on a microscope slide and defibrinated with a toothpick or the corner of another slide. The slide is dried in a horizontal position, protected from direct sunlight, and is stained without fixation by Giemsa or Field stain prior to microscopic examination at 100 × 10 magnification. Proper preparation of the sample is important to avoid artefacts: correct thickness and defibrination, purity and quality of the staining reagents and cleanness of the slides. The microscopist should pay attention to the morphology of the trypanosomes (long-slender, short-stumpy with often serious deformation). The detection limit is about 5000 tryps/ml. In those situations where a centrifuge is not available, thick blood film is the technique of choice for blood examination although it is time consuming (10–15 min per slide). Apart from trypanosomes, other parasites such as microfilaria and *Plasmodium* can be detected.

Microhaematocrit centrifugation technique

Capillary tubes containing anticoagulant are three-quarters filled with finger-prick blood. The dry end is sealed with plasticine. By high-speed centrifugation in a haematocrit centrifuge, trypanosomes are concentrated at the level of the white blood cells, between the plasma and the erythrocytes. The capillary tubes, mounted in a special holder, can be directly screened at low magnification for mobile parasites (Woo, 1971). By preparing more than one capillary tube, fewer than 500 tryps/ml can be detected. Unfortunately, the presence of moving microfilariae in the blood often makes it impossible to observe the much smaller trypanosomes.

Quantitative buffy coat (QBC)

Acridine orange is a fluorescent dye that binds to the nucleus and kinetoplast DNA of mobile trypanosomes in fresh blood, thus allowing discrimination from white blood cells in which only the nucleus becomes fluorescent. The quantitative buffy coat (QBC, Becton-Dickinson), originally developed for *Plasmodium* detection, combines both this fluorescent technique and the concentration of parasites by centrifugation. QBC has been used with success in sleeping sickness diagnosis (Bailey and Smith, 1992). After high-speed centrifugation of the blood in special capillary tubes, coated with ethylenediaminetetraacetic acid (EDTA) and acridine orange and provided with a floating cylinder, mobile trypanosomes can be identified by their fluorescent kinetoplast and nucleus between the white blood cells in the expanded buffy coat. Ultraviolet light is generated by a 'cold light source' connected by a glass fibre to a special objective containing the appropriate filter. This objective can be mounted on almost every microscope. The technique is highly sensitive and robust. Unfortunately, Becton-Dickinson recently decided to stop its production.

Mini anion exchange centrifugation technique (mAECT)

The mAECT has been introduced by Lumsden *et al.* (1979). The technique consists of separating the trypanosomes from venous blood by anion chromatography and concentrating them at the bottom of a sealed glass tube by low-speed centrifugation (3000 rpm). The large blood volume (300 µl) enables detection of fewer than 100 tryps/ml, resulting in high sensitivity, but the manipulations are quite tedious. In the case of low parasitaemia and failure of all other techniques to detect the parasite, mAECT can make the difference. The price may be prohibitive for large-scale use. mAECT columns and accessory materials are available from Institut National de Recherche Biomédicale (Kinshasa, Democratic Republic of Congo).

In vitro *culture*

Detection of trypanosomes through inoculation of culture medium with blood is possible but, due to low success rates, *in vitro* culture is seldom applied for diagnosis. The kit for *in vitro* isolation (KIVI) was originally developed for isolation of trypanosomes under field conditions (Aerts *et al.*, 1992) but can be used for diagnosis when other techniques fail. The technique consists of inoculating aseptically a large volume of venous blood (10 ml) into culture medium whereafter bloodstream trypanosomes transform into procyclics and start to multiply. The culture flask is kept in the dark at ambient temperature (ideally 27°C) and should be examined for the presence of trypanosomes during several weeks. The KIVI can be ordered at the Institute of Tropical Medicine, Antwerp.

Xenodiagnosis

Xenodiagnosis can be performed by inoculating rodents with the blood of the putative host or by feeding teneral tsetse flies on the host blood. Xenodiagnosis is seldom applied for obvious reasons: need for animals, low success rates, long incubation time.

Cerebrospinal fluid (CSF)

Examination of the CSF is usually undertaken for stage determination and not for primary diagnosis, except in cases of strong clinical or serological suspicion in which trypanosome detection in blood or lymph has failed. Parasitological examination of CSF will be discussed below under stage determination.

Serological Diagnosis

Serological indirect evidence for trypanosome infection can be obtained by demonstrating inflammatory responses, specific antibodies or parasite antigens in the putative host. The introduction of simple antibody detection tests for screening the population at risk, thus limiting tedious parasitological examination on the seropositive suspects only, has been a major breakthrough in diagnosis of human African trypanosomiasis. Reliable antigen detection tests remain to be developed. Seropositivity in antibody detection tests must be interpreted with caution, since antibodies can persist for up to 3 years after cure (Paquet *et al.*, 1992). Also cross-reactivity with other parasitoses can occur, particularly at low serum or blood dilutions and when immunoglobulin M (IgM) is participating in the reaction.

Non-specific biological alterations related to inflammation

Numerous non-specific alterations of the blood and the CSF composition have been observed in sleeping sickness patients of which the tremendous increase in total serum IgM, 8–16 times the normal concentration, is still the most suggestive for African trypanosomiasis. Anaemia is also observed frequently in sleeping sickness patients but can have other causes.

Detection of trypanosome-specific antibodies

Several techniques for detection of trypanosome-specific antibodies in blood, serum and CSF exist. The type of antigen(s) employed greatly determines the sensitivity and specificity of the test. For *T. b. gambiense*, the better tests make use of selected variable surface glycoproteins, while for *T. b. rhodesiense* non-variable antigens are preferred for reasons of much higher antigenic variability of this subspecies.

Only rapid agglutination tests are readily applicable in the field while immunofluorescence assays, ELISA, immune trypanolysis and plate agglutination tests are appropriate for remote laboratory testing of samples collected in the field during surveys.

Immunofluorescence assay (IFA)

IFAs have been used with success for *T. b. gambiense* sleeping sickness control in Equatorial Guinea and in Gabon. Immune

fluorescence can be applied on serum and on filter-paper eluates (Wéry *et al.*, 1970). The availability of standardized and stabilized antigen for *T. b. gambiense* at low cost has greatly improved the reliability of the test (Magnus *et al.*, 1978a). For testing of serum, it is important to use strictly IgG-specific fluorescent conjugates, thus avoiding cross-reactive IgM. Research is still needed to identify better antigens for *T. b. rhodesiense* sleeping sickness. The investment costs for a fluorescence microscope can be reduced by recurring to an external light source (e.g. QBC system or similar, or fluorescent light-emitting diodes).

ELISA

Numerous ELISA tests for sleeping sickness have been described. There is a tendency to use purified antigens instead of crude trypanosome lysates but investigations should continue into the use of recombinant or synthetic peptides. ELISA is interesting for those who wish to perform large-scale surveys on serum, filter-paper eluates and CSF with strict standardization and quantification. Furthermore, it is possible to study different immunoglobulin classes and isotypes separately in serum and CSF (Lejon *et al.*, 1998). However, the need for sophisticated equipment and large volumes of pure water remains a serious drawback for widespread application of the test. Alternative formats such as lateral flow tests for blood and serum and testing of saliva are currently being considered in our laboratory.

Immune trypanolysis

This test for antibody detection makes use of live bloodstream trypanosomes and is restricted to laboratories that have facilities to maintain cloned populations. The test is based on recognition of the variable epitopes on the surface of the trypanosomes by the corresponding antibodies resulting in complement-mediated lysis. The test is highly specific and is used in our laboratory as the reference test for evaluation of other antibody detection systems (Van Meirvenne *et al.*, 1995). Unfortunately, the test sensitivity is limited in the case of *T. b. rhodesiense* sleeping sickness, due to the higher antigenic variability of this subspecies compared with *T. b. gambiense*.

CATT/T. b. gambiense

The card agglutination test for trypanosomiasis (CATT) is a fast and simple agglutination assay developed for detection of *T. b. gambiense*-specific antibodies in the blood of sleeping sickness patients (Magnus *et al.*, 1978b). Although its diagnostic performance is not perfect, the test is now widely used in different countries where *T. b. gambiense* sleeping sickness is endemic. The CATT is currently produced in the Institute of Tropical Medicine, Antwerp. Evidence exists for limited sensitivity of CATT in the Ethiope East focus in Nigeria for which solutions have been found (see LATEX/*T. b. gambiense*, below). To overcome complement-mediated prozone effects, the addition of EDTA in the dilution buffer has been proposed thus increasing the sensitivity considerably combined with only minor loss of specificity (Magnus *et al.*, 2002). To reduce costs of reagent in seroepidemiological studies and in passive case detection, a miniature CATT version using dried blood samples on filter paper has been described (Miézan *et al.*, 1991). As with IFA, special attention should be paid to correct sampling and to dry storage of the filter papers. The occurrence of parasitologically non-confirmed CATT seropositives remains a matter of concern (WHO, 1998).

Procyclic agglutination test for trypanosomiasis (PATT)

The PATT has been introduced for detection of *T. b. rhodesiense* or *T. b. gambiense* antibodies in blood (Pearson *et al.*, 1986; Liu *et al.*, 1989). The original test, using live procyclic trypanosomes, has obvious limitations for field application but the modified test with fixed trypanosomes (MOPATT) is being used with success at Kenyan Trypanosomiasis Research Institute (KETRI), Kenya, and deserves thorough evaluation. According to our experience, the sensitivity of the test for *T. b. gambiense* sleeping sickness is low.

Haemagglutination

As early as 1975, a capillary haemagglutination test had been described for field application (Boné and Charlier, 1975), but it silently disappeared due to the complexity of its execution. A plate agglutination format, particularly useful for *T. b. gambiense* sleeping sickness, is still available from Behring (Cellognost-Trypanosomiasis). Its use may be considered for large-scale surveys on serum samples or filter-paper eluates, especially in those cases where sophisticated equipment for ELISA or indirect fluorescent antibody test (IFAT) is not available. The use of other particles instead of red blood cells coated with more defined antigens (*T. b. gambiense* or *T. b. rhodesiense*) might improve the performance of the assay.

LATEX/T. b. gambiense

The latex agglutination test for *T. b. gambiense* (Büscher *et al.*, 1999) has been developed as an alternative to CATT/*T. b. gambiense*. The test is based on the combination of three purified variable surface antigens resulting in high sensitivity, e.g. in the Nigerian samples where CATT/*T. b. gambiense* fails. The test is currently under evaluation and research continues to replace the purified native antigens with recombinant antigens or synthetic peptides.

Detection of trypanosome antigens

ELISA

ELISA tests for trypanosome antigen detection have been developed by Liu and Pearson (1987), Olaho-Mukani *et al.* (1994) and Nantulya (1988), making use of polyclonal and monoclonal antibodies. The tests can be performed on serum and CSF samples from *T. b. gambiense* as well as from *T. b. rhodesiense* patients. Apart from the need for sophisticated equipment, the contradictory results obtained with similar test systems for animal trypanosomiases have diminished the interest in implementation of these tests in sleeping sickness control programmes.

Card indirect agglutination test for trypanosomiasis (CIATT)

The CIATT is the result of the combination of latex agglutination technology with monoclonal ELISA experience (Nantulya, 1997). CIATT should not be confused with CATT, the latter being an antibody detection test for *T. b. gambiense* infections widely applied in the field (see above). In contrast to ELISA, the CIATT is intended to be applied as a pen-side assay generating immediate results. The test is still under evaluation (WHO, 1998) but preliminary results indicate specificities tested in non-endemic areas ranging from only 61% in Côte d'Ivoire to 98% in Tanzania (Meda *et al.*, 1999). It should be noted that a positive result in tests such as the CIATT may result from the presence not only of trypanosome antigens but also of agglutinating anti-mouse-Ig antibodies and of anti-idiotypic antibodies. Furthermore, circulating antigen may be incorporated in immune complexes and not available for the reaction, thus giving false negative results.

Molecular Diagnosis

Molecular techniques can be applied on various sample types to provide evidence of infection. However, and often overlooked, evidence is still indirect and molecular tests can also generate false negative and false positive reactions. The different assays published so far remain to be evaluated on a larger scale. Molecular diagnostic techniques are far from simple and their present application is limited to research purposes. Although molecular techniques are very sensitive, the test sample should be large enough and well stabilized to contain the required minimum amount of template DNA or RNA.

DNA detection

Polymerase chain reaction (PCR) is a molecular technique which theoretically is very sensitive and specific. Several groups have already published research on its diagnostic

potential and use in sleeping sickness (Schares and Mehlitz, 1996; Kabiri *et al.*, 1999). A wealth of variations on the sample preparation and detected sequences exists from which the most convenient can be chosen. However, some practical considerations should be kept in mind. For example, some authors propose the collection of blood on filter paper for PCR testing of the eluted DNA, thus limiting the sensitivity of the PCR to one trypanosome per volume of blood retained in the filter paper. Furthermore, filter papers should be protected from direct sunlight to prevent DNA degradation, unless special filter paper (e.g. Whatman FTA) is used. In our laboratory, excellent recovery of DNA from 180 µl blood samples is obtained with the commercially available QIAamp DNA blood kit.

PCR results should be interpreted with caution. For example, in our laboratory, positive PCR results have been observed in animals long after treatment without evidence of cryptic infection. Furthermore, Garcia *et al.* (2000) observed unexplained false negative and false positive results in CATT seropositive but parasitologically non-confirmed persons and in the CATT negative control group. It is probable that much work still has to be done before PCR can replace parasite detection in sleeping sickness – if it ever does. Nevertheless, some improvements can be expected in the near future, including: (i) simplification of sample preparation; (ii) visualization and quantification of the PCR result by ELISA or fluorescence; and (iii) a reduced need for expensive equipment. PCR tests that are able to distinguish *T. b. gambiense* and *T. b. rhodesiense* from the other Trypanozoon taxa have now been developed (Welburn *et al.*, 2001; Radwanska *et al.*, 2002a).

RNA detection

RT-PCR

Reverse transcriptase PCR is a technique to detect mRNA, which is far less stable than DNA and which is indicative for active transcription of DNA sequences. In this way, the technique is less prone to contamination during sampling and may allow differentiation between an active infection and persisting DNA derived from dead trypanomes. RT-PCR might be of importance in clinical trials on treatment as they are in progress, in drug resistance studies and in vaccination trials. Again, this technique probably will be used exclusively for research purposes, with only indirect impact on control activities in the field.

Hybridization

Fluorescent molecular probes that hybridize with ribosomal RNA have recently been developed for facilitated detection of the parasite in thick blood films or blood smears (Radwanska *et al.*, 2002b).

Stage Determination and Follow-up

Accurate stage determination is essential to select an adapted treatment with minimal risk for the patient. Since there are no exclusive clinical signs, nor any clear changes at blood level, indicating the evolution from the haemolymphatic to the meningoencephalitic stage, stage determination is performed by examination of the CSF obtained by lumbar puncture, assuming that the CSF composition reflects the events going on in the central nervous system.

For follow-up after treatment, the blood and the CSF of the patient should be re-examined on several occasions (ideally at 1, 3, 6, 12, 18 and 24 months). In theory, a patient is considered cured when during this 2-year follow-up period no trypanosomes are detected in the blood, lymph or CSF and when the CSF stays or returns to normal (WHO, 1998). In practice, this strict follow-up is seldom achieved since patients tend to stay away from control lumbar punctures as soon as they feel healthy. Relapsing patients, therefore, are too often in a very advanced stage when recognized. Diagnosing a relapse is not always easy and only a few guidelines for follow-up can be found. Persistence or reappearance of clinical symptoms gives a good indication but is no absolute proof. Serological tests on blood are useless, since

antibodies persist after cure. Trypanosome detection relies on poorly sensitive techniques and often the parasite can only be detected in the CSF. Other CSF parameters sometimes never return to normal values. Finally, differentiation between a relapse and a reinfection is almost impossible.

Following the WHO recommendations for stage determination and follow-up (WHO, 1998), the CSF has to be examined for the presence of trypanosomes for direct diagnosis and white blood cell number and total protein concentration for indirect diagnosis. If at least one of these parameters is beyond the normal value, a patient is considered to be in the meningoencephalitic stage and should be treated accordingly.

Alternative CSF parameters for indirect stage determination and follow-up have been proposed, such as trypanosome-specific antibodies, anti-galactocerebrosides, the total IgM concentration and detection of trypanosomal DNA.

Detection of trypanosomes in CSF

In poorly equipped or non-specialized laboratories, trypanosomes are generally detected by the relatively insensitive but simple direct examination of CSF. Increased sensitivity of trypanosome detection can be obtained by centrifugation of the CSF sample or by *in vitro* culture (Kronenberger and Miezan, 1988). For obvious reasons, the latter technique is restricted to research purposes. Not only for detection of trypanosomes but also for cell count, it is important to examine the CSF immediately after lumbar puncture, because trypanosomes and cells in CSF seem to be more fragile than those in blood and they lyse quickly. The finding of trypanosomes in CSF allows immediate classification of a patient in the meningoencephalitic stage, though in some cases patients with trypanosomes in otherwise normal CSF have been cured with first-stage drugs (Doua *et al.*, 1996).

Detection of trypanosomes in CSF during the follow-up period is the absolute proof of treatment failure. Therefore, the use of the most sensitive techniques is recommended during follow-up, particularly when other CSF parameters remain abnormal or when clinical signs for nervous system involvement are present.

Detection of trypanosomes in the cell counting chamber

When the number of trypanosomes in CSF is very high (at least one trypanosome/µl, depending on the volume in the counting chamber), trypanosomes can be seen during enumeration of white blood cells. This is often associated with pleocytosis.

Single centrifugation of CSF

Several millilitres of CSF are centrifuged at low speed (2000–4000 rpm) and supernatant is removed without touching the bottom of the tube. Part of the remaining drop or sediment (invisible at low cell counts) is transferred to a slide for microscopic examination. The sensitivity of this technique is limited.

Double centrifugation of CSF

The sensitivity of trypanosome detection in CSF can be improved further by double centrifugation (DC) (Cattand *et al.*, 1988). After a first low-speed centrifugation of up to 5 ml of CSF, the sediment is taken up in one or two microhaematocrit tubes which are flame-sealed and centrifuged at high speed, whereafter the bottom of the tube is examined under the microsope. In spite of its high sensitivity and the availability of low-budget centrifuges connected to a car battery through a 12–220 V adapter, DC is not widely applied in the field. This is probably due to the number of manipulations and the need for both an ordinary and a microhaematocrit centrifuge.

Modified single centrifugation

The modified single centrifugation technique is a simple, sensitive, rapid and cheap alternative to double centrifugation of CSF. Up to 2 ml of CSF are centrifuged at low speed in a flame-sealed Pasteur pipette, such as used in the mAECT, and the pipette is mounted in a

viewing chamber (Miézan *et al.*, 2000). The test can be performed within 10 min. Although it remains to be evaluated on a larger scale, sensitivity seems equal to or even better than that of double centrifugation.

White blood cell count in CSF

CSF white blood cell count is without any doubt the most widely used technique for stage determination and follow-up. Miézan *et al.* (1998) stated that 'in poorly equipped laboratories, the diagnosis of CNS involvement in patients with confirmed systemic infection should be based only on the white cell count'. However, the authors disregarded the fact that concurrent infections inducing pleocytosis in CSF may interfere with the interpretation of cell count results.

The upper limit for normal and the cut-off value for the haemolymphatic stage are set at five cells/µl in CSF (WHO, 1998) but it has been suggested that the cut-off should be raised to ten or 20 cells/µl. It is true that *T. b. gambiense* patients with up to 20 cells/µl in their CSF have been successfully treated with pentamidine (Doua *et al.*, 1996) but a considerable amount of relapses has been observed during a clinical study carried out in Uganda on such patients. It should also be noted that, in children, normal leucocyte counts in CSF range from 30 cells/µl when younger than 1 year down to 10 cells/µl in puberty.

Counting chambers with a volume of at least 1 µl should be used, such as the Fuchs-Rosenthal, Nageotte or Neubauer counting chambers. The currently used cut-off value of 5 cells/µl is near the detection limit of most counting chambers, resulting in large variations when counting on samples with low cell numbers is repeated. In addition, due to time constraints or lack of money, rules for cell counting are often not followed, resulting in unreliable cell counts. For example, the rule of taking enough CSF (at least 5 ml), examining it immediately and mixing it before taking the sample for cell counting is often not followed. Other general mistakes are the use of worn-out cell counting chambers, or using ordinary microscope coverslips to replace broken original coverslips, negligent mounting of the coverslip (without visible Newton rings), wrong application of CSF and immediate counting without allowing the cells to settle down in the counting chamber. The use of disposable cell counting chambers such as KOVA slides (ICL), retaining a fixed volume of liquid and eliminating manipulation errors, should be encouraged.

Only a few guidelines can be found for follow-up, although cell count is often the only parameter checked. Pépin *et al.* (1994) considered cell counts higher than the previous determination and above 50 cells/µl to be a relapse, even if the patient is asymptomatic. Cell counts higher than the previous one and between 20 and 49 cells/µl are only considered as relapse when symptoms recur. When cell count is high (> 50 cells/µl) but lower than the previous one, they advised against treatment as it can take months before the cell count returns to normal. It is also known that there can be a significant increase in CSF cell number, also termed 'fluid storm', immediately after Melarsoprol treatment, which is not at all predictive of relapse. According to the guidelines of the Bureau Central de la Trypanosomiase in the Democratic Republic of Congo, a significant increase in cell numbers compared with previous controls, even in the absence of trypanosomes, is indicative of treatment failure.

Determination of CSF total protein concentration

Protein quantification on CSF is not a current practice in sleeping sickness control centres, probably due to the need for more or less sophisticated material (spectrophotometer), the instability of the reagents and the belief that CSF protein concentration is highly correlated with CSF cell number and adds no additional information.

A variety of protein quantification methods for CSF is available: precipitation methods such as sulphosalicylic acid precipitation, the Sicard and Cantaloube method and the trichloroacetic acid precipitation and colorimetric methods such as the Coomassie bril-

liant blue method and the BCA method. Depending on the method applied, largely differing cut-off values are prescribed for sleeping sickness: 250 mg/l (Sicard and Cantaloube method), 370 mg/l (colorimetric methods) or 450 mg/l (sulphosalicylic acid precipitation). Moreover, different results may be obtained depending on the protein standard, IgG or albumin, which is only rarely used in current practice. This leads to contradictory results when the same sample is measured with different methods and when the associated 'normal' cut-off is applied, if a cut-off is available at all. On top of the practical drawbacks, determination of the proteinorachia for stage determination suffers from one major theoretical shortcoming. The baseline protein concentration in CSF is determined by serum Ig and albumin concentrations. Pathological increases are the result of intrathecal Ig synthesis and/or blood–CSF barrier dysfunction. In trypanosomiasis patients without CNS pathology, CSF protein concentrations are relatively high due to the very high Ig concentrations in serum. On the other hand, since blood–CSF barrier dysfunction occurs only in a very advanced stage of sleeping sickness and usually remains moderate, the resulting increase in proteinorachia will also be a late and moderate event. Instead of total protein, the quantification of intrathecal IgM seems more appropriate for stage determination (Lejon *et al.*, 2003; see below).

Detection of trypanosome-specific antibody in CSF

The detection of trypanosome-specific antibodies in CSF of second-stage patients by IFA or ELISA has been described extensively. Furthermore, by ELISA it is possible to study semi-quantitatively the different immunoglobulins in serum and CSF (Lejon *et al.*, 1998), allowing the calculation of intrathecal synthesis of specific antibodies, which indicates an inflammatory response in the central nervous system. However, the need for sophisticated equipment precludes the widespread application of these techniques.

The detection of trypanosome-specific antibodies in CSF by techniques applicable in the field, such as CATT and indirect haemagglutination, has been explored. The sensitivity of CATT appears too low whereas indirect haemagglutination presents the highest sensitivity and specificity, combined with a clear correlation with the CSF cell number. LATEX/*T. b. gambiense* can also be performed on CSF samples for specific antibody detection (Büscher *et al.*, 1999). The reagent seems to be not 100% sensitive, but is highly specific for second-stage trypanosomiasis.

It has been described that CSF specific antibody concentrations drop down quickly after successful treatment (Smith *et al.*, 1989). The decrease of trypanosome-specific antibody concentrations in CSF might therefore be a good parameter for definite cure.

Anti-galactocerebrosides in CSF

Several antibodies directed against brain-specific proteins have been detected in serum and CSF of sleeping sickness patients. The presence of anti-galactocerebroside antibodies in CSF, assessed by ELISA, seems a promising marker for central nervous system involvement (Bisser *et al.*, 2000). Further studies on the applicability of this parameter for stage determination and the development of a simpler assay are necessary before implementation in the field. No data are yet available on the disappearance of these antibodies after cure.

IgM detection in CSF

The CSF of sleeping sickness patients can contain high levels of IgM, which are explained by the high serum levels but also and mainly by the exceptionally strong intrathecal IgM synthesis in patients with central nervous system involvement, and/or by blood–CSF barrier dysfunction (Lejon *et al.*, 2003). The latter two are both indicators of inflammation in the central nervous system due to parasite invasion. However, it should be borne in mind that IgM in CSF is only pathognomic when the CSF has not been contaminated with blood during lum-

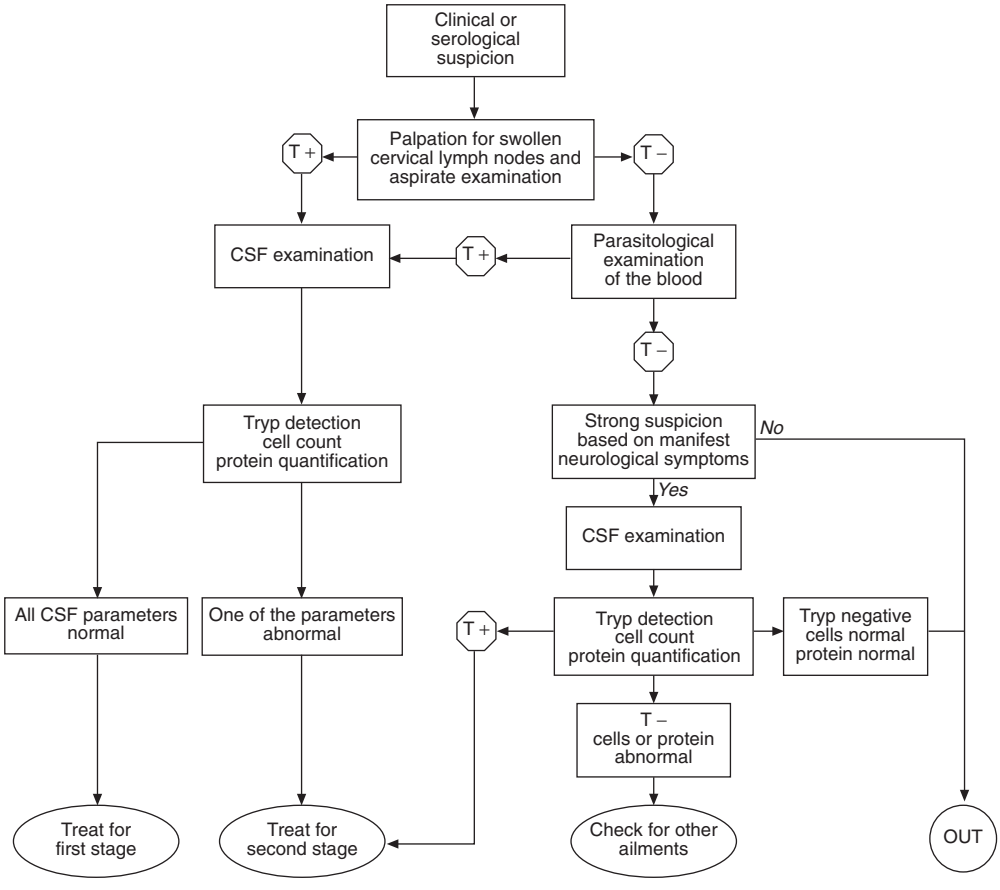

Fig. 10.1. Simplified algorithm for sleeping sickness diagnosis starting from clinical or serological suspicion of infection up to stage determination. T + trypanosomes detected in the sample, infection confirmed; T − no trypanosomes detected in the sample, still suspicion of infection.

bar puncture, since even small volumes of blood will cause abnormally high IgM concentrations in CSF.

Due to the lack of appropriate reagents to quantify IgM in CSF under field conditions, IgM determination is seldom applied for stage determination. A rapid latex agglutination test (LATEX/IgM), which combines stability, sensitivity and simplicity, has been developed (Lejon *et al.*, 2002). Twofold serial dilutions of the CSF are tested and the highest dilution causing an agglutination of the reagent (the end titre) is determined. End titres ≥ 8 have been shown to be 89% sensitive and 93% specific for presence of intrathecal IgM synthesis. Moreover, such high end titres were prognostic for a relapse in sleeping sickness patients with fewer than 20 cells/µl who were treated with the first-stage drug, pentamidine. The test is currently under evaluation in different control centres for sleeping sickness.

Detection of trypanosome DNA in CSF

Theoretically, the detection of trypanosomal DNA in CSF could provide a more sensitive tool for stage determination and follow-up than trypanosome detection but its implementation in the field is not straightforward. PCR with *T. brucei* specific primers on CSF of *T. b. gambiense* patients and suspected sleeping sickness patients has been described to

be 100% sensitive compared with double centrifugation (Truc *et al*., 1999). However, the fact that the number of CSF samples positive in PCR systematically exceeds the number of trypanosome positives, casts some doubts on the relevance of the results. As for trypanosome detection in CSF, one might question the meaning of PCR positivity when other CSF parameters are normal. The detected DNA could originate from the blood or from trypanosomes that have invaded the CSF without actually causing injury or inflammation to the central nervous system. Finally, as for other techniques, it cannot be excluded that PCR also suffers from aspecific reactions.

Although very limited data are available from follow-up studies, it looks as if PCR becomes quickly negative after treatment (Truc *et al*., 1999). No data on relapsing patients have yet been published.

Organization of Diagnostic Activities

The practical set-up of a diagnostic procedure depends on several variables, including the infecting agent (*T. b. gambiense* or *T. b. rhodesiense*), number of expected patients, size of population at risk, access to laboratory facilities, technical skill of health personnel, active or passive case detection etc. However, some general rules apply to all situations where the intention is to treat the patients correctly (WHO, 1998). An example of a simplified diagnostic flow chart is given in Fig. 10.1. First of all, one should know whether *T. b. gambiense* or *T .b. rhodesiense* is involved, depending on the distribution of the subspecies. Next, a brief anamnesis is obtained paying attention to subjective signs and to signs observed by the patient's family, followed by a clinical examination of the patient (neurological signs, palpation of the cervical lymph nodes). In cases of possible *T. b. gambiense* infection, a serological test such as CATT can confirm suspected sleeping sickness.

Clinical or serological suspects undergo parasitological examination following the general diagram in Fig. 10.1. The procedure can be adapted to particular situations, such as mass screening of the population at risk where, in the first instance, a serological test can be performed on the whole population to focus on only the seropositive cases.

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11 Epidemiology of Human African Trypanosomiasis

Susan C. Welburn, Eric M. Fèvre, Paul G. Coleman and Ian Maudlin

Sleeping sickness, a disease thought to have been largely conquered by the 1960s, has re-emerged as a serious public health problem in sub-Saharan Africa (Fig. 11.1). It is estimated that 300,000–500,000 people are currently infected and 100,000 deaths are caused each year by the disease (Cattand *et al.*, 2001). If incidence alone is considered, the disease may appear as a minor problem compared with other diseases, but if disability-adjusted life years (Murray, 1994) are considered, because of its severity (untreated human African trypanosomiasis results in 100% mortality) the social and economic impact of trypanosomiasis ranks third amongst malaria and schistosomiasis in affected parts of sub-Saharan Africa (Cattand *et al.*, 2001).

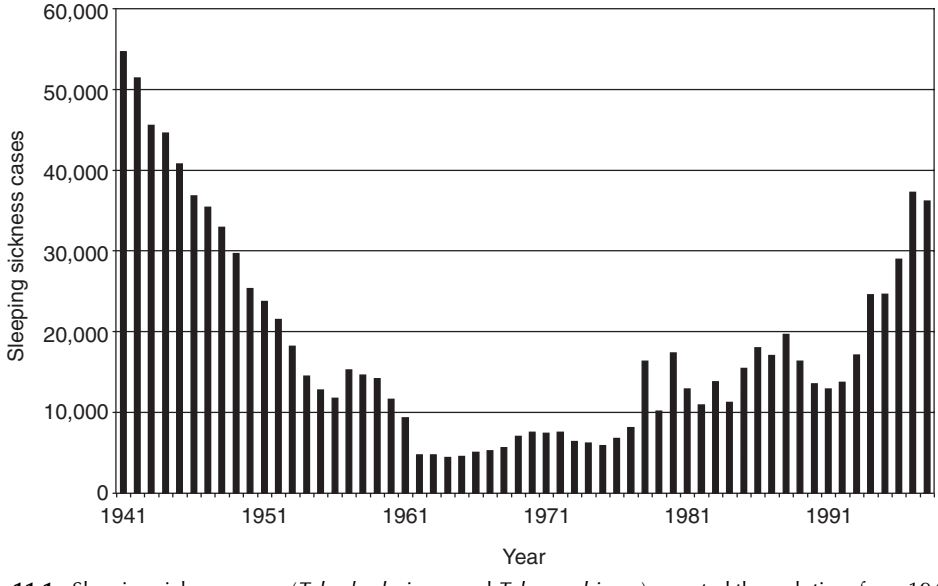

Fig. 11.1. Sleeping sickness cases (*T. b. rhodesiense* and *T. b. gambiense*) reported through time from 1941 to 1998. Reproduced with permission from World Health Organization.

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The history of sleeping sickness is intimately entwined with the history of Africa and in particular with the colonial period of African development. As a result, the epidemiology of sleeping sickness is often studied as a part of African social history. Consequently, our understanding of the origins and causes of the recurrent epidemics of sleeping sickness that the continent has witnessed is based largely on conjecture and inference from social studies rather than the analysis of raw data. The dominance of this socio-economic approach to the nature of sleeping sickness epidemics is understandable as, until recently, the tools that would have permitted a quantitative analysis were lacking.

The interest shown in sleeping sickness by colonial governments is only too easily rationalized. Towards the end of the 19th century there were massive epidemics of sleeping sickness in East and West Africa, which frightened colonial administrations because of the economic consequences that such massive death rates would have on the stability and productivity of their colonial investments. The response of the European colonial powers to this crisis of confidence ranged from short-term measures to restrict or enforce the movements of people in affected areas, to financing longer-term research to understand and control the disease. The parasite and its vector were soon identified and a small army of scientists was attracted to work on this biologically fascinating disease. More than a century later, this enormous research effort has led to a very detailed knowledge of the biology of the trypanosome but, sadly, the understanding of the important aspects of the underlying epidemiology of the disease remains poor.

Sleeping Sickness – a Socio-economic Problem?

Prominent among the many mysteries of sleeping sickness epidemiology has been the unpredictable and episodic nature of recurrent epidemics. Social scientists have tended to step into this quantitative vacuum and have related the great epidemics at the end of the 19th century to social change wrought essentially by the colonial 'scramble for Africa'. The gains from this type of analysis tend, quite properly, to be socio-political so that, for example, the actions of colonial governments can be seen to have had wide-ranging and sometimes unexpected impacts on the peoples of Africa. Generally this approach has not contributed to the promotion of decision-making processes that might lead to the avoidance, or prevention, of future epidemics. Knowing, for example, that one effect of the colonization of Africa in the 19th century was to have promoted sleeping sickness epidemics did not help post-colonial governments to prevent a wave of epidemics sweeping the continent at the end of the 20th century; nor has this knowledge greatly aided organizations involved in controlling the present-day crisis.

It is not the intention of this chapter to describe in detail the socio-economic analyses relating to the history of sleeping sickness epidemics in Africa, which have been so beautifully laid out elsewhere – see, for example, Ford (1971) and Lyons (1992) – but it is useful to outline briefly the thrust of this approach. Colonial scientists were in general agreement that the huge epidemics at the beginning of the 20th century were the result of lawlessness amongst their subject societies. Later, a more sophisticated view was adopted which came to be known as '*Pax Britannica* epidemiology' (Duggan, 1962). In this analysis, sleeping sickness was apparently strictly localized prior to the widespread colonization of Africa by the European powers in the 19th century, having had little opportunity to spread between small fixed centres of population. The slave trade, particularly in West Africa, had conspired to confine people to the security of walled towns; this immobilization was thought to have held sleeping sickness in check. *Pax* (whether British, Belgian or French), by pacifying warring groups, would have encouraged safe travel and promoted farming in more remote areas, thereby bringing humans into contact with tsetse flies, which prefer uncultivated habitats. As communications improved during the colonial advance across Africa, so the disease would have spread with the people; epi-

demics would then have followed the arrival in an area of people carrying trypanosomes in their blood (Morris, 1963). For example, the infamous rubber tax imposed in the Belgian Congo was thought to have been responsible for the spread of sleeping sickness by driving people into tsetse-infested areas in search of rubber to meet the tax demands of the colonists (Lyons, 1992).

Ford (1971), in his seminal study, took an entirely different view of the effects of the colonial period; far from peace, he suggested that war was at the heart of the problem. The 'war' in question was ecological in nature, resulting (in Ford's view) from the biological disruptions consequent on colonization. This analysis – that disturbance of the environment resulting from colonial interventions upset the delicate balance that had evolved amongst flies, trypanosomes, people, flora and fauna – has been largely accepted by subsequent socio-political students of the origins of the 'colonial' disease, e.g. Musere (1990).

It must be stressed that Ford (1971) also perceived that colonization *per se* was not the sole progenitor of disease and predicted that the disappearance of colonialism would not necessarily mean the end of the sleeping sickness problem. This proved to be highly prescient. For example, the sleeping sickness epidemic in southeastern Uganda in the 1980s may be related to the ecological knock-on effects of political instability in that country during the 1970s (Köerner *et al.*, 1995). There is currently a pandemic of sleeping sickness from Sudan in the north to Angola in the south, which may similarly be correlated with the breakdown of civil society in these regions (Fig. 11.2).

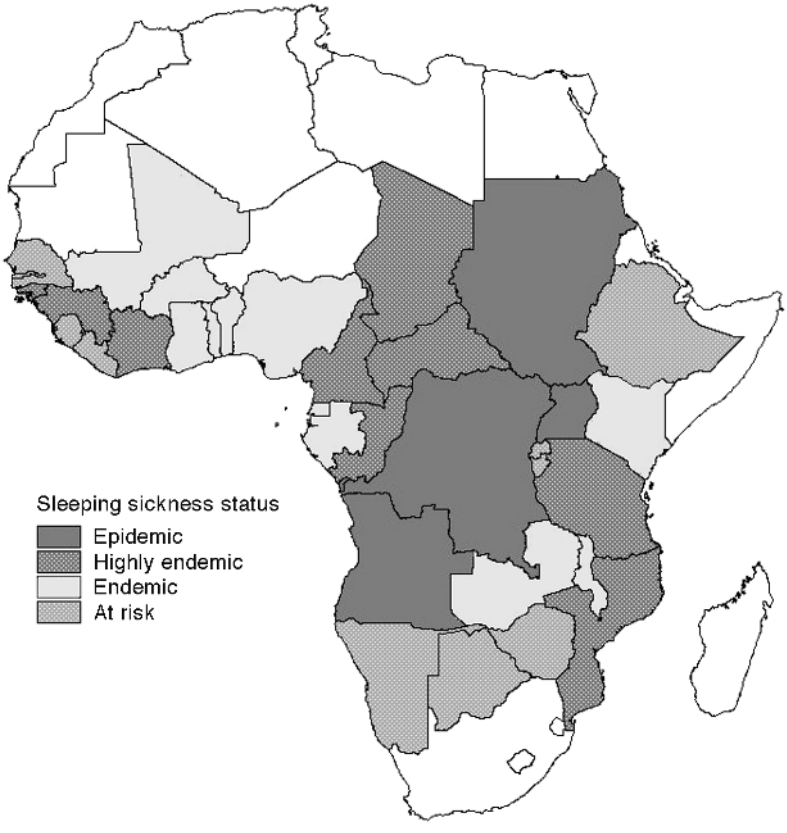

Fig. 11.2. Map of countries currently affected by human sleeping sickness.

Origins of the Disease

Two diseases, three parasites

Sleeping sickness may well have played an important role in the evolution of human populations in Africa (Welburn *et al.*, 2001a). Before considering this, the two quite distinct forms of the disease must first be delineated. The subgenus Trypanozoon is divided into two species: *Trypanosoma brucei* sensu lato (s.l.) and *Trypanosoma evansi*. There are three subspecies of *T. brucei* s.l.: *T. brucei rhodesiense* and *T. brucei gambiense* are both infective to humans and animals, while *T. brucei brucei* is non-human infective. All three subspecies are morphologically indistinguishable, which has, until recently, greatly inhibited the understanding of the epidemiology of sleeping sickness. Gambian (*T. b. gambiense*) and Rhodesian (*T. b. rhodesiense*) sleeping sickness do, however, present distinct clinical and epidemiological pictures resulting in chronic and acute forms, respectively, of the disease. Gambian sleeping sickness has a long asymptomatic stage eventually succeeded by a subacute febrile illness followed by late-stage chronic meningoencephalitis; death may take several years following onset of the disease (Apted, 1970). The Rhodesian form of the disease progresses much more rapidly, most deaths occurring within 6 months of the onset of illness (Apted, 1970; Odiit *et al.*, 1997).

There is a further striking difference between the two human forms of the disease, which is unconnected to medical syndromes but relates simply to the distribution of the disease. A clear-cut geographical division exists between *T. b. gambiense* and *T. b. rhodesiense*, roughly following the Rift Valley, which is also intimately bound up with the evolution of hominids. Following long-term climatic changes, the savannah areas in the east of Africa increased starting around 5 million years ago. Apes ancestral to humans were forced to forage in open country, resulting eventually in the evolution of bipedalism and of hominids in the Rift Valley (Stringer and McKie, 1996; WoldeGabriel *et al.*, 2001).

In their ancestral home in the forested areas to the west of the Rift Valley, the apes would have become adapted to *T. b. gambiense* over evolutionary time. Experimental work with apes bears this out; infections with *T. b. gambiense* usually lead to chronic infections and long-term survival of the animal. *In vitro* experiments using serum from ground-dwelling primates and humans gave similar results, indicating a period of adaptation of the apes to infection with *T. b. gambiense*. By contrast, the death rate for untreated apes infected with *T. b. rhodesiense* reaches 99% (Ashcroft *et al.*, 1959; Baker, 1962, 1968; Godfrey and Killick-Kendrick, 1967; Baker and Taylor, 1971; Yesufu, 1971; Johanson and Edey, 1990). Change of habitat would have brought the apes and early hominids into contact with a new set of trypanosomes circulating in game animals of the savannah and in particular *T. brucei* s.l. At some time in evolution, savannah populations of *T. brucei* resident in the wild animal reservoir will have acquired the gene or genes for human serum resistance and become a threat to the health of early hominids. *T. b. rhodesiense* is known to be a zoonotic parasite, having been transmitted experimentally from both wild (Heisch *et al.*, 1958) and domestic animals (Onyango *et al.*, 1966) to humans. As noted above, *T. b. rhodesiense* remains an acute disease in apes and monkeys as well as in humans and must have been fatal to the evolving populations of early hominids.

Evolution of human serum resistance

Conventional wisdom suggests that parasite–host interactions inevitably evolve to lower and lower levels of pathogenicity. Thus *T. b. gambiense*, as it is a chronic disease, is assumed to be a perfectly adapted parasite (Anderson and May, 1982) while *T. b. rhodesiense*, as it quickly kills its human host, is presumed to be not so well adapted and hence at an earlier stage in its evolution. This conventional view is no longer widely accepted; pathogenicity is not considered a good indicator of the age of a parasitic relationship (Ebert, 1999). *T. b. rhodesiense* may well be as ancient as *T. b. gambiense*, having evolved a

gene for human serum resistance long before it came into contact with human populations. The molecular differences between human infective *T. b. rhodesiense* and non-infective *T. b. brucei* are still not clearly understood, but it has been shown that the expression of a human serum resistance associated (*SRA*) gene allows *T. b. rhodesiense* to survive exposure to normal human serum (Xong *et al.*, 1998). The presence of this *SRA* gene appears to be sufficient within the southeast Uganda sleeping sickness focus (Welburn *et al.*, 2001b) and other East African foci (Gibson *et al.*, 2002) to discriminate between *T. b. rhodesiense* and *T. b. brucei*. These results suggest that the *SRA* gene is sufficient in itself to confer human serum resistance on a *T. brucei* s.l. trypanosome. The question then arises whether *T. b. rhodesiense* and *T. b. brucei* are simply a polymorphism of *T. brucei* s.l. Previous DNA studies based on minisatellite markers (MacLeod *et al.*, 2000) have indicated that human serum resistance may have evolved at least twice in *T. brucei* s.l., resulting in genetically different populations of *T. b. rhodesiense* in southern and eastern Africa, both fatal to humans. Characterization of strains by genetic fingerprinting (Hide *et al.*, 1994) has shown that *T. b. rhodesiense* and *T. b. brucei* fall into two discrete groups; one group is *SRA* positive, the other lacks this gene (Welburn *et al.*, 2001b).

This then raises the question of whether natural populations of *T. brucei* exist in a state of Hardy–Weinberg equilibrium as a result of mating (Tait, 1980; Gibson and Stevens, 1999; Gibson, 2001) or, as others suggest, are simply clonal populations which have mostly given up the mating habit (Tibayrenc *et al.*, 1990). For a detailed review of this complex issue, see Tibayrenc and Ayala (2002).

There is no evidence of any heritable immunity to *T. b. rhodesiense* in humans or their ape ancestors, though studies on the distribution of human immunological markers in relation to sleeping sickness are still lacking. In the absence of evolving immunity, early hominids would have been forced to avoid areas of tsetse infestation, which may in the end have led them out of the Rift Valley and eventually 'out of Africa'. We know that early human populations remained small for long periods of time in Africa and only started to increase rapidly in number on leaving the continent (Hassan, 1981). Humans and their closest relatives are essentially trypanotolerant to *T. b. gambiense* (Paindavoine *et al.*, 1986) and so human populations returning to settle in West Africa would have brought with them their inherited partial tolerance to *T. b. gambiense*, which remains largely a chronic disease. There is also some indirect evidence that the aboriginal Pygmy populations of equatorial Africa are resistant to *T. b. gambiense* infections (Frézil, 1999).

Pre-colonial Period

Huge epidemics of sleeping sickness occurred in both West and East Africa subsequent to colonization by the European powers in the 19th century. As noted earlier, there are sound reasons to suppose that these epidemics were precipitated by the socio-economic changes consequent upon colonization. This analysis has led to the widespread notion that there was a unique and causal relationship between the two. Following on from this assumption, it is further assumed that there had been no serious epidemics of sleeping sickness in Africa prior to colonization. As written records of the pre-colonial period are limited, this is difficult to dispute but movements of people, and more importantly their livestock, were in fact a major feature of African development for thousands of years before the 'scramble for Africa'.

The outpouring of tribes from West Africa across the centre of the continent, which took place at the turn of the first century AD, is considered by some to have been the most important event in African history (McEvedy, 1995). The Bantu opened up equatorial Africa, displacing the hunter-gatherers, San and pygmies, who were no match for the Bantu with their maize and cattle and iron weapons. The pygmies retreated into the forests and the San were pushed off most of their range, except the Kalahari. By the 1st century AD the Bantu had reached the Rift and Lake Victoria and by the 2nd century had completed their progress from Lake

Victoria to the Indian Ocean. The 5th century saw the Bantu expand to the south of the continent and colonize Madagascar.

Two more significant migrations took place in East Africa which will have impacted on the epidemiology of sleeping sickness. Firstly, around AD 1350, two groups of Nilo-Saharan pastoralists (Madei and Kalenjin) moved from the Sudd to the shores of Lake Victoria (Madei) and the Kenya highlands (Kalenjin). The arrival of these Nilo-Saharans will have shifted the balance back towards pastoralism, which the Bantu invaders would have reduced. Secondly, a wave of Nilo-Saharan pastoralists, the Luo, moved up the Nile towards the shores of Lake Victoria around AD 1600. Domestic cattle play a central role in the epidemiology of Rhodesian sleeping sickness. These successive waves of invading pastoralists are as likely to have had dramatic effects on the epidemiology of the disease around the shores of Lake Victoria in pre-colonial times as they have today (Fèvre *et al.*, 2001).

Noting that, in Ford's opinion, European colonization led to ecological disaster, it seems logical to infer that the colonization of East and equatorial Africa by the Bantu, which took place two millennia before the European arrival, was likely to have had similar consequences in terms of sleeping sickness risk. However, population pressure and agricultural practices introduced by the Bantu invasion would eventually have reduced or removed threatening tsetse populations sufficiently to allow long-term settlement there, despite the continued presence of an animal reservoir of *T. b. rhodesiense*. The convergence of Nilo-Saharan pastoralists and Bantu agriculturalists around the Great Lakes region also had dramatic effects on the ecology of that region which, as a result, may well be the primordial focus of Rhodesian sleeping sickness epidemics.

Gambian and Rhodesian Forms of Sleeping Sickness Meet in Uganda

Uganda is the only country to have endemic foci of both infectious agents of sleeping sickness, *T. b. rhodesiense* and *T. b. gambiense* (Welburn *et al.*, 2001a). The present-day distribution of these two forms within Uganda is quite separate, with *T. b. gambiense* in the northwest and *T. b. rhodesiense* in the southeast of the country. This discrete distribution has been observed since the 1940s, when *T. b. rhodesiense* was thought to have been introduced into Uganda (MacKichan, 1944). Prior to this, sleeping sickness in both Ugandan foci was said to have been of the Gambian form (Bruce *et al.*, 1903). As there were no means at that time, other than clinical signs, to differentiate the two forms, it is understandable that Bruce identified *T. b. gambiense* as the parasite responsible for the epidemic in southeast Uganda in 1901.

It is evident even from recent events that, once established as a focus of infection, sleeping sickness is unlikely to disappear unless drastic control measures are put in place. If *T. b. gambiense* had been responsible for the estimated 250,000–500,000 deaths in Busoga in 1901, it would still be expected to be present today as the major parasite species in the area; as Dutton and Todd (1903) observed, 'no district in which sleeping sickness has once existed is known to become entirely free from the disease'. On the contrary, there have been no clinically recorded cases of Gambian sleeping sickness in Busoga since the 1940s. Moreover, since the development of accurate tools for identification of *T. b. gambiense*, not a single case of *T. b. gambiense* has been confirmed in Busoga (Smith and Bailey, 1997; Smith *et al.*, 1998). Over the border in Kenya, in what is essentially an extension of the Uganda focus, this diagnostic confusion persisted even longer. Gambian sleeping sickness was being diagnosed in the Western Provinces of Kenya as recently as the 1960s (Wellde *et al.*, 1989) but again, since the advent of differential tools, Gambian sleeping sickness has not been observed in Kenya.

Similar confusion about causal organisms is seen in discussions of the spread of the disease in Tanzania. The first cases of sleeping sickness are thought to have been due to the spread of *T. b. gambiense* down both sides of Lake Victoria to reach the south of the lake by 1907. Thousands of cases were reported from the shores of Lakes Victoria and

Tanganyika between 1901 and 1907 (Fairbairn, 1948). In 1911 an outbreak of sleeping sickness was reported in southeastern Tanzania but in this case the causal organism was identified as *T. b. rhodesiense*, thought to have spread northwards from Zambia/Mozambique. The disease was seen to spread northwards, reaching the shores of Lake Victoria by 1930. Between 1922 and 1946, 22,955 cases of Rhodesian sleeping sickness were diagnosed in Tanzania. The historical record of the disease in Tanzania parallels that of Uganda and Kenya, starting with the observation of two distinct diseases and ending with the disappearance of *T. b. gambiense* at some time between 1900 and 1940.

DNA profiling studies have shown that some of the *T. b. rhodesiense* populations now present in southeast Uganda are closely related to parasites circulating 30 years ago (Hide *et al.*, 1994). Moreover, the parasites currently circulating in Uganda have been found to bear little relation to those isolated in Zambia from which it is supposedly derived (Hide *et al.*, 1994). These results contradict the notion that *T. b. rhodesiense* entered Uganda from Zambia in a circuitous route via Tanzania (Fairbairn, 1948). Data thrown up by new technology tend to support the hypothesis (Köerner *et al.*, 1995) that the same or very closely related parasites were circulating 100 years ago or more in southeast Uganda. It seems unlikely, on present evidence, that *T. b. gambiense* has ever been involved in the large-scale sleeping sickness epidemics seen in southeast Uganda. A comparison of patient records from the first recorded epidemic with those recently collected in southeast Uganda supports this view (Fèvre *et al.*, 2004).

Animal Reservoir of Rhodesian Sleeping Sickness

The zoonotic nature of *T. b. rhodesiense* was considered likely almost from the outset of studies on this organism but was not confirmed until parasites collected from an infected bushbuck were tested on human 'volunteers' and resulted in sleeping sickness (Heisch *et al.*, 1958). This observation led to an over-emphasis being placed on the wild animal reservoir, and on the bushbuck in particular, in relation to the epidemiology of the disease. Subsequently a similar study involving human 'volunteers' and infected cattle showed that domestic livestock could also be a reservoir of Rhodesian sleeping sickness (Onyango *et al.*, 1966).

From the epidemiologist's viewpoint, what is important about the animal reservoir is not simply its existence (although that is a prerequisite) but the extent of its involvement in transmission. Indeed, long before the molecular tools were available to quantify its extent, Rogers (1988) developed a model suggesting that control of the animal reservoir might offer a cost-effective approach to disease control.

To quantify the role of the animal reservoir in Rhodesian sleeping sickness, the first essential was a tool to differentiate *T. b. rhodesiense* from *T. b. brucei*. The first attempts to do this were based on *in vitro* human serum resistance (HSR) tests (Jenni and Brun, 1982); isoenzyme analyses (Bagster and Parr, 1973; Gibson *et al.*, 1980) and RFLP analyses (Hide *et al.*, 1990) were introduced later. All of these techniques are laborious and time consuming (see Chapter 10). This far from satisfactory state has been changed by the observation that the *SRA* gene (Xong *et al.*, 1998) is responsible for the resistance of *T. b. rhodesiense* to human serum (Vanhamme *et al.*, 2003). The *SRA* gene, which has been identified in laboratory stocks and in field isolates of *T. b. rhodesiense*, is not present in field isolates of *T. b. brucei* (Welburn *et al.*, 2001b). It appears that, at long last, there is a potentially simple diagnostic for *T. b. rhodesiense* that enables the precise estimation of the extent of the animal reservoir. Using the *SRA* gene as a marker, Welburn *et al.* (2001b) found that 18% of cattle sampled in Soroti, Uganda, were carrying *T. b. rhodesiense*.

The existence of animal hosts of *T. b. rhodesiense* has consequences not only for the origins of disease foci but also for the spread of disease. In the past, spread of the disease was usually attributed to the movement of infected humans. Thus Fairbairn (1948) was able to plot the spread of *T. b.*

rhodesiense from Zambia through Tanzania to the shores of Lake Victoria and relate this to the movements of infected persons between villages. Having perceived the mobility of people as the problem, the colonial authorities quickly grasped the idea that the simplest way to halt the spread of disease would be to ban the movements of people or, more drastically, the removal and resettlement of whole populations. To this end, 140,000 Tanzanians were 'resettled' between 1922 and 1945. The failure to eradicate the disease despite these draconian resettlement measures was attributed to the reservoir of disease in game animals. The sequence of events was thought to have been: (i) disease is introduced into a new area by an infected human; and (ii) the disease enters the wild animal reservoir, which then maintains the disease endemically. As Fairburn (1948) concluded 'so long as game are present and act as reservoirs of the parasite, the disease can be reduced in incidence, but not eliminated'. Until Onyango *et al.* (1966) showed that cattle were carrying the human infective parasite, game animals were assumed to be the primary reservoir of *T. b. rhodesiense*. It has been seen from recent studies in Uganda that domestic livestock are far more likely to be the source of infection for a sleeping sickness case than another infected person or indeed game animals (Fèvre *et al.*, 2001). The balance may change, however, in areas where wildlife is more abundant.

The animal reservoir of *T. b. gambiense*

The status of *T. b. gambiense* as a zoonotic disease has remained doubtful, as the definitive 'volunteer' experiments were apparently never carried out. There is indirect evidence based on isoenzymes and DNA that *T. b. gambiense* also has an animal reservoir (Gibson *et al.*, 1980; Mehlitz *et al.*, 1981, 1982; Paindavoine *et al.*, 1986). If a wild animal reservoir for *T. b. gambiense* does exist, it would appear (given the discussion above of the evolution of sleeping sickness) that the logical place to look would be amongst ape populations of West and equatorial Africa. Unlike *T. b. rhodesiense* sleeping sickness, there is no measure of the extent and relevance of the animal reservoir for *T. b. gambiense*. Given that *T. b. gambiense* control has been effective in the past simply by case finding and treatment, this would suggest that the animal reservoir of *T. b. gambiense* is not as important to the maintenance and transmission of Gambian sleeping sickness as it is to the Rhodesian form of the disease.

Modelling Sleeping Sickness

While the work of Ford (1971) had become synonymous with the socio-economic analysis of sleeping sickness, he was also well aware that the majority of studies were based on inadequate data and analysis. Indeed, Ford was anxious that a formal (by which he meant mathematical) model of sleeping sickness be developed along the lines that MacDonald (1957) had developed for malaria.

Many simple epidemiological models describe a population in terms of its immune status. One such type is a compartmental or SEIR model (in which an individual is either *s*usceptible, *e*xposed, *i*nfected or *r*ecovered), where a recovered individual is removed from the pool of potential future hosts due to the development of immunity. Such a paradigm for describing diseases may be attractive but is not applicable to human trypanosomiasis. There is little concrete evidence of a host 'immune memory' towards human-infective trypanosomes (even for *T. b. gambiense*); an individual who has recovered from an infection of trypanosomiasis may develop the infection again, if unlucky enough to be bitten by another infected fly.

The relative importance of the animal reservoir does not change throughout or after an epidemic of *T. b. rhodesiense*. Patients with Rhodesian sleeping sickness are unlikely to be bitten by a fly during the clinical phase and therefore do not act as reservoirs themselves. After an epidemic, the population of susceptibles is just as large as it was before the epidemic, if there is no development of immunity as discussed above.

Differences in the epidemiology of *T. b. gambiense* and *T. b. rhodesiense* could have profound effects on the relative effectiveness

of sleeping sickness interventions. A mathematical model of sleeping sickness transmission has been developed that incorporates an examination of the effectiveness of different interventions (Welburn *et al.*, 2001a) – early curative treatment of humans, chemoprophylactic treatment of animals and vector control – will vary between the two parasite species under different assumptions of the existence of an animal reservoir.

As noted above, Gambian and Rhodesian sleeping sickness show different clinical manifestations, particularly in the duration of infection (and so infectiousness) which affects any model of the disease. It has been seen that, in the absence of treatment, the natural duration of infection of Rhodesian sleeping sickness (about 6 months) is much shorter than the average 2-year duration of untreated Gambian infections. In the model shown here, the basic reproduction number of infection R_0 (that is, the number of secondary infections arising from one infectious host in a population of fully susceptible hosts) (Anderson and May 1992) is linearly related to the duration of infectiousness, such that R_0 increases with increasing length of infectiousness.

In this model, effectiveness is measured as a reduction in the effective reproductive number relative to no intervention (i.e. R_0). The effectiveness of early treatment of human cases (Fig. 11.3a), chemoprophylaxis of domestic livestock reservoir (Fig. 11.3b) and vector control (Fig. 11.3c) are expressed as a percentage reduction in the effective reproductive number, R, of infection relative to no control. For each intervention, the results are shown for *T. b. rhodesiense* infections assuming a livestock reservoir, and *T. b. gambiense* infections with and without a livestock reservoir. This is described in further detail in Welburn *et al.* (2001a).

The model shows that early case treatment is most effective against Gambian infections when there is no animal reservoir (Fig. 11.3a). The presence of an animal reservoir reduces the relative effectiveness more for Rhodesian than for Gambian infections, because the duration of infectiousness is greater in the latter and so transmission in humans accounts for a greater proportion of the total R_0. Chemoprophylaxis of animals is most effective for *T. b. rhodesiense* infections, as the animal reservoir is a more important component of the overall R_0 (Fig. 11.3b). If it is assumed that there is no significant livestock reservoir for Gambian infections, chemoprophylaxis of the livestock population has no effect. Vector control (here simply described by increasing tsetse mortality) is equally effective for all three scenarios, regardless of duration of infection or existence of a livestock reservoir (Fig. 11.3c). The central role of cattle in the spread and persistence of Rhodesian sleeping sickness (Fèvre *et al.*, 2001; Welburn *et al.*, 2001b) provides important and novel opportunities for human disease control by targeting the animal reservoir, e.g. through chemotherapy (Fèvre *et al.*, 2001; Welburn *et al.*, 2001a). Block treatment of cattle, however, might impair the effective veterinary interventions by promoting drug resistance and could also interfere with the efficacy of drugs used for human sleeping sickness cases (Barrett, 2001). Rather than block treat cattle in an epidemic zone, it may soon be possible to target cattle carrying *T. b. rhodesiense* having identified them using molecular markers; this would diminish the likelihood of drug resistance arising (Geerts *et al.*, 2001).

Spread of sleeping sickness

A distinction should be made between the expansion/contraction of foci, which occurs during an epidemic in a sleeping sickness endemic area, and true spread of the disease to new areas (Fèvre, 2002). Looking at recent events in southeast Uganda, Busoga has always been affected to a greater or lesser degree and is a long-term endemic focus. Sleeping sickness moved eastwards into Tororo District in the 1980s, spreading to new habitats, and moved northwards at the end of 1998. The causes of the expansion/contraction are most likely linked to a number of factors such as seasonal rainfall patterns, variations in human encroachment into tsetse habitat, soil moisture and other environmental variables. These factors are mostly linked to vector abundance and human–vector contact.

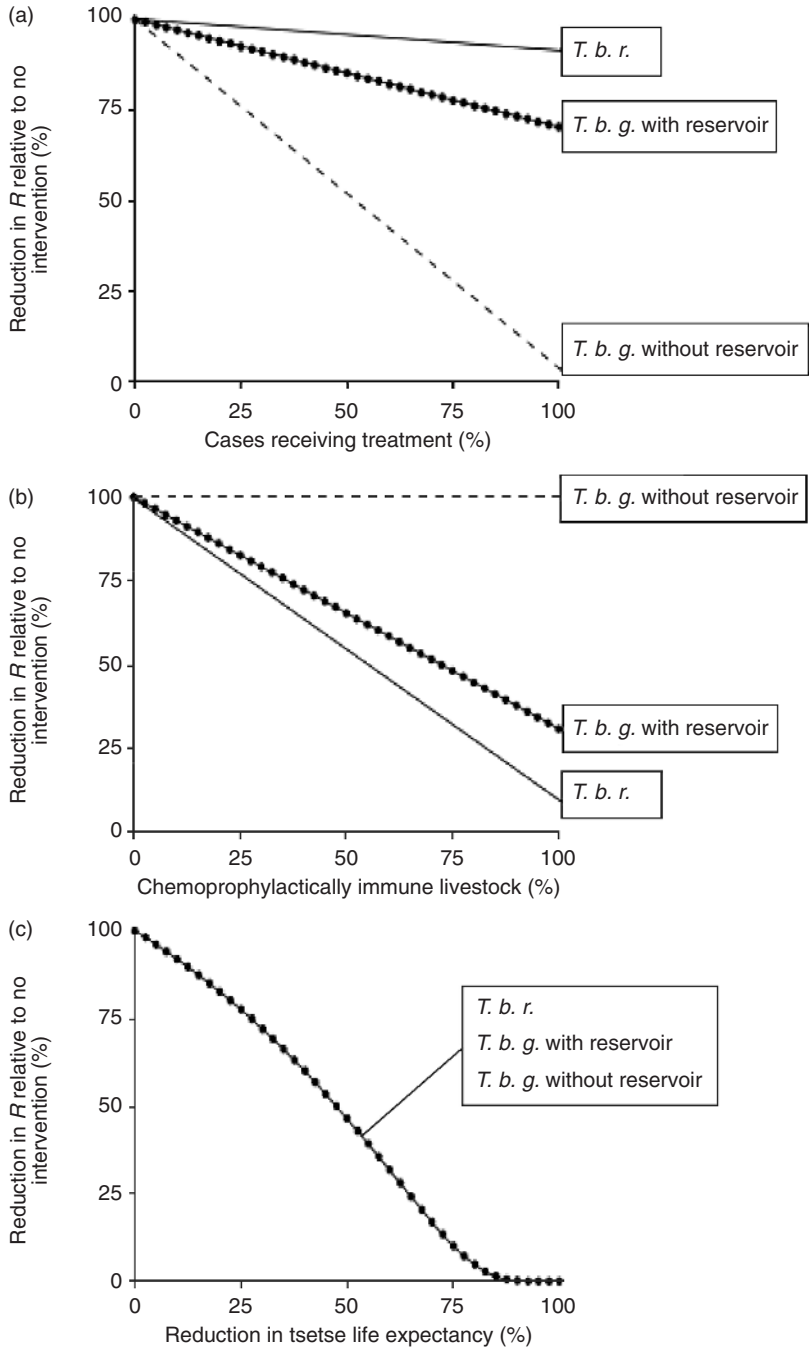

Fig. 11.3. Comparison of different interventions for the control of both forms of sleeping sickness, showing reduction in effective reproduction number (*R*) under different control strategies: (a) following treatment of human cases; (b) following treatment of the domestic animal reservoir; (c) following a reduction in tsetse life expectancy, which is equivalent to vector control. *T. b. g., Trypanosoma brucei gambiense*; *T. b. r., Trypanosoma brucei rhodesiense*. Reprinted from Welburn *et al.* (2001a), © 2001, with permission from Elsevier Science Ltd.

By contrast, the true spread of the disease can only result from carrying parasites to previously unaffected areas where conditions are right (human–vector–reservoir) for disease transmission (Fèvre, 2002). Historically the spread of sleeping sickness has been attributed to movements of infected humans as the source of the disease. For example, Fairbairn (1948) was able to draw a detailed map showing how the disease had spread around Tanzania. Wilde and French (1945) showed experimentally that cattle can maintain *T. b. rhodesiense* infections for more than 6 months and were the first to speculate that cattle might be responsible for the spread of *T. b. rhodesiense* from endemic areas to areas free of the disease. It has been seen how important cattle are in the epidemiology of Rhodesian sleeping sickness as a reservoir of disease in endemic areas (Hide *et al.*, 1996) but it has recently emerged that cattle may also be responsible for the spread of this disease (Fèvre *et al.*, 2001). Looking back in time, cattle movements – rather than movements of humans – may have been largely responsible for the spread of Rhodesian sleeping sickness across East and Central Africa. The spread of *T. b. gambiense*, in contrast, can be understood largely by understanding human population movements, recently demonstrated by events in the Sudan (Moore *et al.*, 1999).

Conclusions

The means to allow a more quantitative approach to the epidemiology of sleeping sickness are now available. Sophisticated GIS analysis based on satellite imagery is now under way which will allow outbreaks of disease to be tracked and related to environmental as well as social change. In the case of Rhodesian sleeping sickness, recent advances in molecular techniques have, at last, enabled us to distinguish *T. b. rhodesiense* from *T. b. brucei* (Welburn *et al.*, 2001b) and so quantify the role of the animal reservoir. It is possible to monitor fly populations with increasingly sensitive traps and attractants (Vale, 1998). Taken together, these advances in the understanding of human sleeping sickness should help agencies involved in the control of the disease, whether at local, governmental or international level, to estimate risk and to plan control campaigns more precisely and cost effectively.

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12 Diagnosis of American Trypanosomiasis

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Diagnosis

American trypanosomiasis in humans is characterized by an acute phase with high parasitaemia followed by a chronic phase with scarce parasites. The acute phase lasts for 2–4 months and is rarely diagnosed, because the clinical manifestations are non-specific or absent. The chronic phase may evolve into different recognized forms – cardiac, digestive or both (associated) – but nearly half of infected individuals remain for life in the asymptomatic, indeterminate form (Rassi *et al*., 1992). Diagnosis of the infection in the asymptomatic individuals is by chance, during routine medical check-ups or due to testing of a blood donation. Apart from the history (born in endemic areas) of those in the indeterminate form, the presence of specific antibodies against the protozoan *Trypanosoma cruzi* is the only key for aetiological diagnosis (Luquetti and Rassi, 2000).

Diagnosis of American trypanosomiasis should be established by: (i) clinical findings (if present); (ii) history; and (iii) laboratory tests.

Clinical findings

Clinical findings include, in the acute phase, a lesion at the portal of entry, present in nearly half of those diagnosed. Romaña's sign (unilateral palpebral ocular oedema) or an exanthematous induration of the skin (chagoma), frequently on the limbs, indicates the place of entry and multiplication of the parasite and may help the diagnosis in endemic areas. Fever is generally present, as well as widespread oedema in some cases. Liver and spleen enlargement may also be present. Myocarditis may be present, and encephalitis, which is seen mainly in children younger than 4 years of age, has a poor prognosis and may lead to death, which ocurrs in fewer than 5% of acute-phase cases.

In the chronic phase, the cardiac form is characterized by slight to severe alterations of the cardiac rhythm and electrocardiographic abnormalities such as right bundle branch block, present in nearly half of the individuals with the cardiac form and seldom observed in other cardiac diseases. Cardiac insufficiency as well as thromboembolic cerebral vascular strokes may also be related to Chagas disease (Rassi *et al*., 1992). Digestive manifestations in American trypanosomiasis may affect the upper digestive tube, at the oesophagus, or the lower tract at the colon, and are due to denervation caused by the parasite, in segments that handle solid material (the food bolus, faeces). The consequences are megaoesophagus and megacolon; in the former, patients describe difficulty in swallowing food, which

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is retained in the oesophagus. The main complaint of those with megacolon is severe constipation, which may continue for more than 30 days (Rezende and Luquetti, 1994).

History

The second clue for a correct diagnosis is history, i.e. the origin of the patient. Contact with triatomine bugs during childhood, relatives known to have Chagas disease or the patient having been a recipient of blood in endemic areas all indicate greater probability of positive diagnosis. Because an increasing number of *T. cruzi* infections are now acquired in non-endemic areas by blood transfusion from donors resident or previously resident in endemic areas, the possibility of American trypanosomiasis should be borne in mind for any transfused individual. Children born of infected mothers may acquire the infection by congenital transmission in 1–12% of deliveries, which is also relevant in non-endemic areas (Carlier and Torrico, 2003).

Laboratory tests

The third issue for aetiological confirmation of American trypanosomiasis is laboratory diagnosis, which may be considered as two different approaches, according to the suspected phase of the infection being investigated. During the acute phase, parasitological tests are preferred, due to the higher parasitaemia. For the more common chronic phase, serological tests should be performed instead.

Parasitological Tests in American Trypanosomiasis

Acute phase

Parasites are more easily demonstrated in peripheral blood in the acute phase than during the chronic phase. Parasitological tests are thus preferred for diagnosis of this phase, irrespective of the mechanism by which the acute infection was acquired (i.e. vectorial, transfusional, congenital, oral, by laboratory accident or as a result of reactivation by immunosupression of a chronic phase infection). Treatment for cancer, organ transplantation and AIDS have all contributed recently to the increased number of reactivated infections, which may be fatal.

For parasitological diagnosis of the acute phase, direct methods are often adequate but when parasites are scarce, concentration methods may be used prior to microscopy. Multiplication techniques, such as haemoculture, xenodiagnosis or animal inoculation may be applied but all have the disadvantage that several weeks are needed before the result is obtained. This will delay the start of specific treatment, which is more likely to be successful if commenced early in the acute phase. PCR with specific probes is available in some advanced centres and may assist definitive diagnosis.

Direct methods

The easiest and cheapest is the wet blood film, performed by placing one drop of fresh blood between a slide and coverslip, and then examining the blood by light microscopy at 400 × amplification. The coverslip should be pressed (e.g. with a ballpoint pen) to obtain a thin layer of red blood cells. If present, parasites will be detected by the rapid movements of the refringent trypomastigote forms and should be clearly visible with low light intensity or with phase microscopy. At least 200 fields should be searched. Cases acquired by blood transfusion often have large numbers of parasites, one per field or more. In these cases, stained thin smears, as used for differential counts of blood cells, may show the stained parasite. Nevertheless, in most cases, with lower numbers of parasites, the wet preparation is preferred, due to its higher sensitivity. For *T. cruzi*, thin smears for staining should not be made with a very acute angle between the slides as this disrupts the fragile organisms.

Concentration methods

Strout's method and microhaematocrit are preferred, according to our experience (Luquetti and Rassi, 2000), and are also the

simplest techniques. With Strout's method, after clotting 3–5 ml of blood, 1 ml of exudated serum is submitted to slow-speed centrifugation (160 *g*); the supernatant is then transferred to another tube and spun at 400 *g*. The serum is discarded and the last remaining drop is observed by microscopy, as for a wet blood film. This method is superior to Ficoll-Triosil separation (Luquetti and Rassi, 2000). Microhaematocrit is preferred when only minute samples of blood may be taken, as in newborns, and parasites may be found at the buffy coat by direct examination of the capillary on the microscope (Freilij and Altcheh, 1995).

Chronic phase

Multiplication methods

These are seldom used for laboratory diagnosis of the acute phase. Haemoculture and xenodiagnosis are currently used in the chronic phase in some circumstances – for example, to confirm a doubtful serological result or, more often, for follow-up of chemotherapy or for isolation of parasites. In this context, selection of patients for trials of new drugs and evaluation of cure after treatment with available drugs are the main applications, nearly always restricted to research institutions. Other situations are related to the isolation of parasites for identification of *T. cruzi* strains by isoenzymes (Luquetti *et al.*, 1986) or for study of strain behaviour in animal models, all research-related activities (Chiari, 1992). In those regions where *T. cruzi* and *T. rangeli* overlap, staining and microscopy may be necessary to distinguish the two trypanosome species.

Xenodiagnosis is performed by rearing nymphs of the vector (*Triatoma infestans, Rhodnius prolixus* or *Dipetalogaster maximus* are most often used) and applying 40 hungry (not fed for 15 days) nymphs, in four boxes of ten bugs each, to the skin of the patient, on the limbs, for 30 min (Cerisola *et al.*, 1974). The intestinal contents of each nymph are examined 30 and 60 days later, to search for *T. cruzi*. Several studies have demonstrated a xenopositivity from 13% to 58% for a single application, depending on the age of the patient, the geographical region etc. Alternatively, artificial xenodiagnosis has been employed with similar results and the advantage of avoiding skin reactions to the triatomine bites, and this is mandatory for those who are immunosuppressed. For artificial xenodiagnosis, 20 ml of heparinized blood is collected and offered aseptically to bugs, through a membrane (condom) at approximately 35–37°C.

Haemoculture is as effective as xenodiagnosis (Minter-Goedbloed, 1978), provided that some details are carefully observed: 30 ml of blood is collected, processed immediately and spun down (at 4°C) to eliminate the supernatant (plasma). Six or more tubes of Liver Infusion Tryptose medium (3 ml each) are prepared and an equal amount of buffy coat plus red blood cells are added, which should be examined monthly for periods of up to 6 months. Luz *et al.* (1994) showed a 79.4% positivity in 34 patients, strictly following this protocol.

Animal inoculation is seldom used, because mice have different susceptibilities to *T. cruzi*. A highly susceptible strain of mouse (e.g. Balb/C) should be used and even then not all *T. cruzi* isolates will produce detectable parasitaemia.

PCR has been used for American trypanosomiasis since 1990 (Avila *et al.*, 1990) and is performed in some laboratories. Appropriate primers are available but PCR is generally employed only for research purposes. Sample contamination is controlled by using separate rooms for each step. PCR has consistently given higher sensitivity than xenodiagnosis and haemoculture when assessed in parallel. Initially 100% positivity with blood from chronic-phase patients (possibly preselected by positive parasitological tests) was reported but more realistic levels of 60% to 96% have since been described, depending on the age and characteristics of the population. Other diagnostic methods include detection of specific antigen in blood, antigenaemia (Araújo *et al.*, 1981) or antigenuria (Corral *et al.*, 1996), with encouraging results but they are not used as routinely.

There are advantages and disadvantages for each of these indirect methods. Xenodiagnosis can be applied in the field and

there is no problem with contamination but examination of bugs is time consuming and hence expensive. Artificial xenodiagnosis requires appropriate infrastructure for membrane-feeding bugs and is also time consuming. Haemoculture requires rapid handling and sterility from the start of the processs until the final examination, 6 months later. These conditions exclude haemoculture from field application. PCR is potentially attractive since collection of blood may be done in the field, it survives transportation at room temperature for more than 1 month in EDTA-guanidine, and the result may be available in a few days. Nevertheless, all these indirect methods are expensive and not commercially available. Comparative studies employing two indirect methods simultaneously show failure of even the most sensitive (PCR), i.e. in a single patient xenodiagnosis may be positive and PCR negative (Junqueira *et al*., 1996). On the other hand the same test performed repeatedly with the same group of patients will display an increasing cumulative rate of positivity. Rassi *et al*. (1991), employing four serial xenodiagnoses in 41 patients, found a positivity of 24.4% with the first xenodiagnosis, which increased to 36.6% with the second application, 39% with the third and 41.5% with the fourth. The same applies to haemoculture performed on three serial tests on 60 patients, with a positivity of 40% with the first, 65% with the second and 70% with the third haemoculture (Castro *et al*., 2002).

It is apparent that the parasitaemia during the chronic phase seems to be scarce and cyclic, or in waves such that repeated examinations are necessary to improve results, if possible with different methods. The other obvious conclusion is that parasites are not always present in each millilitre of blood, so negative PCR results are compatible with the absence of parasites in the blood sample taken for PCR, and the presence of parasites in the bloodmeal of bugs employed for a simultaneous xenodiagnosis (Junqueira *et al*., 1996).

Serological Tests in American Trypanosomiasis

These are essential for the aetiological diagnosis of the chronic phase of the infection. Nevertheless, searching for IgM-specific antibodies may be helpful in diagnosis of the acute phase if clinical and epidemiological suspicion persists even though parasites are not demonstrable by direct methods. Indirect immunofluorescence with anti-human IgM may be used and any titre from 1:5 is considered positive: it is necessary to have positive controls, which are difficult to obtain, as well as good quality anti-human IgM conjugates. False positives may be due to rheumatoid factor, frequently present among the older population, and to avoid false postives adsorption of serum with appropriate reagents is necessary. Other tests for IgM are direct agglutination of epimastigotes with and without treatment of the serum with 2-mercaptoethanol, and ELISA with an anti-human IgM conjugate. Recombinant antigens such as Shed Acute Phase Antigen (SAPA) (Affranchino *et al*., 1989) may be used as well.

For the chronic phase, IgG class specific anti-*T. cruzi* antibodies are sought in serum from suspect cases. In contrast to parasitological tests, which once positive give a definitive diagnosis, positive serological tests only indicate probable infection. Problems of cross-reaction with other protozoa, concentration of antibody conjugates and skill in performing the tests are among the pitfalls with serological assays. Apart from difficulties inherent to serological tests, quality of available reagents and failure of good laboratory practice account for the majority of common problems with serology. In an attempt to minimize these problems, two separate official initiatives were recently implemented in Brazil and are currently in use in other Latin American countries. One was aimed at improving reagents and the other at improving technical skills in serology for the entire network of laboratories involved. The first was organized by the Ministry of Health and the National Health Foundation, through which all available kits for indirect haemagglutination (IHAT) were purchased and distributed to five reference laboratories in different Brazilian states. These laboratories selected 90 sera and used the same sera independently with the same range of kits. When the results were

decoded, four kits were found to be of good quality, four reasonable and three inadequate (Saéz-Alquézar *et al.*, 1997). The second initiative, by the coordination of blood and the AIDS programmes in Brazil, was the elaboration of a videotape and manual (Ministério da Saúde, 1998) for distance learning on standardized procedures by all Brazilian blood banks and diagnostic laboratories. All relevant technicians are required to complete the course.

Several serological tests have been used in the past but three are now routinely peformed in most South and Central American laboratories: namely IHAT, indirect immunofluorescence (IIF, or the indirect fluorescent antibody test – IFAT) and ELISA. All these tests are commercially available, with different sources; all have been extensively tested for more than 25 years in different countries and under diverse conditions, and all have proved to be reliable. These three tests use crude antigens. Although specificity is not absolute, it is possible to diagnose more than 95% of infected individuals, if two tests are used in parallel and titres are measured. These assays are called 'conventional techniques'. Non-conventional, new-generation assays are mostly not yet available commercially, are used by a single or few research workers and are yet to be accepted by the community. The majority of new-generation tests use recombinant antigens or synthetic peptides in an ELISA. Other proposed tests have used purified antigens (Schechter *et al.*, 1985) and chemiluminescence (Almeida *et al.*, 1997) or Western blotting (Mendes *et al.*, 1997) or even flow cytometry (Martins-Filho *et al.*, 1995). Complement-mediated lysis has been used mainly for control of cure in treated patients (Galvão *et al.*, 1993). Attempts have also been made to employ skin tests of immediate and delayed hypersensitivity.

Earlier tests included the complement fixation reaction (CFR or CFT) or Guerreiro and Machado (1913) reaction. This test has been withdrawn for routine diagnosis in Brazil because of its complexity but is still used in some research laboratories. Direct agglutination (DAT) after reduction with 2-mercaptoethanol has been used mainly in Argentina (Vattuone and Yanovsky, 1971) but is not available. Latex tests have low specificity and are no longer used. A promising flocculation test is not available, mainly due to its cost.

Conventional tests

The principles and results obtained with conventional tests are described here briefly.

Indirect haemagglutination (IHAT)

IHAT has been in use since 1962 (Cerisola *et al.*, 1962), with good results. Red blood cells (of different animal species) are sensitized with antigens of a strain of *T. cruzi* (strain 'Y' and strain 'Tulahuen' are the most used). This antigen is stable for several months and different batches maintain their performance. The assay is performed in disposable microtitre polystyrene plates, V- or U-bottomed, by adding the diluent, serial dilutions of sera and the antigen. After mixing, the plate is left static and a visual reading is taken after 2 h. In our experience this test is specific (> 95%) but the sensitivity is around 93–95%. Relatively low concentrations of antibody (dilutions of 1/16) may be indicative of infection. The main advantage of this test is its simplicity, with only two steps, sample dilution and antigen distribution. Occasionally a false positive result is obtained, which can be abrogated by incubation of sera with 2-mercaptoethanol, for 30 min at 37°C. Another source of false positive reactions may be serum with antibodies against the red blood cells used. This uncommon (0.5% of negative sera) complication is resolved by incubating serum first with non-sensitized red blood cells, which are usually included in the kits.

Indirect immunofluorescence (IIF)

IIF, or IFAT, is very popular in many routine laboratories, since the same conjugate may be used with different parasites for serological diagnosis of several infectious diseases. Cultures of epimastigote forms of *T. cruzi* ('Y' strain or other strains) are deposited in

round wells on microscopy slides and left to dry. Sera at different dilutions are added to each well and incubated for 30 min. After washing, a further incubation with anti-human IgG conjugated to fluorescein isothiocyanate is performed. Results are read under an ultraviolet (UV) light microscope and fluorescent epimastigotes indicate positive sera (Camargo, 1966). This technique is extremely sensitive but has low specificity with cross-reactions with sera from *Leishmania* (both cutaneous and visceral) infections as well as an array of low titre reactions (1/20–1/40) with other sera. It is appropriate for blood banks when maximum sensitivity is requested. One of the main disadvantages is the subjective reading.

ELISA

ELISA was described for American trypanosomiasis by Voller *et al.* (1975) and it is widespread, mainly because of the possibility of automation and the objective reading by optical density in a spectrophotometer. The principle is similar to IFAT, with particulate antigen bound to a flat-bottomed microplate, followed by incubation with sera and a second incubation with an anti-human IgG conjugated with an enzyme (e.g. peroxidase). A colourless substrate gives a coloured product in the presence of the enzyme and intensity is measured by a spectrophotometer. This is a very sensitive technique but specificity may be less than 95% and borderline results may be obtained, which require retesting.

Combined testing

A World Health Organization Experts Committee (WHO, 1991) recommended use of two different serological tests to obtain a more reliable result. By employing a combination of any two of the three tests described, the sensitivity rises to 98% and specificity to 96% – levels that are acceptable for the serological diagnosis of any infectious disease. Less than 5% of sera from endemic regions have problems of interpretation and they should be processed with other additional methods.

Non-conventional tests

Since 1989, several purified or recombinant antigens and synthetic peptides have been described and the majority of them compared in multicentric studies (Moncayo and Luquetti, 1990; Levin *et al.*, 1991). Even though some single antigens proved to have a good performance with a limited number of sera, there is a growing consensus to use a mixture of antigens to improve sensitivity. The great advantage of nearly all these recombinant antigens, in comparison with crude preparations, is the specificity. The majority of recombinant antigens do not cross-react with sera from visceral leishmaniasis and other infections. Sensitivity is high but, in order to avoid false negative reactions, the recommendation is to use these new tools always in parallel with a conventional method of high sensitivity such as ELISA or IFAT with crude antigens (Franco da Silveira *et al.*, 2001).

Some recombinant assays are already marketed, such as the immunoassay of Gador, Argentina (Pastini *et al.*, 1994), the line immunoassay INNO-LIA of Innogenetics, Belgium, and the PaGIA of Diamed, Switzerland (Rabelo *et al.*, 1999). The latter employs red beads sensitized with three synthetic peptides, which are spun down on a gel.

Other assays currently under evaluation are a Western blot with trypomastigote shed antigens (TESA blot) (Umezawa *et al.*, 1996) and the transialidase inhibition assay (TIA) (Leguizamón *et al.*, 1994). New developments are being tested as single-step reactions on appropriate membranes, as Chagas Stat-Pak (Luquetti *et al.*, 2003), with results read in a few minutes. All these new tests are so far only available for research.

Laboratory Diagnosis in Different Contexts

Laboratory diagnosis of American trypanosomiasis may be required in different circumstances and situations, for which the approach may differ:

- For diagnosis of a suspected case in an outclinic or hospital, the physician asks for

serology to confirm or exclude aetiology. The laboratory should employ very specific tests and adequate sensitivity to include as chagasic only those with significant titres of antibody by at least two techniques. False positive results may be deleterious for the non-chagasic patient. The belief that Chagas disease will end in early death is strong in Latin American countries and, based on false positive serology, the physician may propose specific treatment with drugs that can have severe side effects.

- For donor blood the haematologist should provide safe blood (Schmuñis, 1991). Upon minor suspicion, the unit of blood should be retested and, if not unequivocally negative, be discarded. Under these conditions highly sensitive tests are preferable and one of them should be an ELISA. Cut-off titres are lowered to ensure a safe product. There may be a number of false positives. Those donors excluded should be referred to a reference centre for Chagas disease to confirm or exclude the infection.
- For epidemiological surveys, easily performed tests are preferred. Filter paper blood samples are appropriate (Zicker *et al.*, 1990). The survey by Camargo *et al.* (1984) was performed using IFAT on more than 1 million samples. Other purposes of surveys are to certify that an area has been succesfullly treated with insecticides and transmission interrupted. In this case, the target population is children born after interventions began.
- Investigation of congenital transmission starts by confirming that the mother is seropositive. If she is negative, there is no possibility of congenital transmission. If positive, the baby should be examined for parasites after birth. As some infants are infected during delivery, to avoid losing these cases, children may be examined for IgG anti-*T. cruzi* antibodies when 6 or 9 months of age, by which time the antibodies are likely to indicate active infection and suggest treatment (Carlier and Torrico, 2003).
- For treatment follow-up, serological tests are used to check for fall in antibody titre or elimination of antibody. Several tests should be used and the patient followed up for several years (see Chapter 22). Parasitological tests used to confirm therapeutic failure may be used if available (Galvao *et al.*, 2003).

To summarize, diagnosis of American trypanosomiasis differs according to the clinical phase of the disease: for rare cases in the acute phase, parasitology is preferred; the more common chronic phase should be diagnosed by a combination of clinical, epidemiological and laboratory tests. The latter are reliable if good quality kits are used and good laboratory practice followed. Three tests are widely used and recognized (IHAT, IFAT and ELISA). Diagnosis may be even more specific with the aid of a new generation of reagents based on recombinant proteins and synthetic peptides, some of which are already marketed. Finally, in special situations, such as follow-up of specific treatment, parasitological multiplication methods may be used, such as haemoculture, xenodiagnosis and PCR. The PCR diagnosis has advantages of high sensitivity and the short time to produce a result but is not commercially available. With improvement of technology, the future trend will be to use single-step devices and peptide mixtures, yielding a result in few minutes.

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13 Epidemiology of American Trypanosomiasis

Michael A. Miles, Matthew Yeo and Michael W. Gaunt

Introduction

Chagas disease (American trypanosomiasis) is the most important parasitic infection in Latin America, where more than 10 million people carry the protozoan agent, *Trypanosoma cruzi*. The infection is a zoonosis with a complex and poorly understood epidemiology. About 30% of *T. cruzi*-infected individuals develop chronic chagasic cardiomyopathy, which in some regions is associated with megaoesophagus and megacolon. Pathogenesis is not fully understood.

The species *T. cruzi* is genetically diverse. Two principal subspecific groups have been identified and named *T. cruzi* I and *T. cruzi* II, the latter with five subgroups (a–e). *T. cruzi* I predominates in the Amazon basin and in endemic countries north of the Amazon; *T. cruzi* II is the predominant cause of Chagas disease throughout the Southern Cone countries of South America. This chapter proposes that *T. cruzi* I may have evolved in the palm tree ecotope associated with the opossum *Didelphis* and *Rhodnius* vectors, and that *T. cruzi* II may have evolved in the terrestrial ecotope with edentate hosts and *Triatoma* vectors. Phylogenetic analysis based on DNA sequence data indicates that genetic exchange has contributed significantly to the evolution of *T. cruzi*. We have recently generated *T. cruzi* I hybrids in the laboratory from clonal parental genotypes. This capacity for genetic exchange may facilitate the spread of virulent or drug-resistant strains, or the extension of host range. With the forthcoming advent of the *T. cruzi* genome sequence, comparative genomics of diverse *T. cruzi* strains may give insight into the differential pathogenesis of severe and benign Chagas disease. Molecular taxonomy and population genetics research on both *T. cruzi* and its triatomine vectors can already be applied to define where domestic and silvatic transmission cycles overlap, and thus contribute to the design of disease control strategies. Priorities for control of Chagas disease are improved vector control, screening of blood and organ donors, and the development of new drugs to eliminate the present burden of human infection.

Geographical Distribution

Chagas disease (American trypanosomiasis) is considered to be the most important parasitic infection in Latin America (in terms of disability-adjusted life years, DALYs). The agent of Chagas disease is the protozoan parasite *Trypanosoma cruzi*. It is estimated that more than 10 million people carry *T. cruzi* infection in the Americas. The geographical distribution of *T. cruzi* in vertebrates and tri-

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atomine bugs is far more extensive than the range of the human disease, in part due to vector behaviour and the socio-economic status of human populations (Miles, 1998). *T. cruzi* infection of vertebrate hosts and triatomine vectors is recorded from between 43° S in Argentina to 42° N in the USA. Human infections are less widely distributed and are rare in the USA and relatively uncommon in the Amazon basin. Vector-borne cases of *T. cruzi* infection are not known outside Latin America. Nevertheless, sporadic cases are occasionally recorded far beyond the endemic region, due to either blood transfusion-associated transmission or congenital transmission. Severity of Chagas disease is said to vary geographically (see below).

Vertebrate Hosts

All mammal species are thought to be susceptible to *T. cruzi* infection, whereas birds and reptiles are not. More than 150 species of mammal, comprising 24 families, are recorded as infected with *T. cruzi* or *T. cruzi*-like trypanosomes. Prevalence rates for silvatic vertebrate hosts have seldom been determined accurately. Many host records are derived from single or small numbers of reports. Nevertheless, it is clear that some mammal species, such as the marsupials *Didelphis marsupialis* and *Philander opossum* and the edentate (armadillo) *Dasypus novemcinctus,* may have high infection rates. A morphologically distinct trypanosome, *Trypanosoma rangeli*, is also transmitted to vertebrates in Latin America by triatomine bugs. The vectors of *T. rangeli* are bugs of the genus *Rhodnius*. In contrast to *T. cruzi, T. rangeli* invades the salivary glands of *Rhodnius*, so that transmission is by the bite and not by contamination with infected bug faeces. A common host of both *T. rangeli* and *T. cruzi* is the opossum, *Didelphis* spp. Molecular comparisons suggest that *T. rangeli* may be closely related to *T. cruzi. T. rangeli* infects humans but is considered to be non-pathogenic.

T. cruzi-like trypanosomes, of the same subgenus as *T. cruzi* (*Schizotrypanum*) are cosmopolitan in bats.

Triatomine Vectors

The insect vectors of *T. cruzi* are reduviid bugs of the subfamily Triatominae (Hemiptera: Reduviidae: Triatominae). New species of Triatominae continue to be discovered and described. To date there are approximately 138 species, though a few may be synonymous. The vast majority of these species occur only in the Americas. Of the 13 species recorded from the Old World, seven are closely related to *Triatoma rubrofasciata*, which is thought to have spread around the world with shipping and the ship rat, *Rattus rattus*. The remaining five species belong to the genus *Linshcosteus,* for which the origin is less certain: some authorities believe it to have an independent Old World origin; others consider that it may be derived from the New World tribe Triatomini. *T. rubrofasciata* carries *Trypanosoma conorhini*, which is transmitted to rats via infected triatomine faeces. Rarely *T. rubrofasciata* is found infected with *T. cruzi* in Latin America but not elsewhere. It is fortunate that triatomine bugs have not spread to rural Africa, where the structure of local houses is similar to that of infested dwellings in Latin America.

The natural ecological niches (ecotopes) of triatomine bugs are wide ranging. Natural habitats include palm trees, rockpiles, burrows, hollow trees, tree holes, beneath tree bark, epiphytes and nests of some bird species. With few exceptions silvatic bugs seem not to fly to a moving target to take a bloodmeal. Rather, they infest the refuges and nesting sites of their vertebrate hosts. A few species have adapted to infest houses and peridomestic dwellings. These species may form large thriving colonies, with thousands of bugs in a single house, feeding from people and their domestic animals, such as dogs, cats and guinea pigs. *Triatoma infestans* is the main vector in the Southern Cone countries of South America (Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay) and in southern Peru. *Rhodnius prolixus* and *Triatoma dimidiata* are principal vectors in northern South America and Central America. *Panstrongylus megistus* is an important vector in eastern and central Brazil, and *Triatoma brasiliensis* is domestic in northeastern Brazil (Gaunt and Miles, 2000).

Transmission Cycle Types

Triatomine species are often designated as 'domestic', 'peridomestic', or 'silvatic', to indicate their biological behaviour and to represent the risk that they pose as vectors of *T. cruzi* to humans. Most triatomine species are entirely silvatic, with little or no significance to the epidemiology of Chagas disease. Some species have quite specific habitat and host associations; others are eclectic and can feed from several different host species. A few, such as *Eratyrus mucronatus*, will take invertebrate blood, at least in the nymphal stages. *Panstrongylus lignarius* will descend tree trunks to approach potential hosts, presumably attracted by vibration and movement. Only *Rhodnius brethesi* appears to pose a silvatic threat, as it may contaminate forest workers gathering fibre from the piassaba palm, which is its natural habitat. Several silvatic species may be light attracted into houses as adults. Sporadic cases of Chagas disease may result, either when an adult bug attacks inhabitants who are unlucky enough to become contaminated with infected bug faeces, or when the bugs contaminate food. Examples of species that fly to lights in the Amazon basin are *Panstrongylus geniculatus*, *Rhodnius pictipes* and *E. mucronatus.* About half of the known cases of human Chagas disease in the Amazon basin are due to small epidemic outbreaks of orally transmitted infection (Coura *et al.*, 2002). Light may be used to illuminate palm or sugarcane presses at night; rarely bugs contaminate such presses and may transmit *T. cruzi* infection to those who consume the juice. Regions where *T. cruzi* is abundant in silvatic mammals and silvatic triatomine bugs but which have only sporadic cases of Chagas disease and no domestic bug colonies are referred to as having enzootic transmission cycles. Even in the Amazon basin a few triatomine species show a tendency towards domiciliation. For example, *P. geniculatus* has been reported from pigsties abutting houses. Such species are sometimes considered to be candidate domestic vectors.

Silvatic and domestic transmission are considered to be continuous or overlapping transmission cycles when the same triatomine species is found in houses and adjacent silvatic habitats. Examples of such overlapping transmission cycles are found in northeastern Brazil, where *T. brasiliensis* infests houses and adjacent rockpiles, and probably also in some regions of Venezuela, where *R. prolixus* infests houses and adjacent palm trees.

At least four of the five highly domiciliated triatomine species have spread beyond their original silvatic range. The most notable example is *T. infestans*, which may have originated from rocky areas in Bolivia and spread to all six Southern Cone countries and to southern Peru. Similarly, *R. prolixus* is thought to have spread to Central America, *T. dimidiata* from Central America to parts of northern South America and *P. megistus* from southern Brazil to central and northern Brazil. Another example of a species that has extended its range in this way is *Rhodnius ecuadoriensis* in Peru and Ecuador. Localities with a domestic triatomine species confined to houses have discontinuous or separate transmission cycles (Miles, 1998; Miles *et al.*, 2003).

Definition of whether domestic and silvatic transmission cycles are overlapping or separate is crucial to the planning of control campaigns. If domestic colonies are replenished from silvatic foci, spraying might be necessary more frequently and follow-up surveillance may need to be prolonged and more rigorous. If there are no relevant silvatic populations, reinvasion from silvatic foci does not need to be considered. Molecular genetic analyses of triatomine populations and of *T. cruzi* strains can help to define whether there is continuity between domestic and silvatic transmission cycles (Miles, 1998).

Not all *T. cruzi* transmission is vector borne. Blood-transfusion transmission is a significant cause of human infection and has given rise to tens of thousands of cases of Chagas disease. Serological screening of donor blood for antibodies to *T. cruzi* is now a fundamental requirement of control programmes and a legal requirement in many endemic countries. Similarly, unscreened organ donors can transmit *T. cruzi* to recipi-

ents of organs. Immunosuppression may exacerbate the acute phase in such recipients, or may reactivate infection in recipients who were already chronic carriers to produce a secondary acute phase infection. Immunosuppression associated with HIV and AIDS may also reactivate chronic infection, with severe sequelae such as meningoencephalitis, which carries a poor prognosis. Congenital *T. cruzi* infection may occur in a small proportion of infants born of seropositive mothers.

Either blood-transfusion transmission or, more rarely, congenital transmission may occur far from endemic areas. The risk of blood-transfusion transmission outside endemic areas is increasing with migration of populations from Latin America. Migrants who have been long-term residents in rural endemic areas should be screened for antibodies to *T. cruzi* and excluded as blood donors if they are seropositive. Consumption of blood or raw meat from reservoir hosts may also give rise to oral infection. Anal gland infections can occur in the opossum *Didelphis* and gland secretions may therefore also be a source of infection.

It is likely that the oral route of infection is extremely important in silvatic cycles involving insectivorous hosts such as marsupials, rodents, edentates and primates, all of which may eat infected triatomine bugs.

Disease Types

The initial acute phase of Chagas disease is often asymptomatic but up to 10% of acute-phase infections may be fatal. In the absence of successful drug treatment, infection is usually retained for life, as is seropositivity. Spontaneous elimination of infection and reversion to seronegativity are rare. Following the acute phase most patients enter an indeterminate phase with no overt symptoms of disease, which in most cases lasts for life. However, around 30% of infected individuals develop chronic Chagas disease with electrocardiogram abnormalities, sometimes associated with megaoesophagus or megacolon. The geographical distribution of these latter signs of chronic Chagas disease is striking and enigmatic. It appears that megaoesophagus and megacolon are well known as a consequence of *T. cruzi* infection in central and eastern Brazil but virtually unknown in northern South America and Central America. This is one of several features of the biology and epidemiology of the *T. cruzi* zoonosis that has led to the suggestion that *T. cruzi* may be heterogeneous, with benign and virulent genotypes (Miles, 1998).

Genetic Diversity of *T. cruzi*

The concept of heterogeneity of *T. cruzi* arose not only from the differential geographical distribution of chronic disease but also from varied success with chemotherapy, from antigenic comparisons, and distinct behaviours of *T. cruzi* strains in mice and triatomine bugs. The advent of multilocus enzyme electrophoresis (MLEE) enabled a systematic comparison of *T. cruzi* phenotypes, and genotypes (by interpretation of isoenzyme profiles). In 1977 an analysis of domestic and field isolates from São Felipe, Bahia State, Brazil, revealed distinct *T. cruzi* strains in domestic and silvatic transmission cycles (Miles *et al.*, 1977). This accorded with the discovery of *Triatoma tibiamaculata* in opossum refuges in bromeliad epiphytes. Not only were the domestic and silvatic strains of *T. cruzi* distinct, they were distinct by 11 out of 18 enzyme profiles, i.e. more distinct than biologically and clinically separate species of *Leishmania*. The two *T. cruzi* strains were designated zymodeme 1 (Z1) and zymodeme 2 (Z2). Subsequent studies demonstrated that *T. cruzi* Z1 was not confined to domestic cycles elsewhere, in fact it was the cause of sporadic acute cases of Chagas disease in Amazonian Brazil, and predominated in domestic transmission cycles in Venezuela (Miles *et al.*, 1978, 1981). Wider studies demonstrated that *T. cruzi* Z2 was common in domestic transmission cycles in the Southern Cone countries of South America, whereas *T. cruzi* Z1 predominated in endemic countries north of the Amazon. This led to the simplistic

but unproven hypothesis that *T. cruzi* Z2 was the virulent agent of chronic Chagas disease and that *T. cruzi* Z1 was more benign, although both zymodemes were associated with similar acute phase infections and with chagasic cardiomyopathy (Luquetti *et al.*, 1986). Furthermore, the isoenzyme phenotypes reinforced that idea that domestic and silvatic transmission cycles were largely separate in the Southern Cone region but might be overlapping in at least some parts of Venezuela.

MLEE is still an important method of characterizing *T. cruzi* isolates but it has been supplemented with a wide range of DNA-based techniques. These include: restriction fragment length polymorphism (RFLP) analysis of kinetoplast DNA (kDNA, schizodeme analysis); random amplification of polymorphic DNA (RAPD); comparison of ribosomal and mini-exon DNA sequence polymorphisms; microsatellite analysis; and RFLP analysis of two internal transcribed spacer regions (ITS1 and ITS2). All these approaches support the subdivision of *T. cruzi* into at least two major phylogenetic groups, which have now been named by international consensus as *T. cruzi* I and *T. cruzi* II. *T. cruzi* I corresponds with Z1 and *T. cruzi* II incorporates Z2 and four other subgroups of *T. cruzi* II, designated *T. cruzi* IIa–e (Tibayrenc *et al.*, 1993; Fernandes *et al.*, 1998; Oliveira *et al.*, 1998).

Remarkably, the genotypic subspecific groups of *T. cruzi* coincide with phenotypic groups originally defined by isoenzymes. A relatively consistent and coherent picture has emerged. Thus the current view of the subspecific taxonomy of *T. cruzi* is as follows (Brisse *et al.*, 2000):

- *T. cruzi* I, equivalent to isoenzyme phenotype Z1;
- *T. cruzi* II;
- *T. cruzi* IIa, isoenzyme phenotype Z3;
- *T. cruzi* IIb, isoenzyme phenotype Z2;
- *T. cruzi* IIc, isoenzyme phenotype Z3/Z1 ASAT (aspartate aminotransferase);
- *T. cruzi* IId, isoenzyme phenotype Bolivian Z2;
- *T. cruzi* IIe, isoenzyme phenotype Paraguayan Z2.

The position of Z3, as *T. cruzi* IIa, within *T. cruzi* II, is somewhat controversial – some authors consider Z3 to be more closely related to *T. cruzi* I than *T. cruzi* II (Fernandes *et al.*, 1999). The latter authors also separate Z3 into two subgroups, which may correspond with the groups designated *T. cruzi* IIa and IIc.

A significant weakness of this view of the subspecific taxonomy of *T. cruzi* is that it is based on isolates collected sporadically across vast geographical distances. The lack of large numbers of isolates from single localities has limited genetic analysis of *T. cruzi* population structures.

Genetic Exchange in *T. cruzi*

Karyotype analysis indicates that *T. cruzi* is at least diploid and this has generally been presumed to be the case in genetic analysis of *T. cruzi* population structures. Tests for random mating (panmixia) in *T. cruzi* populations by using the Hardy–Weinberg equilibrium test and for departure from panmixia using linkage disequilibrium have consistently indicated that *T. cruzi* is substructured into asexual clonal populations. These data are not ideal because sample sizes are small, geographically dispersed and not from single transmission cycles. Furthermore, most studies are based on MLEE, which is less sensitive than nucleotide sequencing approaches. Population genetic analyses initially led to the conclusion that genetic exchange was extremely rare or entirely absent from *T. cruzi* populations.

In contrast to the population genetic studies, some of the early isoenzyme profiles tantalizingly looked like typical heterozygous phenotypes. Some patterns in *T. cruzi* biological clones (populations derived from a single organism) were triple banded for glucose phosphate isomerase (GPI), a dimeric enzyme, and double banded for the monomeric enzyme phosphoglucomutase (PGM). These patterns were typical of those expected for heterozygotes and in some localities at least one corresponding homozygous pattern was seen. In particular, such heterozygous profiles were abundant among isolates of Bolivian Z2 (*T. cruzi* IId)

and Paraguayan Z2 (*T. cruzi* IIe). This further suggested that some *T. cruzi* strains might be the product of genetic hybridization (Miles, 1985). Strikingly, experimental studies by Dvorak and his collaborators had revealed wide variation in DNA content among *T. cruzi* strains, even in clones derived from a single strain *in vitro*. Dvorak suggested that hybridization events might give rise to *T. cruzi* strains with increased DNA content (McDaniel and Dvorak, 1993).

Carrasco *et al.* (1996) described putative parental and hybrid PGM phenotypes of *T. cruzi* I from a single locality in the Amazon basin of Brazil. The frequencies of the PGM alleles appeared to be in Hardy–Weinberg equilibrium, though this was not statistically significant. Attempts were made to generate hybrids in the laboratory by passaging together *T. cruzi* clones with the two putative parental genotypes. Clones of the putative parents were first genetically (episomally) transformed to be drug resistant to either hygromycin or G418. After passage through the entire life cycle, resultant populations were selected for double drug resistance, i.e. for the ability to grow in the presence of both hygromycin and G418. A small number of double drug-resistant biological clones were obtained (Stothard *et al.*, 1999). Analysis of the double drug-resistant biological clones (Gaunt *et al.*, 2003) demonstrated the following characteristics: (i) episomal constructs derived from both parents; (ii) a combination of both parental PGM phenotypes; (iii) a combination of both parental cysteine protease genotypes (CP); (iv) sharing of RAPD bands between hybrids and clones; (v) hybrid profiles at several microsatellite loci; (vi) apparent uniparental inheritance of kinetoplast maxicircle DNA; (vii) evidence of allele loss, and of homologous recombination; and (viii) strong genetic parallels with genotypes among natural isolates of *T. cruzi*.

The mechanism, of aneuploidy through hybridization, explains the wide range in DNA content of *T. cruzi*, and reconciles the paradox between apparent clonal propagation, yet occurrence of recombination. Determination of frequency of recombination will require distinct genetic models and predictions.

Additionally, Machado and Ayala (2001) used maximum likelihood phylogenetic analysis to re-examine the relationships between *T. cruzi* I and *T. cruzi* II, including its subgroups. They concluded that the nuclear genomes of *T. cruzi* isolates representing groups IId and IIe were indeed hybrid, and that they might be derived from an ancient hybridization event between parental strains similar to *T. cruzi* IIb and Ilc as supported by Gaunt *et al.* (2003) and by the sequencing and karyotype analysis of Brisse *et al.* (2003). Machado and Ayala (2001) concluded that genetic exchange events across and within subgroups of *T. cruzi* II have significantly contributed to the genetic diversity and evolution of *T. cruzi* (see also Tibayrenc and Ayala, 2002). However, our experimental findings, summarized above and as described in Gaunt *et al*. (2003), lead us to conclude that genetic hybridization is also still active and generating genetic diversity in *T. cruzi*, with potential epidemiological consequences.

Host Associations and Evolution

T. cruzi I predominates in domestic transmission cycles in all the endemic countries north of the Amazon basin. It is also the most abundant form of *T. cruzi* isolated from enzootic transmission cycles in the Amazon basin, and it is found in some silvatic transmission cycles further south.

The common opossum, *Didelphis*, is associated with *T. cruzi* I over a vast geographical range. Although many mammal species are recorded as infected with *T. cruzi*, prevalence rates in *Didelphis* seem particularly high. Interestingly, it has been suggested that anal gland infections in *Didelphis* might represent a primitive *T. cruzi* life cycle, although it is generally assumed that trypanosomes have evolved from insect kinetoplastids.

The fact that many *Rhodnius* species commonly carry *T. cruzi* I, and also transmit *T. rangeli*, which is often isolated from *Didelphis*, led us to speculate on the evolutionary history of *T. cruzi* I. We propose that *T. cruzi* I has evolved with *Didelphis* and *Rhodnius* in palm trees, which are the pre-

ferred habitat of most *Rhodnius* species, though there are exceptions such as *R. domesticus,* found in bromeliads and hollow trees, and *R. paraensis*, described from an arboreal tree hole. It is not clear how far back in time this host–vector association may hold. Palms are thought to have arisen around 90 million years ago and marsupials were present in South America about 65 million years ago (Gaunt and Miles, 2002).

T. cruzi II is the predominant cause of Chagas disease throughout the Southern Cone countries of South America, and has presumably been disseminated by the spread of *T. infestans*. It is less easy to speculate on the evolutionary history of *T. cruzi* II. One suggestion is that *T. cruzi* II arose by transfer from marsupials into rodents and primates. However, rodents and primates are thought to have arrived in South America 25 million years later than marsupials. Speculating on a more ancient evolutionary history, we have proposed that *T. cruzi* II arose in edentates (armadillos) and reached rodents later by sharing of terrestrial habitats (burrows and rockpiles). Interestingly, at least 20 species of *Triatoma* have terrestrial rocky habitats (Gaunt and Miles, 2000).

Not surprisingly, the host and vector associations that we have suggested do not hold rigidly. The *T. cruzi* I/*Didelphis*/*Rhodnius*/palm tree, and *T. cruzi* II/edentate/*Triatoma*/terrestrial associations are temptingly simplistic. Nevertheless, perceptions may change with fuller studies of the *T. cruzi* zoonosis. Data sets are inadequate at present to test fully the evolutionary significance of these associations. Furthermore, the position of *T. rangeli* and bat trypanosomes is unclear.

Genotypes and Disease Prognosis

It seems inevitable that the distinct *T. cruzi* genotypes are linked both to the pathogenesis of Chagas disease (Vago *et al.*, 2000) and to transmission cycles involving different vertebrate hosts and vectors. Recently *T. cruzi* I-specific and *T. cruzi* II-specific epitopes of a mucin-like antigen (trypanosome small surface antigen, TSSA) have been described, and used to devise a strain-specific serological test by ELISA (Di Noia *et al.*, 2002). All chronic Chagas disease was associated with *T. cruzi* II seropositivity. Note, however, that this study was confined to patients in the Southern Cone countries and did not evaluate human sera from endemic countries north of the Amazon, such as Venezuela, where *T. cruzi* I predominates, yet where infection is still associated with myocardiopathy, though not with mega syndromes.

The *T. cruzi* genome project is at present focused on the strain CL Brener, which has a *T. cruzi* IIe (hybrid) genotype (Andersson *et al.*, 1998; Machado and Ayala, 2001). Comparative genomics across the two major subdivisions of *T. cruzi* and the subgroups of *T. cruzi* II may give insight into the differential pathogenesis of severe and benign Chagas disease. Epidemiological proof of virulent and avirulent *T. cruzi* strains is another matter and would require a large and rigorous investigation, covering confounders such as human genetic diversity, coinfections, nutritional status and other factors. Simplification of genotyping methods will help to unravel the complexities of the *T. cruzi* zoonosis but only if they are applied as part of in-depth field studies with statistically significant data sets.

Concluding Remarks

Molecular taxonomy and population genetics can make a fundamental contribution to defining where silvatic transmission cycles are relevant to designing control strategies. Such methods are applicable to both the disease agent and the triatomine vector. Genetic hybridization of *T. cruzi* may have yielded recombinant genotypes with enhanced vigour and may continue to facilitate the spread of virulence, drug resistance, or the extension of host range. Molecular phylogenetics is likely to give further insight into the evolutionary history of both *T. cruzi* and Triatominae. The latter should not divert attention and resources from the priorities of perfecting vector control strategies and of devising new drugs to eliminate human infection.

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14 Diagnosis and Epidemiology of African Animal Trypanosomiasis

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Introduction

The diagnosis of African bovine trypanosomiasis has been the subject of extensive research over the century following David Bruce's discovery of the associations between nagana (a wasting disease of cattle in Zululand), the tsetse fly and the trypanosome. The need for improved diagnostic methods has long been recognized for a variety of reasons, but in recent decades this need has almost been translated into a quest for its own sake, so that the reasons for it are sometimes forgotten. As a result, some researchers have perhaps lost sight of the conditions under which their diagnostic methods might be used and have invested considerable time and expenditure developing systems unlikely ever to be applied in clinical situations. Nevertheless, these systems may comprise useful research tools for the epidemiology of the disease and occasionally find usage in field conditions or at least on material collected from the field. Some of the methods for diagnosis of African animal trypanosomiasis, and their relative importance as practical field methods for disease control and as research tools, are shown in Fig. 14.1.

There can be few who would disagree that the primary reason for diagnosis of bovine trypanosomiasis is for the appropriate application of therapeutic and sometimes prophylactic measures. Hence, to be useful, the methods available for diagnosis must be suitable for those individuals actually conducting these measures, who generally operate under the most adverse physical and economic conditions. One reason for this situation is that, while highly contagious epidemic diseases such as foot-and-mouth disease, contagious bovine pleuropneumonia and rinderpest are being increasingly recognized as transboundary diseases and diseases of trade, and therefore worthy of major investment at national and international level for their control, endemic diseases such as trypanosomiasis and tick-borne diseases are relegated to the category of production diseases for which the farmer must bear the costs.

Other reasons for diagnosis include the need to target and monitor tsetse control or eradication operations, investigations into the efficacy of chemotherapy and particularly into trypanocidal drug resistance, and pathophysiological, epidemiological and socio-economic studies. Individuals involved in these activities frequently (but not always) enjoy the luxury of better physical and economic working conditions than those engaged in diagnosis for clinical purposes. The focus on development of improved diagnostic methods that might

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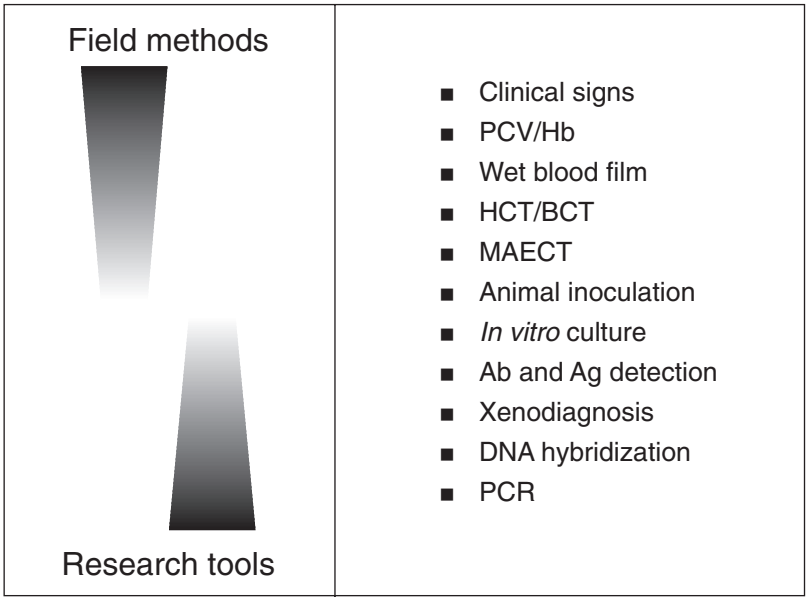

Fig. 14.1. Methods for diagnosis of African animal trypanosomiasis and their relative importance as practical field methods for disease control and as research tools. PCV/Hb, packed red cell volume/haemoglobin; HCT/BCT, haematocrit centrifugation technique/buffy-coat technique; MAECT, miniature anion exchange centrifugation technique; Ab and Ag, antibody and antigen detection (usually by ELISA); PCR, polymerase chain reaction.

be suitable for both clinical diagnosis and more academic studies might appear to be a noble aim, but could be argued to have seriously detracted from the effective development of either.

The diagnosis of trypanosomiasis in domestic animals, including tsetse-transmitted bovine trypanosomiasis, has been the subject of a number of extensive reviews (Molyneux, 1975; Stephen, 1986; Nantulya, 1990; Luckins, 1992, 1993). A new edition of *A Field Guide to the Diagnosis, Treatment and Prevention of African Animal Trypanosomiasis* (Uilenberg, 1998) gives a good general overview of the techniques in current use.

This chapter will mainly consider the diagnosis of African bovine trypanosomiasis, since cattle are the domestic species in which the disease is most frequently diagnosed and treated. However, similar considerations apply to diagnosis in small ruminants and to a lesser extent in pigs. Diagnosis of non-tsetse-transmitted trypanosomiasis is considered in Chapter 15.

Clinical Diagnosis

It is probably fair to say that at present the majority of individuals involved in the treatment and indeed prevention of African bovine trypanosomiasis have no access to any diagnostic methodology whatsoever, other than that of physical examination of the animals under their care. This fact alone means that while the clinical signs of bovine trypanosomiasis are widely regarded as not being specific for that disease, the ability of those involved to recognize these signs is arguably as important as any laboratory or technology-based methodology that might be developed.

Clinical signs of acute bovine trypanosomiasis include anaemia, weight loss, roughness of the hair coat, enlargement of peripheral lymph nodes, pyrexia, abortion, reduced milk yield and, in the absence of treatment, death. Cases progressing to a more chronic disease state may be characterized by anaemia, cachexia, poor produc-

tivity and infertility. The clinical picture depends to some extent on the species of infecting trypanosomes and the geographical location. Hyperacute disease associated with *Trypanosoma vivax* may resemble an acute septicaemia or result in a haemorrhagic syndrome, cases of which may often be found dead.

Classically in West Africa, acute *T. vivax* has been considered more important than *Trypanosoma congolense*, whereas in Central or East Africa *T. congolense* was considered the more important parasite. This is a rather simplistic view, as evidenced by outbreaks of acute haemorrhagic disease associated with *T. vivax* in cattle in coastal Kenya. *T. congolense* infections tend to be less acute and less dramatic, and in that sense are less pathogenic than *T. vivax* infections, although the end result is almost as lethal (Stephen, 1986). *Trypanosoma brucei* infections are generally regarded as being of low pathogenicity for African cattle, which may be infected without showing overt clinical signs, but exotic breeds of cattle and their crosses may be more susceptible (Jordan, 1986). These *T. brucei* infections are nevertheless important, since a proportion of them may express human serum resistance and the affected cattle therefore represent a reservoir for *rhodesiense* sleeping sickness. As such their diagnosis and elimination may be an important challenge in the control of that disease. Long-standing *T. brucei* infections in cattle may also be associated with neurological signs, as described in western Kenya (Wellde *et al.*, 1989), but these may occur only rarely as a terminal event and are therefore of limited diagnostic value (Stephen Angus, unpublished data).

Under most circumstances, a presumptive diagnosis is made on the basis of clinical signs alone, followed by administration of trypanocidal drugs, with a positive response to treatment being interpreted as confirmation of the diagnosis. However, these signs are all non-specific and a number of other disease states that occur in the endemic area may result in the same clinical picture. Hence ideally diagnosis should be confirmed by the use of more specific diagnostic tests.

Parasitological Diagnosis

Blood films

The examination of wet blood films and Giemsa-stained thick and thin fixed blood films with the aid of the light microscope have been used as diagnostic methods ever since they were first used to identify the aetiological agents of trypanosomiasis. With the wet blood film, a drop of blood can be examined next to the animal, provided that a microscope is available. Thin and thick blood smears fixed in methanol or acetone and stained with Giemsa may be used in the laboratory to detect blood parasites and determine the trypanosome species involved, respectively. However, these techniques are not sensitive enough to detect the low parasite levels, characteristic of the disease in large animals. In order to have a higher chance of detecting parasites it is often considered best to collect blood samples early in the morning and from peripheral capillaries, e.g. from the ear or the underside of the tail.

Although glandular fluid collected from the prescapular lymph node and fluid expressed from the chancre may also contain trypanosomes (Robson and Ashkar, 1972), it is often more practical to collect blood in sufficient quantity to allow the use of a concentration method.

Concentration techniques

A practical method using centrifugation of microhaematocrit capillary tubes containing the blood sample and examination of the buffy coat/plasma junction under the microscope was described by Woo (1970). This method, known as the haematocrit centrifugation technique (HCT), was subsequently improved in the buffy coat technique (BCT) by cutting the capillary tube, expressing the buffy coat/plasma interface on a microscope slide and using dark-ground or phase-contrast illumination (Murray *et al.*, 1977). The advantages of these two methods are that diagnostic sensitivity is increased, due to a concentration of parasites following centrifugation, and that

at the same time the packed red cell volume (PCV) can be determined as a measure of anaemia. Paris *et al.* (1982) compared the effectiveness of some of these newer diagnostic methods with the traditional blood films and found the BCT to be the most sensitive technique, followed in order of decreasing sensitivity by the HCT, Giemsa-stained thick film, Giemsa-stained thin film and wet blood film. The analytical sensitivity of the BCT depended on the species of trypanosome, with the smallest numbers detectable per millilitre of blood being 2.5×10^2, 5×10^2 and 5×10^3, for *T. congolense*, *T. vivax* and *T. brucei*, respectively. On the other hand, the HCT proved to be the most sensitive microscopic technique to detect *T. brucei* in bovine blood. Both techniques can be used under field conditions, provided that a microscope, a haematocrit centrifuge and a small generator are available. In practice, this usually means that a four-wheel-drive vehicle is also required to transport the necessary equipment. The BCT method has additional advantages in that the three most important trypanosome species in ruminants can often be identified, due to their characteristic movement patterns, and that an estimation of the parasitaemia can be made using a scoring system (Paris *et al.*, 1982).

A method using miniature anion-exchange columns for the separation of trypanosomes from erythrocytes prior to concentration by centrifugation (Lumsden *et al.*, 1979) has not seen widespread application under field conditions. Other concentration techniques such as the capillary concentration technique have been described but are less sensitive and less practical for detecting trypanosomes in animals under field conditions.

Sub-inoculation Methods

Sub-inoculation methods are those by which trypanosomes are demonstrated by transferring infection from the suspected case to another vertebrate host, to an invertebrate host or to an *in vitro* culture system. These methods have the additional advantage that stabilates of the isolated parasites may be prepared for further investigation.

Animal sub-inoculation

Sub-inoculation of blood from suspected cases into another species (especially laboratory rodents) has been widely used, though not all trypanosomes are infectious for these species. Immunosuppression of laboratory rodents either by irradiation or using chemical immunosuppressants such as cyclophosphamide may increase the proportion of trypanosomes, resulting in infection (F.W. Jennings, personal communication, 1992 Glasgow). Inoculation of susceptible rodents may be more effective for some trypanosome species, particularly *Trypanozoon* spp., than others. Robson and Ashkar (1972) found far more cases of *T. brucei* infection in Kenya using mouse sub-inoculation than using blood examination but for *T. congolense* infections mouse sub-inoculation revealed only half as many positive animals as were identified using blood examination. Mouse sub-inoculation failed to pick up any *T. vivax* infections. For this species, sub-inoculation of domestic ruminants (usually sheep or goats on grounds of expense) rather than rodents may be recommended. *T. vivax* may vary in its ability to give rise to parasitaemias in goats, depending on the geographical origins of the parasites (Peregrine *et al.*, 1991), and to maximize the chance of detecting this species of trypanosome it may be necessary to sub-inoculate calves.

Xenodiagnosis

Xenodiagnosis is the feeding of a clean susceptible vector species on a suspected case of trypanosomiasis, after which it is either dissected and examined for the presence of infection, or allowed to feed on a clean animal which is itself examined for the development of infection. Because of the scarcity of laboratory-reared *Glossina* spp. in Africa, this method of diagnosis is rarely attempted for tsetse-transmitted bovine trypanosomiasis. However, it is an extremely sensitive method and may be warranted in particular circumstances if the presence of a trypanosome infection in a particular animal

or cattle population is suspected on the basis of surrogate tests (i.e. tests that do not conclusively demonstrate the presence of the parasite, such as antigen-ELISA, see below) but cannot be conclusively demonstrated by other means. The differential susceptibility of different species of *Glossina* should be taken into account if this technique is used; for example, *Glossina palpalis* is unlikely to become infected with *T. congolense* (Stephen, 1986).

In vitro culture methods

The first *in vitro* cultures of bloodstream-form trypanosomes were initiated from an intermediate rodent host in which parasites were isolated. Zweygarth and Kaminsky (1990) described a method for isolating *T. brucei brucei* and *T. evansi* directly in culture from host animals with low parasitaemias, which were in some cases not detectable on wet blood film or HCT. A kit for the *in vitro* isolation of trypanosomes (KIVI) has been developed as a means of isolating *Trypanosoma gambiense* from humans and has also been shown to be useful in diagnosis, as it may demonstrate the presence of this organism at very low levels in patients' circulation. The method has also been assessed for use in domestic animals and shown to detect more *T. brucei* infections than conventional parasitological techniques (McNamara *et al.*, 1995a). However, this method is relatively expensive and not practical for routine diagnosis of bovine trypanosomiasis.

Immunological Diagnosis

Methods of demonstration of trypanosomes, either directly in the blood or indirectly by sub-inoculation of animals, culture media or xenodiagnosis, all have their limitations in terms of sensitivity and practicability. This, together with recognition of the invaluable contribution of serological methods to the control of diseases of viral and bacterial aetiologies, has led to considerable research into the development of immunological methods for the diagnosis of trypanosomiasis.

Complement fixation test

The complement fixation test (CFT) was used extensively and exclusively in North America in the successful campaign for the eradication of equine dourine (*Trypanosoma equiperdum* infection), in which trypanosomes are rarely demonstrable in blood or body fluids. Although it has been compared favourably with the indirect fluorescent antibody test in the diagnosis of *T. congolense* infections in cattle (Lotzsch and Deindl, 1974), this test suffers problems in reagent preparation, standardization and anticomplementary activity in sera (Luckins, 1993) and has not found a useful role in the diagnosis of bovine trypanosomiasis. The indirect haemagglutination (IHA) test has also found some use in the diagnosis of *T. evansi* infections but is known to be unreliable for detecting *T. vivax* infection.

Indirect fluorescent antibody test

The development of the indirect fluorescent antibody test (IFAT) a primary binding assay and thus one in which the antibody–antigen reaction is measured directly, was a significant step forward in the detection of anti-trypanosomal antibodies. In this test, blood films from infected animals with high parasitaemias are fixed and used as a source of trypanosomal antigens to which anti-trypanosomal antibodies in test sera may bind specifically. Bound antibodies are visualized using anti-host species immunoglobulin (e.g. anti-bovine immunoglobulin) conjugated to a fluorescent dye, which may be observed using an ultraviolet microscope. The IFAT has been shown to be both sensitive and specific in the detection of bovine anti-trypanosomal antibodies (Luckins and Mehlitz, 1978). Although a degree of cross-reactivity between *T. brucei*, *T. congolense* and *T. vivax* means that the IFAT is not reliably species specific, cross-reactivity is not complete and thus all three antigens must be used for maximum efficiency (Luckins, 1993). Other disadvantages of IFAT are the requirement of an expensive ultraviolet microscope, the subjectivity of the interpretation of the results and the lack of quantification of the antibody response.

Card agglutination trypanosomiasis test

Another simple antibody detection method that is particularly suitable for field use in Africa is the card agglutination trypanosomiasis test (CATT), which has found widespread application in the diagnosis of *T. gambiense* sleeping sickness (see Chapter 10). The antigens originate from particular variable antigenic types (VATs) of *T. gambiense* that are highly conserved across the range of this species, and thus the majority of infected individuals develop antibodies that cause visible agglutination when whole blood or serum is mixed with the antigen on a card. Although this test has been adapted for use for diagnosis of *T. evansi* in animals, it is less likely to be applicable to *T. congolense* or *T. vivax* than the *Trypanozoon* species because of the difficulty in identifying suitable VATs in these species (Luckins, 1992).

Enzyme-linked immunosorbent assay (ELISA)

The ELISA, another primary binding assay, was developed in the early 1970s and has since, in various forms, become one of the most widely used techniques in biomedical science. In the ELISA, in its original form, antigens are immobilized by passive adsorption on to a solid-phase and frequently 96-well polystyrene microtitre plate, and detected using specific antibody labelled with an enzyme that catalyses the conversion of a colourless substrate to a visible coloured product. A more useful variant, the indirect ELISA, is analogous to the IFAT, in that specific antibody in a test sample, if present, binds to the immobilized antigen and is itself detected by means of enzyme-labelled anti-species (e.g. anti-bovine) conjugate and conversion of a substrate–chromogen mixture to a coloured product. In practical terms the attraction of the ELISA is twofold. Firstly, the test may be performed without specialized equipment, so that it may be adapted to inexpensive versions for use in the field or under-resourced laboratories. Secondly, with suitable equipment various degrees of automation are possible, allowing a high throughput of test samples in suitably equipped laboratories (Luckins, 1992). Moreover, the ELISA gives quantitative responses that are directly (or indirectly) related to the level of analyte in the sample, making it particularly amenable to quantitative epidemiological studies. Interfacing of ELISA reading equipment to personal computers is now commonplace, facilitating the use of appropriate software for rigorous quality assurance and data analysis.

Antibody-detection ELISA (Ab-ELISA)

The indirect ELISA was adapted for a number of protozoan diseases, including trypanosomiasis (Voller *et al.*, 1976), and it was shown to be capable of detecting specific antibodies in trypanosome-infected cattle (Luckins and Mehlitz, 1978). It was also shown to detect more serologically positive cattle than the IFAT. As with the IFAT, cross-reactivity between the three major tsetse-transmitted trypanosome species occurs in the indirect ELISA using crude antigen preparations, and sera must be screened against all three antigens for optimum sensitivity (Luckins and Mehlitz, 1978; Luckins, 1992). The fractionation of trypanosomal antigens by column chromatography has been claimed to increase the species specificity of antibody detection by ELISA (Ijagbone *et al.*, 1989), but this approach has yet to see widespread application. Similarly, although the genes for a number of candidate trypanosome antigens have been cloned, no suitable recombinant products are widely available for detection of species- or subtype-specific anti-trypanosomal antibodies.

Interest in the Ab-ELISA for diagnosis of bovine trypanosomiasis declined during the late 1980s and much of the 1990s because of the availability of the apparently more useful Ag-ELISA (see below). However, for various reasons the Ag-ELISA failed to live up to its early promise and hence there has recently been a resurgence of interest in the Ab-detection ELISA.

As with most serological tests for disease diagnosis, a single positive serological result cannot be used to demonstrate the presence of active infection, since antibody frequently persists far longer than does the infectious

agent within the host. In cattle, this has been shown to be the case for up to 6 and possibly as long as 13 months following clearance of trypanosome infection (Luckins, 1992; Van den Bossche *et al.*, 2000). This has sometimes been viewed as a major drawback to the usefulness of the Ab-ELISA for bovine trypanosomiasis and this would indeed be a serious disadvantage were the assay to be used as a basis for diagnosis and treatment of individual animals. However, the usual scenario is that ELISA results from field samples become available far too late to influence the management of individual clinical cases. Indeed these results are generally obtained in the context of research projects and rarely, if ever, reach those responsible for the clinical welfare of affected cattle.

In the last decade (1990–2000) interest in the Ab-ELISA for bovine trypanosomiasis has focused on its use as an epidemiological tool for mapping and quantifying trypanosomiasis prevalence and risk. In this context, the persistence of antibody may be regarded as advantageous, since the Ab-ELISA will provide information on the aggregated level of trypanosome challenge over a prolonged period prior to the survey. The technique has recently found use in many regions of sub-Saharan Africa, for example, under a Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA) Co-ordinated Research Programme (Dwinger, 2000), and there is an increasing body of data attesting to its reliability and robustness. While most workers continue to use crude trypanosomal antigens, modifications to the original method include the use of dried blood spots on filter papers rather than conventional serum samples (Hopkins *et al.*, 1998), thus obviating the requirement for cold chain facilities, the adoption of rigorous quality assurance based on the use of reference sera (Hopkins *et al.*, 1998), the use of heat/detergent-denatured antigen and the use of ELISA plates pre-coated with antigen (Rebeski *et al.*, 2000).

Antigen-detection ELISA (Ag-ELISA)

Demonstration of the parasite itself rather than antibodies is necessary for the confirmation of active infection, though it is widely recognized that the conventional parasitological techniques are relatively insensitive. Modifications of the ELISA have enabled the technique to be used for the detection of antigens, which may be a better indicator of active infection than the detection of antibodies. The double-sandwich ELISA method (Voller *et al.*, 1976) was shown by Rae and Luckins (1984) to detect antigens in animals within 10–14 days of infection with *T. congolense* and *T. evansi*, and these antigens were shown to disappear within 21 days of trypanocidal drug treatment. In this ELISA, polyclonal antibodies raised against crude trypanosomal antigen preparations were used to coat microtitre plates, and antigen present in test sera bound to the antibody. The bound antigen was then detected using the same antibody conjugated with enzyme and a suitable substrate.

Ag-ELISAs for tsetse-transmitted trypanosomiasis were developed at the International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya, using the sandwich ELISA methodology, but incorporated trypanosome species-specific monoclonal antibodies (Mabs), which reacted with determinants of *T. brucei*, *T. congolense* or *T. vivax* (Nantulya *et al.*, 1987). The aim of the development of these tests was to increase the sensitivity of diagnosis, in both the analytical sense (i.e. a smaller quantity of the analyte – trypanosome – can be detected) and in the epidemiological sense (i.e. a greater proportion of infected animals react in the test). In addition, it was hoped to maximize the specificity of the tests (i.e. to minimize the number of false positive results). In practice this was equated to lack of cross-reactivity to other protozoon pathogens (Nantulya *et al.*, 1987) and lack of cross-reactivity of each species-specific test to the other two trypanosome species (Nantulya and Lindqvist, 1989).

MONOCLONAL ANTIBODY SPECIFICITY A number of ostensibly species-specific Mabs directed against salivarian trypanosomes are shown in Table 14.1. Of these, some have been tested in IFAT (Nantulya *et al.*, 1987), some in Ag-ELISA using procyclic

Table 14.1. Monoclonal antibodies (Mabs) to trypanosome invariant antigen used in IFAT and Ag-ELISA.

Mab identification	Immunogen	Mab class	Subgroup specificity
TB7/8.1.48[b]	*T. b. brucei* LUMP 427 pt	IgM	*Trypanozoon*
TB7/8/13.12[a]	*T. b. brucei* LUMP 427 pt	IgM	*Trypanozoon*
TR7/47.34.16[a,c,e,f]	*T. b. rhodesiense* B704 pt	IgM	*Trypanozoon*
TC3/17.1.13[a]	*T. congolense* STIB 212 pt	IgG_1	*Nannomonas*
TC6/25.25.4[b]	*T. congolense* STIB 212 pt	IgG_3	*Nannomonas*
TC40/31.15.45[c]	*T. congolense* KILIFI/83/IL/97 pt	IgM	*Nannomonas*
TC39/30.38.16[d,e]	*T. congolense* bf	IgM	*Nannomonas*
TV8/8.33.42[b,c]	*T. vivax* IL 1392 pt	IgG_3	*Duttonella*
TV8/8.5.38[a]	*T. vivax* IL 1392 pt	IgM	*Duttonella*
TV27/9.45.15[e]	*T. vivax bf*	IgG_1?	*Duttonella*

[a]Reactivity tested in IFAT but not Ag-ELISA against procyclic lysates (Nantulya *et al.*, 1987).
[b]Reactivity tested in IFAT and Ag-ELISA against procyclic lysates (Nantulya *et al.*, 1987).
[c]Reactivity tested in Ag-ELISA against infected cattle sera (Nantulya and Lindqvist, 1989).
[d]Used in Ag-ELISA for *T. congolense* in goats and cattle (Masake and Nantulya, 1991).
[e]Used in Ag-ELISA for field diagnosis in cattle at Nguruman, Kenya (Nantulya *et al.*, 1992).
[f]Used in Ag-ELISA for *T. brucei* infections in cattle (Masake *et al.*, 1995).
bf, bloodstream forms; pt, procyclic trypomastigotes.

lysates (Nantulya *et al.*, 1987), some in Ag-ELISA on samples derived from experimental infections of cattle and goats, some with (Nantulya and Lindqvist, 1989) and some without treatment (Masake and Nantulya, 1991), and some in Ag-ELISA on bovine field samples (Nantulya *et al.*, 1992). Ag-ELISA results from various species, including cattle, pigs, monkeys and humans, are reported in numerous publications without mentioning the precise identity of the Mabs used. Clearly, it is difficult to assess the performance of an immunoassay technique in which there is such variability and uncertainty regarding the primary immunological reagents.

ANTIGEN RECOGNITION The earliest group of anti-trypanosomal Mabs to be described for use in Ag-ELISA (Nantulya *et al.*, 1987) were shown by IFAT to react with molecules on the plasma membrane of live and formaldehyde-fixed homologous procyclic forms, but not bloodstream forms. In the same work, ELISA reactions were obtained with supernatants of procyclic lysates, procyclic culture supernatants and, significantly, lysates of bloodstream forms prepared from stocks isolated in various countries. This work suggested that the antigens detected were water soluble, present in more than one stage of the life cycle, and relatively conserved over the parasites' geographical ranges. However, with regard to the bloodstream forms, neither the details of the species, number and origins of the trypanosome isolates tested, nor which Mabs they reacted with were described in detail.

FAO/IAEA AG-ELISA KIT The antigen-detection ELISA developed at ILRAD was introduced in the form of a standardized kit to a number of African institutes involved in tsetse and trypanosomiasis control during the period 1987–1995 under a Coordinated Research Programme of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. The FAO/IAEA trypanosomiasis Ag-ELISA kit used three species-specific Mabs, TR7/47.34.25, TC39/30.38.11 and TV27/9.45.15, directed against invariant surface antigens of *T. brucei*, *T. congolense* and *T. vivax*, respectively, and being of the IgM, IgM and IgG_1 antibody classes, respectively. Of these Mabs, only the IgG_1 used for detection of *T. vivax* was clearly identified as being the same as one of those featured in the published literature (see Table 14.1).

The introduction of the Ag-ELISA for diagnosis of bovine trypanosomiasis was nothing if not controversial. Highly polarized views on the effectiveness of the technique ranged from the Ag-ELISA being regarded as 100% sensitive and specific on the one hand, to it being regarded as worthless on the other. These views were debated in numerous fora, notably the biennial meetings of the International Scientific Council for Trypanosomiasis Research and Control (ISCTRC), often heatedly and with little scientific basis to many of the arguments used. The reality probably lay somewhere between these two extremes.

Although a large body of work was conducted using the Ag-ELISA, very few of these studies were designed so as to allow an impartial and objective evaluation of its usefulness. Moreover, frequent changes in the Mabs used in the Ag-ELISA and in the assay protocol itself added to the confusion, resulting in capricious and often unexplainable results. Ultimately, definitive double-blind trials were conducted at the International Livestock Research Institute (ILRI), Nairobi, on *T. congolense* and *T. vivax* experimentally infected cattle, which formally demonstrated that the version of the Ag-ELISA in use at that time was insufficiently sensitive to be of diagnostic value (Eisler *et al.*, 1998). Moreover, significant cross-reactivity was demonstrated between the *T. brucei* Ag-ELISA and sera from *T. congolense*-infected cattle.

The outcome of this was that by the late 1990s both ILRI and the Joint FAO/IAEA Division abandoned work on the Ag-ELISA. It is worth noting that the ELISA and related immunoassay techniques are widely applied and accepted in other areas of biomedical science for the detection and quantification of antigens, hormones and drugs and that perhaps in the fullness of time this approach can be re-evaluated in the context of diagnosis of African animal trypanosomiasis. Once laboratory-based ELISAs are developed, pen-side versions of these immunoassays should be technically feasible and would have the advantage of providing immediate results on which therapeutic decisions could be based. However, even if they were to be developed, they would be unlikely to become available in tsetse-infested areas of Africa in the foreseeable future for economic reasons, since even in the developed world the prohibitive expense of pen-side immunoassays generally precludes their routine use on farm animals.

Molecular Methods for Detecting Livestock-infective Trypanosomes

Accurate diagnosis of animal trypanosomiasis and definitive identification of the causative trypanosome species are clearly useful objectives that may contribute to the epidemiology and ultimately the long-term control of the disease. While these two simple objectives remain elusive with all the aforementioned parasitological and immunological technologies, molecular methods based on the detection and amplification of nucleic acids (DNA and RNA) certainly have the technical potential to achieve them.

For the convenience of medical and veterinary practice, the African trypanosomes have been classified into species or subspecies, based upon morphometric characteristics and the diseases they cause in their respective hosts. These criteria can be compounded by the complex interactions between the genetic background of the host on the one hand and the genotype of the parasite on the other.

The detection and identification of trypanosomes by molecular means should be based upon stable, parasite-specific genetic characteristics specific to that parasite that can withstand environmental influences exerted by either the host or the vector. These markers should be able to reveal the presence of the trypanosome irrespective of the developmental stage of the parasite at the time of identification.

Found within the genomes of the different trypanosomal species are unique regions of highly repetitive, multicopy DNA sequences as demonstrated by Sloof *et al.* (1983) with the identification of a 177 bp repeat of which more than 10,000 copies existed within the haploid genome of *T. brucei*.

Species-specific probes are now available for specific identification of each of the known species of trypanosomes by DNA hybridization (Majiwa, 1998). These regions were first demonstrated as providing ideal targets for the PCR in 1989 (Moser *et al.*, 1989). Since then a great deal of work has been carried out to optimize protocols for the continual identification of these sites by specific PCR primers (e.g. Reifenberg *et al.*, 1997). Both DNA hybridization and PCR have been applied to great effect to characterize specific trypanosome species infections within both host and vector, with probes often being used to validate PCR amplifications and to confirm the identity of the amplified targets.

When using PCR to identify multiple-copy segments of DNA, the presence of parasite DNA equivalent to one trypanosome in 10 ml of host blood can be detected (Masake *et al.*, 2002). This is due to the very high abundance of target sequence within the genome, only a very small percentage of which is required for successful detection. Furthermore, in experimental studies, the PCR can detect trypanosomes in cattle as early as 5 days after an infective tsetse bite (Masake *et al.*, 2002).

The kinetoplast DNA (kDNA) minicircles of trypanosomes have also been used as a source of DNA probes for their specific identification. The extent to which the minicircles drift in nucleotide sequence is unknown; however, the nucleotides at the origin of replication of the minicircles are conserved in all the Kinetoplastida where minicircles exist. The kDNA minicircle *ori* sequences have therefore been exploited as a means of identifying the trypanosomatids by PCR. In particular, two types of *T. evansi* have been identified on the basis of differences in their kDNA minicircle sequences (Borst *et al.*, 1987). A major disadvantage of using kDNA minicircles in the detection of trypanosomes is that they vary in sequence and proportion among different trypanosomes; furthermore, there are some non-tsetse-transmitted trypanosomes that lack kDNA and thus would not be detected by probes based upon the minicircle sequences.

Species-specific diagnosis

African pathogenic trypanosomes fall into three subgenera: *Duttonella*, *Nannomonas* and *Trypanozoon*. The identification of a universal marker by which to identify members of the *Duttonella* subgenus was hampered by the isolation of a number of different strains, each with their own specific markers. However, the discovery of an antigen found within the bloodstream of infected animals (Nantulya *et al.*, 1992), the protein function of which remains unknown, has proved to be an ideal genetic marker and has been shown to be conserved in isolates of this parasite from across Africa and South America (Masake *et al.*, 1997).

Within the *Nannomonas* subgenus there are three main species: *T. congolense*, *T. simiae* and *T. godfreyi*. It is possible to detect the general presence of *T. congolense* using the glutamic acid/alanine-rich protein (GARP), which has been successfully applied to the savannah, forest and Kilifi subgroups (Asbeck *et al.*, 2000), though no laboratory strain of the Tsavo subgroup was available for this work. To identify clearly between the specific species of *Nannomonas* it is necessary to screen with six separate PCR targets (Masiga *et al.*, 1992, 1996; Majiwa *et al.*, 1993). It was previously thought that certain *Nannomonas* species were limited to particular ecological niches but it has been proved that this is not the case, as revealed when these probes are used in epidemiological studies (e.g. McNamara *et al.*, 1995b).

The identification of members of the *Trypanozoon* group can be confirmed by targets such as the *ingi* element (Kimmel *et al.*, 1987), ribosomal inserted mobile element (RIME) (Hide and Tilley, 2001) and expression site-associated genes 6 and 7 (Kabiri *et al.*, 1999). These genomic regions are present in all members of this subgenus. However, until recently, species-specific identification has been problematic. This is of particular importance for the potentially human-infective parasites. With the identification of the serum resistance-associated (*SRA*) gene, which is confined to *T. b. rhodesiense* (De Greef *et al.*, 1992), it is now possible to differentiate *T. b. brucei* and *T. b.*

rhodesiense from one another using this gene as a stable molecular marker (Welburn *et al.*, 2001). This has found particular application for ascertaining the importance of domestic cattle as a reservoir for human-infective trypanosomes. The *SRA* gene has since been found within *T. b. rhodesiense* stocks from throughout its range in the eastern and southern areas of the African continent (Gibson *et al.*, 2002).

Multiple species detection by PCR

Screening through field samples with a minimum of seven primer pairs in order to differentiate between the various trypanosome species present can be time consuming and costly, with the final result often indicating a limited range of positives within the sample set (Mugittu *et al.*, 2001). In order to maximize the potential of PCR while limiting the actual number of amplifications required, recent research has focused on multiple species identification with a single primer set. The use of ribosomal RNA gene sequences for the finer discrimination and distinction of different species of trypanosomes is attractive (Desquesnes *et al.*, 2001). Given the heterogeneity in the nucleotide sequence composition of both the internal transcribed spacer (ITS) and the external transcribed spacer (ETS) of rDNA (Urakawa and Majiwa, 2001) and the conserved nature of ribosomal RNA gene sequences, this locus is ideal for designing oligonucleotide primers, for the discrimination of the different trypanosomes by PCR. Critical empirical evaluation and validation of the primers based on these sequences will have to be performed in order to demonstrate their utility.

Epidemiological application of PCR to animal trypanosomiasis

A number of important epidemiological issues have been addressed through the application of PCR and DNA probe technology to the problem of animal trypanosomiasis. These include the population structure of trypanosomes, trypanosome interaction with the tsetse vector in different ecosystems (Solano *et al.*, 1995), the characterization of trypanosomes in natural infections of livestock kept under different management systems (Nyeko *et al.*, 1990; Reifenberg *et al.*, 1997; Clausen *et al.*, 1998; Mugittu *et al.*, 2001), the response of trypanosome populations to therapeutic drugs administered to experimentally infected animals (Clausen *et al.*, 1999), monitoring of livestock and tsetse vectors for presence of trypanosomes during trypanosomiasis control programmes, the assessment of trypanosome challenge to wildlife relocated to national parks for game conservation (Mihok *et al.*, 1992), detection of mixed infections in both vectors and vertebrate hosts, and if assessing the extent of spread of human infective trypanosomes in the reservoir populations (Welburn *et al.*, 2001).

These applications have clearly demonstrated the potential for wide-scale application of DNA-based diagnostics in the epidemiology of trypanosomiasis.

Other genetic methodologies

Individual *Trypanozoon* strains have been characterized using primers based on the nucleotide sequence of the mobile element RIME (Hide and Tilley, 2001). Phylogenetic analysis was used to group strains based on similarities and differences in the resulting banding profiles. This technique has yet to be applied to mixed infections, which at present require a more involved analysis of minisatellite markers for effective separation.

Other approaches to investigating the molecular variation between the various *Trypanozoon* species include restriction enzyme fragment length polymorphisms (RFLPs) (Hide *et al.*, 1994), randomly amplified polymorphic DNAs (RAPD), and amplified fragment length polymorphisms (AFLP) (Masiga *et al.*, 2000). While these are effective methods for characterization of trypanosomes, they have not been applied broadly in the detection of parasites because they generally require large amounts of purified parasite DNA.

Although RAPD analysis does not require such large quantities of DNA, it is susceptible to contamination with other nucleic acids and hence results may have low levels of reproducibility.

Finally, the use of quantitative PCR techniques has been shown to be of potential value for other types of parasitic infections in domestic animals (Zarlenga and Higgins, 2001). Whereas conventional PCR techniques simply indicate the presence or absence of parasite DNA, quantitative techniques have the advantage of giving an indication of the level of parasite burden. This may be important with trypanosome infections in terms of their effect on the productivity of livestock.

Limitations in the use of DNA technology

The rate of adoption of diagnostic DNA technology by laboratories in developing countries appears to be limited not only by cost but also by a widespread perception that it is highly complex. However, once established, these diagnostic techniques do have the potential to function efficiently even in the hands of modestly trained technical staff. Moreover, if cost constraints can be overcome, diagnostic PCR technology has the advantage that it can be totally automated, thereby minimizing steps involved in sample handling and decreasing the possibility of contamination. Finally, although PCR is usually conducted within the confines of well-established laboratories, efforts are under way to develop PCR equipment that can be used outside the confines of laboratory buildings.

Conclusion

There now exists a reasonable collection of methods and reagents from which scientists in well-equipped laboratories may choose the one most appropriate for diagnosis of trypanosomiasis in any given situation (see Fig. 14.1). These methods will continue to improve with the availability of complete genome sequences of these fascinating yet debilitating parasites. In the future, diagnostics may be developed that detect important phenotypic characteristics such as virulence of the parasite to the host and resistance of the parasite to trypanocidal drugs. It should be remembered that these methods are unlikely to become available to the majority of individuals conducting disease control activities in the field, who will for the foreseeable future continue to use clinical diagnosis, occasionally supplemented by parasitological techniques and ancillary methods such as haemoglobin determination. This is particularly relevant in the context of integrated disease control, which is increasingly recognized as being of importance in tsetse-infested areas of sub-Saharan Africa. A diagnosis of trypanosomiasis is just one of many differential diagnoses that may be of equal importance to primary animal health care providers working in these areas, others of note being various tick-borne diseases, helminthiases and malnutrition. In this context it is worth noting that transfer of generic technologies such as an inexpensive method of haemoglobin determination, albeit per se unable to provide a specific aetiological diagnosis, may be just as useful as immunological and molecular technologies that are unlikely to be affordable to resource-poor farmers in Africa.

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15 Non-tsetse-transmitted Animal Trypanosomiasis

Antony G. Luckins and Ron H. Dwinger

Introduction

The dependence of African pathogenic trypanosomes on a cyclical mode of transmission in an insect vector has limited their geographical distribution and the diseases they cause to regions infested by tsetse flies. The transmission of trypanosomiasis by other species of haematophagous biting flies in these areas is infrequently reported and, where tsetse flies have been eradicated, there is little evidence that trypanosomiasis persists in their absence. Nevertheless, biting flies such as *Tabanus, Haematopota, Chrysops* and *Stomoxys* spp. can transmit bloodstream trypomastigotes to mammalian hosts and this has enabled some species of pathogenic trypanosomes to spread widely beyond Africa into Asia and Latin America. This non-cyclical or mechanical means of transmission has been acquired by only two species of trypanosomes. *Trypanosoma evansi*, a member of the *Trypanozoon* group, has evolved from *Trypanosoma brucei* and lost the ability to develop cyclically in tsetse flies. *Trypanosoma vivax* is morphologically and biologically similar to the tsetse-transmitted African forms of the parasite but is unable to develop in tsetse flies and is confined to South America. These two species present a considerable challenge to the scientist, the veterinarian and the policy maker. They occur in many different ecological regions in the Old and the New World and although they affect several domesticated livestock species information on their distribution, prevalence and economic significance is lacking. Reproductive wastage is sometimes identified as a source of economic loss but often the impact of non-tsetse-transmitted trypanosomiases on productivity is recognized only if epidemic outbreaks of disease cause considerable loss. Investigations into whether economically significant production losses occur when milder forms of disease exist have been addressed rarely. Knowledge and understanding of the natural history of the diseases they cause is limited compared with the tsetse-transmitted trypanosomiases and the absence of accurate epidemiological data, and the lack of effective monitoring and surveillance, precludes implementation of cost-effective strategies for control.

Epidemiology of *T. evansi* and *T. vivax*

Geographical distribution

T. evansi is the most widely distributed of the pathogenic animal trypanosomiases, affecting domesticated livestock in Asia, Africa and Central and South America. Serious epidemics of surra recorded towards the end of

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the 19th century and in the early years of the 20th century in Indonesia and the Philippines suggest that it could have spread into these regions within the last 100 years (Lun *et al*., 1993; Luckins, 1999a). This pathogenic form of disease now occurs less frequently and, while epidemic outbreaks of disease do occur, *T. evansi* causes primarily a disease with a high morbidity and low mortality.

All species of domesticated livestock can be infected with *T. evansi* but the principal host varies geographically. In northern Africa and in parts of eastern Africa camels are the most important host, whilst in Central and South America the horse is affected. In Asia a much wider range of hosts is involved, including Bactrian and dromedary camels, cattle, water buffalo, horses and pigs. In Africa and South America domesticated livestock other than camels and horses are rarely affected clinically with *T. evansi* but there is serological evidence of infection in goats and sheep in the Sudan and in cattle from Brazil (Luckins, 1999a). *T. vivax* was probably introduced into Latin America in a shipment of zebu cattle imported from Senegal. It was first described in French Guyana in 1919, as an epizootic in dairy cattle with a mortality of more than 52%. The disease was reported to be widespread along the Atlantic coast in Colombia, more commonly occurring in the dry season of the year and in lowland areas of the country. It was also reported from Venezuela, Panama and Surinam and from two islands in the Lesser Antilles, Guadeloupe and Martinique. In a serological survey using the indirect fluorescent antibody test, antibodies against *T. vivax* were detected in serum samples originating from El Salvador, Costa Rica, Colombia, Ecuador, Peru, Brazil and Paraguay, providing evidence that the distribution of *T. vivax* in South America extended from 12° N to the Tropic of Capricorn. Outbreaks of trypanosomiasis due to *T. vivax* have been reported from the Pantanal region in Brazil, from lowland provinces of Santa Cruz Department and from provinces of Beni Department in Bolivia. Prevalence in cattle ranged from 34% in the Pantanal to 86% in Laguna Concepción, Bolivia. The spread of the disease was due to cattle movement and an increase in cattle trading between Brazil and Bolivia.

Horses with fulminating infections with *T. evansi* are a potential source of infection for cattle or water buffalo and these in turn can act as symptomless carriers of infection. It is not clear how important is infection in wild animals and their relationship with domesticated animals in the farm/ranch situation. In some regions the ecological conditions preclude any close contact with wild animals, e.g. in densely populated, intensively farmed regions (Indonesia, Vietnam, Thailand, the Philippines, southern China). *T. evansi* infects various wild animals, including capybara (*Hydrochoerus hydrochoerus*), wild dogs (*Canis azarae*) and deer (*Axis axis, Rusa timorensis* and *Cervus* spp.) but there has been little attempt to investigate the transmission of infection between wild and domesticated livestock. *T. evansi* was introduced throughout its geographical range as a parasite of domesticated livestock and, since infections in wild animals are acquired secondarily, the disease is often fatal. However, *T. evansi* shows a wide range of pathogenicity, and chronic infections in wild animals do occur, as in capybara in South America (Franke *et al*., 1994). The relationships between wild and domesticated livestock, the feeding preferences of flies and the characteristics of the strains of trypanosomes isolated from different hosts require further investigation. Deer, such as the cariacou (*Odocoïleus gymnotis*), can serve as a wild reservoir of *T. vivax* in Venezuela but there is no conclusive proof that deer act as a reservoir from which cattle can become infected.

Phylogenetic relationships and molecular characterization

Isoenzyme studies and characterization of nuclear and kinetoplast DNA support the hypothesis of a limited evolutionary origin for *T. evansi*. In spite of this, the parasite has achieved a wide geographical distribution, infects numerous host species and shows a range of pathogenicity that indicates the possibility of a range of genotypes. Stocks of *T.*

evansi isolated from several different areas of Kenya were found to conform to a homogeneous pattern of zymodemes and were similar to the patterns observed in isolates from South America, Nigeria and Sudan. Similar work on isolates from water buffalo, camels and horses from different parts of China confirmed that *T. evansi* formed a homogeneous group (Lun *et al*., 1992a). This homogeneity was also seen among stocks of trypanosomes that were isolated from dogs and capybara in South America and differed in their virulence. Although there were marked behavioural differences between the stocks examined, all were found to have similar zymodeme patterns and to resemble zymodemes from Africa and other parts of South America (Franke *et al*., 1994). In South-East Asia, stocks of *T. evansi* from Java were shown to form a homogeneous group with similar characteristics to stocks isolated elsewhere.

A greater level of discrimination can be achieved by use molecular karyotyping; stocks that were indistinguishable by isoenzyme analysis were found to fall into several karyotype groups (Lun *et al*., 1992b). An isolate of *T. evansi* from a camel in northwest China differed from the karyotype expressed by other stocks, suggesting that *T. evansi* might have been introduced into China on more than one occasion. Characterization of stocks from China, Africa and South America and the Philippines by restriction fragment length polymorphism (RFLP) revealed that the Chinese stocks were identical but differed from isolates from elsewhere (Zhang and Baltz, 1994). Infraspecific differences amongst stocks of *T. evansi* have also been found in Thailand (Watanapokasin *et al*., 1998). In camels in Kenya, eight different karyotypes were identified amongst trypanosomes isolated from two herds that had been under different therapeutic regimes (Waitumbi and Young, 1994). In a herd treated prophylactically with quinapyramine prosalt, all but one isolate conformed to a single karyotype. In contrast, in a herd where treatment had been administered to camels when they became parasitologically positive, eight different karyotypes were present. *T. evansi* resistant to the effects of quinapyramine were found to express two different karyotypes (Waitumbi *et al*., 1994). No polymorphisms in *T. evansi* stocks from camels in Kenya were revealed by random amplified polymorphic DNA (RAPD-PCR), even though karyotype differences were observed.

The characterization of *T. vivax* isolates from Colombia in South America showed that although minor phenotypic differences existed between them, these parasites are antigenically related and belong to a single serodeme. Characterization by isoenzyme assay, karyotyping and DNA probe analysis showed the Colombian isolates to be more similar to the West African than to Kenyan *T. vivax* (Dirie *et al*., 1993).

Clearly, molecular characterization has potential in determining the relationship between strains isolated from domesticated livestock and the putative reservoir hosts. It could help to define the origin of strains and their spread amongst individuals in a herd, their association with disease, the effects of trypanocidal treatment and the presence of drug resistance. Since relatively few isolates have been examined comprehensively, our knowledge of the molecular characteristics of *T. evansi* or *T. vivax* in relation to their overall epidemiology is limited.

Transmission of Infection

The relationship between *T. evansi* and its mammalian hosts is a complex process influenced by host species and age, previous experience of infection and physiological condition. The degree of infection in the host population, the stage of infection (chronic or acute) and the level of parasitaemia in individual animals influence transmission by insect vectors. Seasonal and environmental influences affect the population density of the insect vectors and the opportunities for transmission. Morbidity is influenced by husbandry practices such as the removal of sick animals that act as reservoirs, the introduction of infected animals that might lead to outbreak of disease in the herd where they are placed, or introduction of susceptible livestock into infected herds. The link between the disease, surra, and the presence of large biting flies was well known

to livestock owners long before the causal agent and its vector were identified. Experimental evidence for the role of the tabanid fly was first reported in 1901 and since then many experiments have been made to transmit *T. evansi* with *Tabanus, Lyperosia, Haematopota, Chrysops* and *Stomoxys* spp. (Luckins, 1999a). Most studies have been carried out with *Tabanus* spp. and this genus contains the most important vectors, though there are relatively few field data – apart from strong circumstantial evidence – to support this contention. Tabanid flies are aggressive feeders, and their vigorous attacks on the host cause defensive reactions that disturb the flies so that they attack other hosts in order to complete their blood meal. This interrupted feeding enables transmission of the trypanosomes; flies initially feeding on an infected animal may complete feeding in an uninfected host. More than 20 different species of *Tabanus* have been shown experimentally to transmit *T. evansi*. *Stomoxys* spp. have been implicated in the dissemination of infection in Mauritius and the Philippines and are also associated with transmission amongst camels in Africa. Transmission by biting flies is not the sole means by which infection is perpetuated; ingestion of meat from infected carcasses by carnivores can result in infection. In South America, vampire bats are said to be of importance both as reservoirs of infection and as vectors. However, there has never been any definitive study to confirm this and it is not clear how important they are.

Mechanical transmission of *T. vivax* from one host to another has been successfully achieved with the tabanids *Cryptotylus unicolor* and *Tabanus importunus* in French Guyana and with *Tabanus nebulosus* in Colombia. The level of parasitaemia of the infected donor is an important factor influencing the success rate of mechanical transmission. There is also indirect evidence of mechanical transmission from epidemiological studies in Colombia, which demonstrated a significant temporal relationship between the feeding activity of tabanids and *T. vivax* incidence, and which showed that *T. vivax* infections were associated with low-lying swampy areas, where tabanids breed (Otte *et al.*, 1994).

Pathology and Pathogenesis of Disease

Trypomastigote forms of *T. evansi* induce the formation of a cutaneous oedematous lesion, known as a chancre, when parasites are inoculated intradermally into the mammalian host. Since the numbers of trypanosomes carried on the proboscis of biting flies is low, it is possible that an early phase of multiplication in the skin serves to increase both the population and the antigenic diversity of the parasites that enter the bloodstream. The multiplying trypanosomes and the resultant intense cellular infiltration in the skin may promote the induction of immunity. In Indonesia, *T. evansi* infections in cattle and water buffalo are typically chronic with associated weight loss and anaemia. A wide range of other clinical signs have been attributed to *T. evansi* infections in cattle and water buffalo, including fever, salivation, diarrhoea, oedema, jaundice, conjunctivitis, lacrimation, mucopurulent nasal discharge, dyspnoea, alopecia, urticaria, swelling of superficial lymph nodes, abortion and infertility, decreased milk yield in cattle, weakness, incoordination and paralysis. Antigen–antibody complexes formed during the host response to *T. evansi* infection may cause inflammatory reactions in the central nervous system, myocardium and skeletal muscle. Although less common, acute disease and high mortality have been reported in Indonesia following movement of livestock between areas with different endemic strains of *T. evansi* (Luckins, 1999b) and after importation of naive stock. Factors such as the stress of movement, adverse weather conditions, nutritional deficiencies, physiological changes and concurrent disease may result in clinical trypanosomiasis in previously latently infected animals (Luckins, 1999a). The effect of *T. evansi* infection on fertility is not fully understood but there have been reports of abortion and cessation of oestrous activity, possibly due to body weight loss. *T. evansi* has been implicated as a predisposing factor for disease outbreaks caused by *Clostridium perfringens* in camels. Immune responses to *Pasteurella multocida* are also depressed, possibly explaining the poor level

of protection against haemorrhagic septicaemia in water buffalo in Vietnam (Holland *et al*., 2001b). In a study of natural *T. evansi* infections in local and imported water buffalo in Indonesia, non-specific gross pathological findings included carcass emaciation, pale mucous membranes and haemorrhagic or congested lungs. Histopathological lesions of non-supportive interstitial myocarditis, multifocal necrosis of spleen and liver, and interstitial pneumonia were considered specific for *T. evansi* infections. In *T. evansi*-infected dromedary camels, complement fixing (CF) antibody titres and circulating trypanosomal antigens rise considerably following infection, leading to complement depletion, a condition reversed by treatment. Depletion in complement level may have important implications in the pathogenesis of trypanosomiasis. Cases of cerebral trypanosomiasis have been reported in dairy cattle in Thailand, the animals showing signs of circling, excitation, aggressive behaviour, ataxis, paresis of hind limbs, lateral recumbency, convulsion and finally death. *T. evansi* was detected on impression smears of organs from cattle that died with nervous symptoms and also in smears made from their cerebrospinal fluid. In hog deer (*Cervus porcinus*), histopathological observations have revealed a generalized non-suppurative meningoencephalitis affecting the white and grey matter at all levels of the brain. Typically, there were broad perivascular cuffs of mononuclear inflammatory cells, including lymphocytes, and some Mott cells.

There have been few studies to determine the underlying process of pathogenesis in non-tsetse-transmitted infections. A series of experiments in sheep gave some insight into the changes that occur during infections with *T. evansi* (Onah *et al*., 1997, 1998a,b, 1999). T and B cell subsets showed significant alterations during the course of infection. In infected sheep $CD8^+$ cells showed little change, but $CD4^+$ cells showed a small decrease; some animals self-cured and in these $CD8^+$ cells decreased. Circulating B cells increased in numbers and anti-*T. evansi* antibodies were produced but their levels were higher in self-cured sheep. When infected sheep were vaccinated with *Pasteurella haemolytica* antigen there was a reduction in the development of a local skin reaction and a decrease in serum antibody responses.

Fever, parasitaemia, suppression of milk yields, abortion and occasional deaths accompany the disease caused by *T. vivax* in adult cattle in South America. Anaemia, lethargy and loss of condition lead to the characteristic 'fly-struck' appearance. Camus and Martrenchar (1990) described weight loss of 10–17 kg in 1 month compared with controls during an experimental infection of zebu cattle in French Guyana. Perinatal infection has been reported in Venezuela but the transplacental route is probably an abnormal method of transmission and unlikely to play an important role in the epidemiology of the disease. Animals that recover from the disease can become reservoirs, showing low and undetectable parasite levels in the blood. Thus, aparasitaemic phases can be part of a *T. vivax* infection in animals and the parasites have been shown to establish in extravascular locations such as the choroid plexus and the aqueous humour of the eye. However, latent infections can be reactivated due to stress.

Diagnosis of Infection

Detection of the trypomastigotes of *T. evansi* or *T. vivax* in the blood of animals provides an absolutely verifiable measure of their infection status. Unfortunately, it is often difficult to demonstrate them in this way, since infections are often chronic and few parasites are present. No single parasitological detection test is totally effective in identifying all infected animals. For example, in horses infected with *T. evansi* the haematocrit centrifuge technique (HCT) is only 71% sensitive compared with mouse inoculation (MI), which is 88% sensitive. In water buffalo experimentally infected with *T. evansi* the HCT was only 13% sensitive and MI 43% sensitive. The difficulty in detecting trypanosomes has created a need for serological tests that can accurately predict the infection status of an individual animal. Over the past 30 years numerous attempts have been made

to produce assays that are more diagnostically sensitive than parasitological diagnosis. Nevertheless, there is still potential to increase the sensitivity of the standard parasitological tests. For instance, use of the buffy coat layer instead of whole blood can increase diagnostic sensitivity tenfold.

Many serological assays have been developed for the diagnosis of *T. evansi* and *T. vivax* but few have found practical application in the field. Complement fixation tests were used extensively in the Philippines in the early years of the 20th century and large-scale surveys have been done using this test in China. However, the tests that have been developed recently, based on enzyme immunoassays, have not been widely applied. There are a number of reasons for this: most have not been properly validated and standardized and so reference materials are not available; the necessary training and support has not been provided to enable diagnostic laboratories to adopt the technologies; and, finally, there has not been sufficient demand from livestock owners for provision of such a service. In addition, few of the tests are able to provide rapid results, as most require sophisticated laboratory facilities and few attempts have been made to design tests that can be used under field conditions. One test that does encompass some of these requirements is the card agglutination test for trypanosomiasis (CATT). This test is available commercially, uses standardized reagents and can be used under field conditions for detecting antibodies to *T. evansi* in a number of animal species (Songa *et al*., 1987). In spite of this, the test has not been applied extensively in the field.

Antibody detection ELISAs (Ab-ELISA) utilize crude sonicated antigens, but there has been little attempt to determine if there are differences in the type of antigen preparation or the particular isolate from which the antigen is derived. Although antibody responses in individual animals vary quite considerably, it is possible in several different host species to detect antibodies 14–21 days after infection. Antibodies decline after treatment but can still be found up to 3 months or longer. In Indonesia, surveys using Ab-ELISAs have confirmed the widespread distribution of *T. evansi* in water buffalo (Davison *et al*., 2000). The test has also been used for detecting *T. evansi* in camels and a number of reports are available from the Sudan, India, Kenya and Mauritania. Ab-ELISA was found to be a useful diagnostic tool in determining the spread of *T. vivax* in the eastern Caribbean, where it was considered that screening tests using ELISA should be carried out periodically for surveillance.

The persistence of antibodies after treatment is considered a major impediment of serological assays, since it is difficult to distinguish between active infections and past infections, thereby making it difficult to determine whether treatment is required. Nevertheless, a study in camels using the Ab-ELISA did provide evidence to the contrary (Rae *et al*., 1989), and studies in water buffalo in Vietnam also confirmed that Ab-ELISA could be used to identify infected animals (Verloo *et al*., 2000). There has been considerable interest in developing tests that do provide evidence of current infection, and for this enzyme immunoassays assays that detect circulating trypanosomal antigen were considered ideal. Antigen detection ELISAs (Ag-ELISAs) are based on the double-antibody-sandwich technique: capture antibody is adsorbed to a microtitre plate and, after reacting with the test sera, the same antibody conjugated with horseradish peroxidase is added as an indicator. Initial tests with Ag-ELISAs were encouraging and it was suggested that these assays had a sufficiently high degree of sensitivity and specificity in studies on cattle, camels, horses and water buffalo to enable discrimination between infected and uninfected hosts (Nantulya *et al*., 1989). Later more critical evaluation of the assays showed that the tests did not come up to expectations. For instance, South American strains of *T. vivax* were not recognized by monoclonal antibodies that detected African strains and the assay was found to have a low sensitivity to Guyanese strains of *T. vivax* (Desquesnes, 1996).

In studies on experimentally infected water buffalo in Indonesia, considerable variation in the patterns of antigenaemia

was detected in individual buffaloes using two Ag-ELISA assays that recognized different antigenic determinants on *T. evansi* (Davison *et al.*, 1999). Antigens appeared 7–42 days after infection and in some water buffalo similar antigenaemia profiles were shown by the two assays. Peaks in antigen production were detected from 42 days after infection, with declining levels thereafter. The highest sensitivity estimates were obtained from 57 to 64 days after infection. The differences in sensitivity reflect the underlying fluctuations in serum trypanosomal antigens that occur throughout the course of infection. After treatment with trypanocidal drug antigen, clearance was variable: in some individuals antigen disappeared rapidly but in others an antigenaemia persisted for up to 74 days after treatment from a primary infection and up to 8 months after secondary infection.

For use in a field survey in Indonesia the two Ag-ELISAs, an Ab-ELISA and the CATT were fully validated in terms of their diagnostic sensitivity and specificity. Response-operating characteristic curves were constructed, and optimal ELISA cut-off values, which minimized the number of false-negative and false-positive results, were chosen. The Ab-ELISA had the highest sensitivity (89%) and the CATT had the highest specificity (100%). There was a significant difference between the sensitivities (71 and 81%), but not between the specificities (75 and 78%), of the two Ag-ELISAs. The four tests were further compared by calculation of post-test probabilities of infection for positive and negative test results using a range of prevalence values and likelihood ratios. The results suggested that the CATT was the best test to 'rule-in' infection (i.e. the highest probability of infection in test-positive animals) and the Ab-ELISA was the best test to 'rule-out' infection (i.e. the lowest probability of infection in test-negative animals). Similar critical comparisons need to be done with other host species in order to evaluate fully the diagnostic tests currently available.

The prevalence and incidence of *T. evansi* infections was estimated in village water buffalo in central Java using these tests and conventional parasitology (Davison *et al.*, 2000). Over 2000 water buffalo were tested in five districts; 4% were positive with the HCT, 58% were positive with the one Ag-ELISA and 70% were positive with the second Ag-ELISA. An increasing prevalence with age was found and the proportion of antigenaemic water buffalo was highest in these aged over 60–84 months old. Parasitaemic water buffalo were found in more than half of the villages visited. Corrected village-specific prevalence values obtained with the two Ag-ELISAs ranged from 0% to 100%, and prevalence differed significantly between villages in four of the five districts. Incidence rates varied according to the test used and ranged from 0.22 (95% CI: 0.09, 0.44) to 0.44 (95% CI: 0.24, 0.76), per animal-year at risk, in two villages.

Diagnosis using the PCR could offer a very precise method for detecting infection and discriminating between infected and non-infected animals. The analytical sensitivity of such tests is high but in experimental situations they have not always given high diagnostic sensitivity. In *T. evansi*-infected water buffalo, PCR was found to have a sensitivity of 78%, similar to that of MI (Holland *et al.*, 2001a). At this level of sensitivity, enhanced parasitological tests could provide more effective diagnosis than PCR. PCR techniques have been evaluated for a South American *T. vivax* stock and gave excellent results in comparison with other diagnostic techniques (Desquesnes, 1997). Under experimental conditions the technique proved consistently more sensitive than parasitological techniques in detecting parasite DNA in dried blood samples (de Almeida *et al.*, 1997). However, the primers used for PCR do not amplify the DNA of all *Duttonella* stocks circulating in a particular region (Lefrançois *et al.*, 1998). A *T. vivax*-specific PCR based on spliced leader intergenic sequences was able to amplify DNA from isolates of *T. vivax* from Colombia, Brazil and Bolivia. The assay was highly sensitive and detected trypanosomes in animals with non-patent infections. Preparations of blood collected on filter paper or slides could also be used, increasing the potential of this test for diagnosis (Ventura *et al.*, 2001).

Control

Preventive measures used by livestock owners to discourage attacks by biting flies include stabling animals, use of smudge fires and installation of netting to protect dairy cattle. It is not known how effective these measures might be in reducing transmission of trypanosomiasis. Strategies for control have relied heavily on the use of chemotherapy to treat infected animals. It is generally recognized that chemotherapy is most effective in the early stages of infection. Four compounds, namely suramin, diminazene aceturate, isometamidium and quinapyramine, have been used to treat camels, cattle, water buffalo, horses and pigs infected with *T. evansi* for many years. Indeed, suramin has been the mainstay of treatment for all host species for over 70 years, even though its intravenous route of administration can be problematic in the field. Suramin binds to serum proteins and can confer a limited protection of about 1 month to treated animals, though there are reports of protection for as long as 3 months. Melarsenoxide cysteamine has been introduced for the treatment of camel trypanosomiasis and has been used in water buffalo (Luckins, 1999b). Although these drugs have been used widely, there are often conflicting reports of their efficacy, suggesting that there might well be differences in sensitivity amongst isolates of *T. evansi* from different regions. Suramin is universally effective at a dose rate of 10 mg/kg. Diminazene aceturate has been used at dose rates from 3.5 mg/kg by intramuscular injection to cure *T. evansi* in cattle, water buffalo and donkeys in India, Thailand and the Philippines and doses as high as 15 mg/kg have been used in water buffalo in India. Quinapyramine is used at 2–5 mg/kg by subcutaneous injection in cattle, camels and horses and is the preferred drug in India.

Drug resistance is known to occur amongst *T. evansi* isolates and there have been reports of its occurrence in several different countries in Africa and Asia. It is not known how extensive its occurrence is, or whether it is (or is likely to be) a serious problem for the control of the disease. A number of different approaches have been used to detect drug-resistant trypanosomes, including *in vivo* tests in rodents or *in vitro* assays. The latter are particularly useful, since *T. evansi* can be cultured in the absence of feeder cell monolayers that could affect the metabolism of the drugs used. Drug resistance has been reported from Kenya, where epidemiological studies using *in vitro* techniques revealed stocks of *T. evansi* resistant to suramin and quinapyramine (Maina *et al*., 1996). *In vitro* testing also showed that, in China, stocks of *T. evansi* from water buffalo were innately resistant to diminazene aceturate (Brun and Lun, 1994), and in Vietnam, isolates of *T. evansi* resistant to isometamidium were found in water buffalo. In Sudan, *in vitro* studies showed that suramin resistance persisted even though the drug had been absent from the market in Sudan for over 20 years.

The efficacy of using diminazene aceturate to control *T. evansi* in draught water buffalo was evaluated in villages in central Java, Indonesia (Luckins, 1999b). From a population of 800 animals, 258 were selected for study. These animals were subdivided into four groups. Group A consisted of water buffalo that were positive by both Ab- and Ag-ELISA and/or positive by MI or microhaematocrit centrifuge technique (MHCT); Group B consisted of water buffalo that were positive only by Ag-ELISA; Group C comprised animals that were positive only by Ab-ELISA; and Group D was a control group of parasitologically negative water buffalo that had no evidence of trypanosomal antigen or antibody. Group A animals were treated at a dose rate of 7 mg/kg body weight. Throughout the period of study there was a gradual decline in the point prevalence of infection in Group A water buffalo. In Groups B and C the prevalence increased after 3 months. In Group D this rise was slower, probably due to the fact that these animals did not initially harbour infection. Although there was a difference in prevalence between the treated and the untreated groups, there was no evidence that the incidence of infection was reduced in the treated animals. In Group D, the cumulative incidence (CI) was 0.77; in Group B it was 0.75

and Group C, 0.68; whereas in Group A, the treated animals, the CI was 0.77. There was no statistically significant difference between these values. This failure to reduce incidence of infection was due to the high prevalence of *T. evansi* infection (30%) in the village herd. Since only a small proportion of the water buffalo were targeted for treatment, a considerable reservoir of infection was still available, emphasizing the need to ensure that all infected animals in a herd are identified if control is to be successful.

Isometamidium chloride, diminazene aceturate and quinapyramine have all been found effective against *T. vivax* infections in South America. A strain of *T. vivax* resistant to diminazene has been found in Guyana but it was fully sensitive to isometamidium chloride. There is no indication that such resistance is widespread or is likely to affect livestock production adversely (Desquesnes *et al*., 1995).

Treatment of proven infected or clinically affected animals fails to address the underlying problem of carrier animals. The low sensitivity of parasitological techniques and the unreliability of clinical signs ensure that this approach cannot decrease the number of cases as effectively as treating all infected individuals. If wildlife reservoirs are of no importance, and if small ruminants and dogs present little or no problem, then it should be possible to effect control based solely on the use of chemotherapy (Lun *et al*., 1993; Luckins, 1999a). It is unlikely that any new drugs will be introduced for the treatment of surra; indeed, the range of options for treatment will decrease as some drugs are withdrawn. It is therefore expedient that the available drugs are administered correctly to mitigate factors that are likely to increase the emergence of drug resistance. Hence it is essential that proper criteria be used to determine which animals should be treated and cognizance taken of the ecological factors that might influence the outcome of control.

Economic Impact

There have been few studies on the effects of non-tsetse-transmitted trypanosomiases on productivity but the information available shows that infections with both *T. evansi* and *T. vivax* have serious economic implications for livestock and farmers in South America and South-East Asia.

In southern Brazil, the Pantanal (a tropical seasonal wetland) contains approximately 1100 cattle ranches, holding some 3 million head of cattle, 50,000 horses and a wide diversity of wildlife, including capybara. Horses are indispensable to the cattle-ranching industry and *T. evansi* affects over 6000 animals/year. Estimates have been made of the risk of infection, costs of diagnosis, different treatments and costs of animal losses (Seidl *et al*., 1998). The total impact of *T. evansi* was about US$2.4 million/year. Results indicated that the most financially acceptable strategy for control was for a curative treatment regime using diminazene aceturate employed year-round. This treatment provided an annual net benefit of more than US$2 million, or US$1845 per ranch, and saved 5783 horses. It represented an annual net benefit of over US$200,000 and 600 horses.

In a study in central Java (Luckins, 1999b) 127 adult female water buffalo kept by smallholder farmers were monitored for 8 months to measure the impact of *T. evansi.* The water buffalo were divided into three groups according to the presence or absence of infection with *T. evansi,* and whether or not they were treated with trypanocidal drug. Group A consisted of 45 water buffalo that were serologically positive for *T. evansi* at the onset of the monitoring period and were treated initially with diminazene aceturate and, if required, during the monitoring period. Group B consisted of 37 untreated water buffalo that were positive for *T. evansi* throughout the monitoring period. Group C comprised 45 animals that remained negative for *T. evansi* throughout the study. A partial budget was constructed to measure the impact of infection on those items of input and output from buffalo production that were anticipated to alter through *T. evansi* infection. Data on calving rates, body weight, death and sale rates, and animals sold through ill health were collected. Only input costs relating to veterinary treatments were recorded, as these were the only inputs that

were expected to change with infection. On average, 30% of the female adult herd was sold in a year. Mortality rates (including those animals sold due to ill health) were low at 3%, as were calving rates that averaged an estimated 10% for the year. Despite low calving rates, the village herd appreciated in value by an estimated US$321,534 over the year. Most of this growth (98%) occurred through weight gain that was either sold or retained in the herd for future use. The female buffalo were thus essentially being fattened for sale, during which time they provided valuable manure and draught power for crop cultivation.

Animals infected with *T. evansi* were more likely to die or be sold through ill health compared with uninfected animals. The combined mortality/emergency sales rates for infected and uninfected animals were 22% and 0%, respectively (Table 15.1). Calving rates were also marginally lower among infected animals. These lower calving rates, higher mortality and emergency sales rates resulted in lower herd growth rates: infected herds produced some US$27-worth less weight per animal per year compared with *T. evansi*-free animals. Most of this reduction was attributed to mortality losses and emergency sales rather than a reduction in growth rates. The productivity of animals that were treated with diminazene aceturate approximately matched that of the *T. evansi*-free animals, yielding weight gains valued at US$24 per animal. Mortality and emergency sales were greatly reduced from 22% for the infected group to 8% for the treated group but still exceeded the loss rates for the uninfected group. However, treated animals that were not lost or sold due to disease appeared to gain weight more quickly than the uninfected group. The average cost of treatment per animal amounted to approximately US$10/year. This cost was based on the price that farmers would pay for the drug procured through private channels. Net returns to an annual course of treatment thus amounted to US$14 per infected animal per annum. This is equivalent to a benefit-to-cost ratio of 2.4, or a return of 135% on treatment costs. Comprehensive treatment of all animals at risk (herd treatment) would only be economically justifiable in this village context if *T. evansi* prevalence rates exceeded 40%; this rarely happened and in most instances prevalence was < 30%. The average cost of the disease per animal was just US$3.40, or 36% of the cost of treatment. At these levels of disease prevalence, farmers could only reap the benefits of treatment if they were able to target infected animals. For this to be economically feasible it would be necessary for infected animals to be identified for the farmer at a reasonable cost (i.e. less than US$14) and with relative ease (i.e. pen-side diagnosis).

There have been few studies to estimate the impact of *T. vivax* among livestock in Latin America. Control of *T. vivax* is based on a combination of restriction of movement of infected animals, treatment of infected animals, epidemiological monitoring of the distribution and severity of the disease and on

Table 15.1. Productivity of water buffalo under village conditions in Indonesia in water buffalo uninfected, infected with *T. evansi* but left untreated, and infected with *T. evansi* and treated with diminazene aceturate.

	Uninfected	Infected and treated	Infected and not treated	Herd average (13% infected)
Mortality rate	0%	4%	9%	1%
Emergency sale rate	0%	4%	13%	2%
Calving rate	11%	7%	9%	10%
Value of weight gain per animal left in herd at end of year	US$384	US$445	US$458	US$393
Increase in value per animal of initial herd stock over the year, through births or weight gain.	US$405	US$403	US$378	US$401

vector avoidance and/or control. In Colombia, trypanosomiasis of domestic animals has been ranked third in economic importance, after ticks and tick-borne diseases and distomatosis. On a regional scale, even the inapparent losses of subclinical infestations could be considerable (Otte *et al*., 1994). The financial impact of *T. vivax* on the Brazilian Pantanal and Bolivian lowlands was recently assessed (Seidl *et al*., 1999). It was estimated that the cost of an outbreak on seven farms in the Poconé region of the Pantanal was equivalent to approximately US$15 per breeding cow (4% of the total value of the animal). If the outbreak had gone untreated the estimated losses would have been nearly US$64 per breeding cow (or 17% of the total value of the animal). In view of the recent introduction of bovine trypanosomiasis to the Pantanal and lowland Bolivia, and the economic losses associated with the disease, investment in improving the diagnosis and control of the disease in Latin America becomes an economic option.

Concluding Remarks

Quantitative data on disease distribution and prevalence of infection of *T. evansi* and *T. vivax* in different livestock species in different production systems is lacking. There is a need for information on the ecology and natural history of non-tsetse-transmitted trypanosomiases to provide greater understanding of the factors that affect transmission and maintenance of infection; the dynamics of mechanical transmission by biting flies is unknown. There is little convincing evidence that wild animals play an important part in the ecology of disease in domesticated livestock. Much information could be acquired using the technologies currently available. This would require application of validated, standardized diagnostic tests at local and regional levels in order to provide an estimation of the national and regional occurrence of *T. evansi* and *T. vivax*. It would then be possible to determine the economic costs of trypanosomiasis on productivity in different production systems and assess the cost effectiveness of chemotherapy. Such strategies would require: continued development of diagnostic tests, particularly simple pen-side tests; identification of the principal vector species responsible for transmission in different ecological situations; determination of the efficacy of different trypanocidal drugs, the extent of drug-resistance and its importance; and critical assessment of the role of wild animals.

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PART 4.
PATHOGENESIS

16 Pathogenesis of Human African Trypanosomiasis

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Introduction

In describing the pathology of African trypanosomiasis it is practical to adhere to the conventional geographical distinction between *Trypanosoma brucei rhodesiense* and *T. b. gambiense* as causing, respectively, the acute and chronic forms of sleeping sickness. However, it is clear that these divisions lie across a continuum of generally similar pathological processes, which in turn reflect a complex range of parasite strains of differing and rapidly adapting forms of virulence. An obvious distinction of the two types is that the Rhodesian forms may be fatal within a few months from damage to the heart or viscera, whereas the Gambian forms may progress for years, with death (if untreated) from additional causes including central nervous system (CNS) damage and intercurrent infections. The unusual neurological manifestations and immune changes of the chronic forms of the disease have attracted much experimental analysis.

The following account requires an initial qualification regarding the balance of the knowledge summarized. The bulk of the mechanistic information concerns studies made on the laboratory model (i.e. mice and rats) infected with *T. b. brucei*, which is non-infective to humans. In the experimental models the parasite strains frequently cause diseases that run rapid courses, with high parasitaemias, compared with the human diseases. These studies have provided much valuable understanding about the disease process, which in many cases appears very relevant to the human infection. However, this chapter attempts to distinguish clearly between the human and relevant animal data, avoiding premature conclusions about the applicability of the latter to humans.

Stages in Disease Pathology

The disease pathogenesis is traditionally divided into two stages. The first or early phase is associated with the establishment of the parasite in the lymphatics, blood and other body systems, excluding the CNS. The early stage is also loosely referred to as the phase of haemolymphatic involvement. During this stage there are no obvious signs of CNS pathology, nor alterations in the cellular composition, protein content or parasites present in the cerebrospinal fluid (CSF). Pathology of the late stage(s) expands when the parasite damages the CNS and this may be diagnosed by increases in the white blood cell (WBC) count, protein levels and the presence of parasites in the CSF (Molyneux *et al*., 1996).

The CNS involvement can commence within a few weeks to several months or, more commonly, years after the infection.

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However, it usually develops insidiously, with a range of neurological signs and symptoms, and mental disturbances that may occur long before there are diagnostically detectable changes in the CSF. Thus there are no clear staging posts in the disease pathogenesis. A major reason for differentiating the two stages lies in the very different drug treatments necessary to effect a cure, which can be difficult once the parasite has entered the CNS. In addition, the pathology in the different body systems of the untreated patient progresses inexorably, with no single cause of death.

Initial Responses and Pathologies in Non-nervous Body Systems

Chancre

At the site of the tsetse bite, where parasites become lodged in the subcutaneous tissues, a hard, painful, red nodule appears in the skin. It arises 4–14 days following the bite and is several centimetres in diameter (usually 2–3 cm, but chancres up to 10 cm have been recorded). In a further 2–3 weeks the chancre is normally resolved, leaving slight scarring. The chancre may be more prevalent in the acute Rhodesian infections. However, the chancre may frequently be disregarded and forgotten by the time the disease has progressed to the more serious stages (Apted, 1970).

Morphologically, the chancre is a localized inflammatory site, with oedema, infiltrations of macrophages and lymphocytes, and multiplying parasites, but without pus as occurs in boils due to many other microbial infections. The parasite spreads from the chancre into the lymphatics and blood system. Parasite multiplication will occur before the host's humoral response is fully effective but at this time antigenic variation will already have been initiated. No detailed studies of the immune responses in the chancre in humans have been undertaken. In experimental laboratory models, inoculation is normally into the blood system via a syringe, but animals will develop a chancre if the inoculation is given into the skin.

Lymph glands

Swelling of the lymph glands occurs as the parasites multiply and spread from the chancre. In the chronic Gambian form the glands of the posterior cervical triangle become very prominent and this important diagnostic feature is known as Winterbottom's sign (Apted, 1970). The germinal centres are expanded with cells of the lymphocyte–plasma cell series, macrophages and general oedema. It is to be noted that the lymph tissue is a preferred site for the parasite, except when a new variant is rapidly multiplying, which occurs predominantly in the blood. This in turn leads to further dissemination away from the lymph tissue, when again the trypanosome seeks out the extracellular spaces in the tissues. In chronic infections, when there may be long periods between peaks of parasitaemia, parasites may be undetectable in peripheral blood. On the other hand the swollen lymph glands become relatively painless and aspiration can be a useful way of isolating trypanosomes and confirming the infection.

Blood

The high metabolic demands of the rapidly dividing new variants of trypanosome which periodically arise during the infections are fulfilled in the blood. During these periods there are major changes in glucose and a range of other substrates and catabolites (Jenkins and Facer, 1985). The waves of parasitaemia are associated with attacks of high fever, which may last from a day to a week, separated by intervals of several days to a month throughout the infection.

The blood composition becomes profoundly disturbed, reflecting the physiological mechanisms associated with the pathological changes in most body systems, together with the attempted repair processes. There is an overall reduction in both erythrocytes and white blood cells. However, during the rapid development of a new variant surface glycoprotein (VSG) determinant B cells are stimulated through both T cell-dependent and T cell-independent pathways. The dramatic increase in immunoglobulins (espe-

cially IgM) succeeds in eliminating the majority of the parasites by immune lysis and their destruction by the Kupffer cells in the liver. The heterologous antigenic variants survive to repopulate the blood and other tissues. When a particular population is eliminated the variant antigenic type (VAT)-specific IgG and IgM levels decline to low concentrations but the levels of the invariant trypanosome antigens remain high. There are also high levels of polyclonal immunoglobulins directed against a large range of host autoantigens, including components of the nervous system such as myelin. Although the responsiveness of B and T cells is generally increased, there is a paradoxical progressive immunosuppression due to impairment of T-cell responses (e.g. helper, suppressor and cytotoxic functions) to the persistent trypanosome antigens (Vincendeau *et al.*, 1999).

In addition to the immunopathology, the blood provides a window on a range of other host–parasite interactions. These include blood coagulation factors, complement, electrolyte balance, potential parasite toxins and other microbial products (including the breakdown products of Gram-negative bacteria), hormones, and cytokine/mediator substances (including nitric oxide products and prostaglandins). Aspects of these, where relevant to the disease pathogenesis, will be described in subsequent sections.

Spleen

The spleen becomes enlarged with the increase relating to the anaemia. The sinusoids become packed with active macrophages (Apted, 1970).

Heart and blood vessels

The heart is considered to be a chief organ at risk, especially in the acute Rhodesian disease forms. In many patients there is evidence of damage from a range of abnormalities, which include tachycardia or bradycardia, cardiac murmurs, myocarditis with congestive heart failure, pericarditis, pericardial effusions and pancarditis (Apted, 1970). Radiologically, the heart commonly appears enlarged and electrocardiographic studies have shown changes that include lengthening of the P–Q segment, flattening or inversion of the T wave, conduction defects and ischaemia.

Another characteristic of the disease in humans and experimental animals is increased capillary fragility and permeability with extensive petechial haemorrhaging. This in turn is associated with a generalized oedema, especially of the extremities and face, with the peculiar sub-orbital puffiness once considered to be a key diagnostic feature. Further analysis in the rodent model has shown that a vasogenic oedema is also present in the CNS in the late disease stages. The cause of the oedema is not known but obvious possibilities are the increased cellular trafficking (chiefly macrophages) across the capillary endothelium, substances released by the parasite (e.g. proteases) or a range of substances released from tissues by the parasite, including vasoactive amines and peptides, cytokines and nitric oxide (Boreham, 1985; Pentreath, 1999). Tissue barrier damage is also present in the kidney glomeruli, allowing the pathological loss of a number of substances from serum.

Skin

Skin rash is a common feature in fair-skinned patients. It is most commonly observed in the shoulders, trunk and thighs, appearing as oval, erythematous areas about 10 cm in diameter with clear centres. The rash is accentuated by heat, sweating or cold, does not itch, and is not obviously correlated with other disease features such as the fever episodes. It has been considered a cardinal diagnostic symptom of infected Europeans (Apted, 1970).

Pruritus is another common peripheral disorder, occurring in about 50% of the cases. However, it should be borne in mind that this is common to a wide range of infections and disorders where foreign substances or altered levels of endogenous mediator chemicals may affect sensory nerve endings.

Skeletal muscle

A generalized skeletal muscle atrophy with patchy cellular infiltration, with such alterations quite obvious in the diaphragm, has been noted. A dramatic characteristic that occurs in a small proportion of patients is a sensation of deep hyperaesthesia, with the pain that shortly follows even a mild blow out of all proportion to the force of contact. This phenomenon was described by Kérandel early in the 20th century and subsequently became known as Kérandel's sign (Apted, 1970). The underlying mechanisms are not known but again are likely to be associated with the altered chemical milieu around the sensory nerve endings.

Eyes

There are several descriptions of eye disease associated with trypanosomiasis. These include conjunctivitis, keratitis, iridocyclitis, and choroidal atrophy (Duggan and Hutchington, 1966; Apted, 1970). The problem here is that it is not clear whether the changes are due to the parasite infection or to other causes, such as localized infections resulting from generalized patient immunosuppression.

Intestinal damage

Although the early pathological reports contained no mention of gross intestinal abnormalities, the presence of elevated levels of circulating endotoxins, originating from Gram-negative bacteria in both patients and animals, prompted studies in the laboratory model with *T. b. brucei*. The infection causes some marked structural changes, with reductions in villous height, wall thickness and oedema, particularly in the jejunum (Nyakundi and Pentreath, 1999). The changes are associated with increased intestinal leakage, measured by urine recoveries of orally administered inert sugar probes lactulose and mannitol, which in turn correlate with high serum endotoxins.

Hormone and Endocrine Changes

Endocrine dysfunction occurs in a high percentage of patients. In women the changes are manifested by menstrual disorders, with arrest in the advanced stages, sterility and susceptibility to abortion or premature births, with stillbirths and perinatal deaths. These changes are associated with uterine hypoplasia and atrophy of the sexual organs. In men the alterations commonly include impotence and testicular atrophy, with in the late disease stages a state of gynaecomastia, where the fat tissue adopts a feminine distribution, and this, together with the generalized oedema, may give the superficial appearance of a well-nourished person (Apted, 1970).

These dysfunctions are the manifestations of some profound underlying changes in hormonal functions. At a gross level the adrenals and thyroid become infiltrated and progressively atrophy. Some of the changes in the later disease stages may be initiated by dysfunctions at the level of the circadian pacemaker systems. Several types of interaction are known to occur normally between endocrine rhythms and the sleep–wake cycle. Some hormones, such as prolactin, have sleep-dependent secretory patterns, even when sleep patterns are altered in disease states. Substances such as growth hormone and plasma rennin are tightly coupled to the internal sleep structure, whereas other hormones such as cortisol are relatively independent of the sleep–wake rhythm generator. Measurements of the substances in sleeping sickness patients has shown that, despite the disorganization of the sleep pattern (with, for example, daytime somnolence), the relationship between the hormonal pulses and the sleep phases was preserved. Thus the slow-wave sleep (SWS) episodes remained associated with increases in cortisol and growth hormone; and rapid eye movement (REM) sleep associated with the pineal hormone, which can modify the circadian timing system, was unaltered (Radomski and Brandenberger, 1999). These studies demonstrate that some of the endocrine pathologies of the disease, especially in the advanced state, have their

origins in dysfunctions of the circadian pacemaker. The bases for alterations in rhythms at the level of the suprachiasmatic nuclei of the hypothalamus in the rodent model are described in a later section.

Immunopathology in Laboratory Animals and Humans

Most of the information on changes in the immune response and their possible contributions to the pathogenesis have been obtained in the animal models infected with *T. b. brucei*. It must be borne in mind that human serum contains high-density lipoprotein trypanolytic factor against this parasite strain (Hajduk *et al.*, 1994), but not in most populations against the Gambian or Rhodesian forms (a notable exception here is the pygmy population of the Congo basin, whose serum possesses a trypanolytic effect again *T. b. gambiense*). Thus any extrapolation of laboratory animal data to humans must be carefully qualified. Also, consideration must be given to the difficulty in distinguishing potentially protective responses from those that may be actually damaging. This ideally requires understanding of the particular facet of the immunological change being studied in relation to the whole immune response. For example, what may appear a pathological secretion of a particular cytokine from an isolated tissue in the culture dish may, in the intact animal, be beneficial for a distant target; ideally a full knowledge of all the downstream effects of that cytokine is necessary before a decision is made about its outcome. Also to be taken into account is the great range of cellular sources and multiple, sometimes overlapping, activities of the cytokines in different tissues. In the following sections emphasis is given to those areas where there is evidence for a role in the immunopathogenesis. Some other aspects of cytokines in the late stage neuropathology are described under that heading.

The coevolution of the sleeping sickness trypanosomes with their hosts has led to strategies by the parasite for avoiding and altering the host immune responses. The period during which the host may act as reservoir for further transmissions is thus extended. The trypanosomes' major strategy is the sequential expression of antigenically distinct VSGs. The different VSGs evoke major changes in the B- and T-cell populations and immunoglobulin profile, which have been summarized in the earlier sections on blood changes. The VSGs also affect the altered production of cytokines. A range of autoantibodies against host tissues are also produced. Other trypanosome components and bacterial products (see final section) also modulate the immune response. The disease progresses to a profound state of immunosuppression, with reports of a range of other concurrent infections including bacterial, intestinal, viral and other protozoal diseases. However, there is no evidence that any of the different types may have a particular association with trypanosomiasis. Instead their incidences follow the prevalences of the particular geographical area of the sleeping sickness patients. This generality, which extends to HIV, is in itself an interesting disease feature in relation to the profound late-stage immunosuppression (Okia *et al.*, 1994).

Autoantibodies

Autoantibodies have been found in a number of experimental animal studies and in infected humans. The autoantigens include components of blood cells, smooth muscle cells, cardiolipids, liver cells, intermediate muscle filaments, rheumatoid factors, components of CNS myelin (the galactocerebroside fractions and myelin basic protein), nucleic acids (both DNA and RNA), the essential amino acid and serotonin precursor tryptophan, and components of neuronal cytoskeleton filament proteins. The wide range and generality of autoantibody production in many different types of animal and in humans indicate that this is a common feature contributing to the pathology. The different types of autoantibody could prove useful in the diagnosis of the disease stage, with increasing CNS components characteristic of the progression of the CNS

(late-stage) involvement. Their production may be induced by a non-specific activation of B cells, especially a subpopulation which, in humans, expresses high levels of CD5 and which may have an intrinsic capacity for natural autoantibody production. They may also be produced generally by B cells in response to trypanosome components that mimic the host tissues – for example, some of the filament components that are common to both flagellar and neurofilament proteins (Amevigbe *et al.*, 1992; Vincendeau *et al.*, 1999).

Immune complexes

In some studies of animal and human tissues, deposits of immunoglobulins have been noted. These immune complexes may comprise both the anti-VSG antibodies and autoantibodies. However, these complexes can activate complement and such activation may be involved in some pathological effects, in particular the thrombosis and localized kidney damage, where the complement-fixed immune complexes may be phagocytosed by the glomerular endothelial cells, leading to infection-related glomerulopathy. It should also be noted that the complexes are not consistently found in either the human patient or in animal studies (Lambert *et al.*, 1981).

Cytokines, mediators, cells and immunosuppression

A substantial number of studies have been directed at different facets of the immune response and their possible contributions to the immune dysfunctions. It should be noted again that most of this work has been carried out in laboratory models, which include a range of inbred, transgenic and knock-out animals, or cattle, with only a small number of studies on the human patient.

In the current field of immunobiology several hundred substances have been identified which participate in the 'cytokine network', in addition to an even greater number of active products which together control the responses of an organism to microbial and parasitic infections. It is also now generally accepted that progressive or prolonged production of some of these substances may subvert certain cellular processes, which were originally beneficial, to become contributory to the pathology within the host. Here again, emphasis is given to the great difficulty in distinguishing the two aspects. In African trypanosomiasis some trends are emerging concerning the immunopathology. In this section the relevant information on the cytokine/mediator and cellular changes are grouped together because of their interdependent and often inseparable roles in the disease pathogenesis.

The immunosuppression is associated with the polyclonal B-cell activation and with the generation of suppressor macrophages and suppressor T cells. Despite the large increases in B cells and plasma cells with hypergammaglobulinaemia, specific antibody responses to successive waves of trypanosome antigens, whilst still capable of controlling successive parasitaemias, become reduced. This suppression of antibody response extends to a wide range of other protozoal and/or microbial infections that may challenge the infected host. In studies with animal models the immunosuppression is caused by the generation of suppressor macrophages and T cells in association with the polyclonal B-cell activation (Vincendeau *et al.*, 1999).

The immunosuppression can be transferred to the spleens of uninfected mice via a relatively small number of peritoneal or splenic macrophages, which in many respects have the characteristics of activated macrophages. The cells are subject to complex changes in phenotype with alterations in receptors, including the expression of major histocompatibility complex (MHC) class II molecules. The altered expression varies with the strain of trypanosome, but can be associated with inadequate presentation of trypanosome antigens (Ia antigen presentation). The cells secrete an altered profile of prostaglandins, with excessive production of prostaglandin E_2 (PGE_2), and release increased levels of interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α). The TNF-α production induced by the

parasite and other products associated the infections, including elevated endotoxins, is beneficial because of its trypanostatic effects, but in the late disease stages its prolonged presence correlates with the disease severity (e.g. anaemia and cachexia) in both animals and humans (Rhind and Shek, 1999). Indeed, as a note of historical interest, TNF-α (then cachectin) was first isolated from rabbits infected with *T. b. brucei* which were in a severe state of anaemia and emaciation (cachexia; Cerami and Beutler, 1988). Another active product released from macrophages is nitric oxide (NO), important in host defence and parasite killing locally and at a distance via the generation of nitrosylated compounds. Elevated nitrate concentrations have been observed in *T. b. rhodesiense*-infected patients and this is likely due to the increased production of NO. Its overproduction may also initiate generalized tissue damage, especially to the vasculature.

T-cell activities are also centrally involved in the immunosuppression. The different T-cell subsets secrete a range of cytokine profiles, which include interferon-γ (IFN-γ), TGF-β, IL-2, IL-4, IL-6, IL-10 and IL-12. IFN-γ is a potent activator of macrophages. One of the fascinating features demonstrated for *T. b. brucei* is that it can utilize some host substances, notably IFN-γ and epidermal growth factor, to promote its own growth. To do this the production of IFN-γ by $CD8^+$ T cells (cytotoxic T cells) is triggered by a factor released from the parasite, termed trypanosome-derived lymphocyte-triggering factor (TLTF) (Eneroth *et al.*, 1992; Bakhiet, 1993). TLTF also induces TGF-β release from $CD8^+$ T cells, which has immunosuppressive effects. The T-cell proliferative response to trypanosomal and other heterologous antigens becomes severely attenuated. This is caused, in part, by macrophage-derived substances (especially prostaglandins and NO) and IFN-γ from $CD8^+$ T cells, which suppresses IL-2 receptor expression on both the $CD8^+$ and $CD4^+$ (T helper) cells. Another contributing factor to the reduced T-cell response is the depression of IL-2 production by macrophages, which in turn may correlate with the high levels of 1L-1 (Vincendeau *et al.*, 1999).

Other accumulating evidence indicates that the immune changes associated with progression to the chronic disease state include a tendency towards the expression of the Th2 subset of $CD4^+$ T cells, whose cytokines include IL-4, IL-5, IL-6, IL-10 and IL-13. In relation to this, IL-4 may be involved in controlling parasitaemia by influencing immunoglobulin synthesis, but may be toxic in excess, while IL-10 may be deleterious if its levels are high enough to downregulate TNF-α and hence its trypanostatic effects. In addition, studies have shown that several of the group of cytokines termed chemokines, which direct the trafficking of different WBC subsets during inflammation in a range of cell types, including endothelial cells and CNS glial cells, are up-regulated in laboratory animals infected with *T. b. brucei* (Sharafeldin *et al.*, 2000).

Thus the immunopathology results from the evasion of antibody-mediated destruction by VSG switching with the suppression of immune responsiveness by processes that are expressed by multiple alterations in lymphocyte activities and the cytokine network. The emerging generalities from the animal models are of great importance, but further studies are necessary to determine whether they are fully applicable to the human patient.

The Nervous System and Neuropathology

Neurological features in humans

Human African trypanosomasis is associated with progressive neurological disturbances. These include a wide range of mental, motor, sensory and circadian pattern (e.g. sleep) disturbances. The changes are progressive, most obvious in the late stages, but in the very great spectrum of alterations that embrace the disease they can be quite early events (Apted, 1970; Atouguia and Kennedy, 2000).

Characteristic mental signs and symptoms are irritability, loss of ability to concentrate, subtle changes in personality and behaviour, indifference, lassitude and anxiety. Later violent mood swings, uncontrolled sexual impulses, delirium, hallucinations, suicidal

tendencies and manic episodes can occur. A substantial literature, often anecdotal, and descriptive of a very great number of cognitive and emotional states, sometimes quite bizarre, has documented the alterations.

The motor involvement is manifested by tremors, often in the fingers and tongue, muscular fasciculations (frequently in the muscles of face, neck, arms and upper trunk) and increasing tonicity, rigidity and paralysis of groups of skeletal muscles throughout the body. Cerebellar ataxia leads to difficulty with walking, and speech becomes indistinct and slurred.

Abnormal reflexes occur and may be used in confirmation of late-stage infection. The noted alterations occur in the pout reflex and palmo-mental reflex. Occasional abnormalities in the Babinski reflex have also been documented.

The sensory changes may include hyperaesthesia, paraesthesia, anaesthesia and intensive, generalized pruritus. Again such symptoms have been considered positive components of late-stage diagnosis in a number of reports.

Sleep disturbances are a cardinal feature of the Gambian disease. Reduced alertness, attention loss and distractibility may be interrupted by narcoleptic crises, with the patient falling prostrate in any situation. The sleep pattern becomes deranged, with daytime somnolence and night-time restlessness. In the late stages the classical picture of almost continuous sleep, which becomes indistinguishable from coma, proceeds to death in the untreated individual. Sleep disturbances also accompany the Rhodesian infections but do not normally become so prominent.

In addition, the disease is recognized for the unusual neurological complications which may be precipitated by the chemotherapeutic regimes employed to attempt cure of the late-stage disease. These are usually associated with administration of the arsenical drug melarsoprol but can occur with other drugs such as diminazine aceturate. The response is commonly known as reactive arsenical encephalopathy and is fatal in about 10% of treated cases (see later).

Study of neuropathology in humans and animals

The complex range of neurological changes with such unusual features has prompted great interest in attempting to explain how the parasite might cause them. Many aspects of the changes bear obvious similarities with some other diseases and syndromes that affect the CNS.

At the onset it is again important to make note of some qualifications about studies of the neuropathogenesis. Firstly, there is the great difficulty in accurately staging the disease according to parasite location. The occurrence of parasites in the CSF is not always a feature of what may otherwise be an obvious late-stage or even terminal chronic Gambian infection. Many of the neurological features can occur very early in the infection of both types of human infection. Secondly, the majority of recent studies have been made on laboratory and other animal models, for which many of the neurological features summarized above are inapplicable.

Nevertheless a start has been made in unravelling the complex neuropathology. In animal models this has revealed some novel, basic nerve mechanisms that may have general application far beyond the disease itself. The information can be described quite appropriately under sections that cover the human patient, the animal models and the post-treatment reactive changes.

Alterations in the human central nervous system

The studies in humans consist of those using non-invasive procedures by computerized tomography (CT), magnetic resonance imaging (MRI) or electroencephalography (EEG), and others involving the macroscopic and microscopic examinations of autopsied CNS tissues from deceased patients. Despite the great difficulties in undertaking or obtaining material for such studies, they have clearly established several of the basic CNS changes.

Neuroradiology and electroencephalography

Although the levels of resolution of CT and MRI images are insufficient to provide information about, for example, trypanosome invasion, the CT scans have shown some heterogeneous areas of reduced density in the centrum semiovale, cerebral oedema and demyelination. MRI has demonstrated lesions of the basal nuclei, brainstem and white matter, sometimes indicative of leucoencephalitis. Because the abnormalities resolve after treatment, it has been suggested that the scans could be useful in assessment of post-treatment improvement (Sabbah *et al.*, 1997).

EEG and polysomnographic recordings have shown that sleeping sickness in both Gambian- and Rhodesian-infected patients involves major alterations in the circadian timing systems, rather than a pathological hypersomnia. Different forms of unusual EEG patterns have been observed, from which some features are fairly consistent. In the late-stage patients the sleep–wake alternations tend to occur in short cycles, with the shortest cycles in the most severely affected patients, but without overall alterations in the total sleep times. Thus the latencies of and between SWS and REM phases become progressively shortened. Patients show marked slow-wave anomalies, with diffuse theta and delta waves occurring in short bursts at all stages of the sleep–wake cycle. The stages intermittent between REM and SWS (i.e. stages 1–4) become difficult to distinguish. Higher-frequency sleep spindles are reduced. Most patients have normal evoked potentials (Hamon *et al.*, 1993; Buguet and Cespuglio, 1999; Tabaraud and Tapie, 1999).

The changes strongly indicate that a major component of the CNS damage involves the circadian timing system. In accordance with this there are major alterations in other biological rhythms, such as temperature, cortisol, prolactin and other hormonal secretions, which have been described above. It must also be noted that the alterations are not pathognomonic for sleeping sickness. Almost identical EEG changes have been observed in a range of other chronic neurological conditions, such as bacterial and viral encephalitis and AIDS.

Morphological changes

Several relatively gross studies of autopsies of Gambian patients in the mid and late 19th century culminated in the detailed studies of Mott (1906). The histopathology consisted of a meningeal and generalized perivascular inflammation within the CNS. The inflammatory infiltrations consisted chiefly of lymphocytes and plasma cells. Mott also described a general distribution of morular cells in the perivascular cuffs, parenchyma, meninges and other body tissues. These large cells are swollen with intracellular accumulations of immunoglobulins of the IgM type. They also occur in other chronic infections that effect the CNS, such as syphilis, but a distinguishing feature of sleeping sickness is their occurrence in relatively large numbers. As a consequence of this, and in recognition of Mott's pioneering work, they have become known in trypanosomiasis as the morular cells of Mott, or Mott cells. Many of the IgM immunoglobulins induced during African trypanosomiasis are directed against autoantigens, including nervous tissue elements such as cerebrosides, gangliosides and myelin basic proteins, in addition to those of the parasite. Such autoantibodies, as well as immune complexes, can be detected in the CSF of many patients with either chronic Gambian or Rhodesian infections, though their possible roles in the pathogenesis are not clear (Lambert *et al.*, 1981; Hunter and Kennedy, 1992).

The microscopic studies continued through the early decades of the 20th century and were extended to the Rhodesian disease form. These repeatedly confirmed the first findings and added further information, some of which seemed to apply to individual cases, but from which the following more general and basic changes apply. The inflammatory alterations appear most obvious in the white matter, occurring in the cerebral hemispheres, but also (according to a number of reports) most obviously in subcortical regions, including the periventricular regions, thalamus, hypothalamus and supraoptic nuclei. With the possible exceptions of some periventricular regions, the

myelin is not disrupted. Evidence of capillary fragility with haemorrhages is given by the occurrence of hemosiderin-filled macrophages in the cuffs around the blood vessels. Damage to choroid plexi and periventricular walls is indicated by thickenings and vascular lesions.

Throughout the brain there is activation of the astrocytes and microglia. However, surprising characteristics of the autopsied material were that the neurons appeared undamaged, with parasites almost entirely absent from the parenchyma.

Many of these general features extended to the spinal cord and roots. Thus the overall picture of the CNS histopathology of the deceased sleeping sickness individual falls, somewhat loosely, into a generalized leucoencephalopathy with predilection for periventricular and basal brain regions. Moreover, these widespread changes do not provide any obvious explanation for the clinical symptoms of the disease (Pentreath, 1995; Kristensson and Bentivoglio, 1999).

The composition of CSF and CNS damage

The CSF forms a reservoir into which a range of substances, including cytokines and mediator substances, may be exchanged between the blood (for example, at the choroid), cells of the nervous tissue and other tissues lining the neuraxis. In pathological states the substances can shed light on the damage and immune changes within the CNS. The details of the exchange of materials with the parenchyma are complex, and in sleeping sickness the likely damage to the choroids, blood–brain barrier and meninges could contribute significantly to its altered composition. The significance of the changes with respect to events in the neurons and glial cells thus becomes difficult to interpret. The presence of parasites in the CSF has traditionally been used as one of the cardinal criteria for diagnosis of late-stage disease, though the WBC count may itself be sufficient to confirm this in the Gambian forms (Miezan *et al.*, 1998). Other evidence has shown that the CSF of late-stage patients is not conducive to parasite survival (Pentreath, 1999).

The CSF of late-stage patients may contain a range of immunoglobulins, immune complexes, light subunit neurofilament and glial fibrillary acidic protein (GFAP), which can be general markers of inflammatory CNS damage with an astrocycte involvement. The fluid has also been shown to contain some very high levels of the potent somnogenic substance prostaglandin D_2 (PGD_2), together with some elevations in the PGE_2 and $PGF_{2\alpha}$, but not IL-1 (Pentreath *et al.*, 1990). The prostaglandins may be derived from astrocytes, meningeal cells or other cells in contact with the CSF, including the infiltrating lymphocytes and macrophages, or the trypanosomes themselves (see final section).

Thus the altered composition of the CSF in the late-stage patient provides evidence of multiple pathological events. However, the cellular origins and exact roles of the substances in the neuropathogenesis are not yet understood.

Animal studies

Different animal species have been studied extensively in order to shed light on the mechanisms underlying the neurological changes in humans. These include monkeys, sheep, goats, dogs, rabbits and a very wide range of different strains or genetically modified laboratory animals. The infections have been made using the human pathogenic trypanosomes *T. b. rhodesiense* and *T. b. gambiense* but more commonly the rodent pathogenic trypanosome *T. b. brucei*. In these studies non-virulent trypanosome strains have frequently been selected in order to produce chronic infections that mimic the human infections. These commonly extend for several months, in a limited number of model situations for over a year, and each has its own particular features in terms of the general pathology and modifications of animal behaviour.

Overall the works have reaffirmed the histopathological changes described in humans. The meningoencephalitis is manifested by infiltrations of lymphocytes and plasma cells in the leptomeninges and perivascularly in the brain parenchyma. Astrocyte and microglial activation become

prominent. Morular (Mott) cells have occasionally been observed. The choroid plexus is an obvious site of damage, frequently with high concentrations of trypanosomes. The choroid may contain deposits of immune complexes. Widespread invasion of the parenchyma by the parasite does not occur. Neuronal or myelin damage is generally absent, although autoantibodies directed against CNS tissue elements such as cerebrosides, gangliosides and myelin basic proteins can be detected in the blood of infected animals. On the basis of the distribution of the lesions in monkeys, parasite access to the CNS has been suggested to occur via the damaged choroid and CSF, or via the Virchow–Robin spaces (Schmidt, 1983).

During the last decade many of the studies in laboratory rats and mice have employed a number of relatively sophisticated cell and molecular biological procedures to analyse the pathology. The distribution of parasites in infected rats, studied by immunohistochemical mapping, has shown that they invade the dorsal root ganglia and some parts of the CNS where, in health, the blood–brain barrier is reduced, allowing the transcapillary movement of quite large molecules, such as peptides and some proteins. These areas, collectively called the circumventricular organs, include the subfornical organ, the organum vasculosum of the lamina terminalis, the median eminence, the subcommissural organ, the pineal gland, the neural lobe of the pituitary gland and the area postrema of the brain stem. The same regions of the nervous system become infiltrated with lymphocytes, predominantly of the $CD8^+$ phenotype, and macrophage-like cells which were immunopositive for MHC class II antigen (Schultzberg *et al.*, 1989). Although there is no obvious damage to neurons or myelin, groups of neuronal perikarya in the supraoptic and paraventricular nuclei of the hypothalamus were induced during infections to express MHC class I antigens. Normally such expression is confined to the choroids, meningeal and vascular linings of the brain. Because both of the brain regions involved project to the median eminence, where parasites and inflammatory cells infiltrate the tissue, the altered MHC expressions in the perikarya may be triggered by retrograde axonal signals (Kristensson and Bentivoglio, 1999).

Because of the central role of the suprachiasmatic nuclei (SCN) of the hypothalamus in the generation and control of circadian rhythms, including hormonal secretions and sleep, in relation to the light–dark cycle, several detailed aspects of its pathophysiology have been studied in rats. The normal diurnal oscillations of the immediate early gene *c-fos* become dysregulated in the SCN neurons, and their normal rhythms in endogenous electrical activity also become markedly altered. The SCN neurons receive some of their input from retinal fibres via the retinohypothalamic tract. The transmitter released from the retinal projections, which signals the light–dark changes, is glutamate. In chronically infected rats the expression of glutamate receptors on the SCN neurons becomes significantly reduced. Other studies have shown that pharmacological blockade of the SCN glutamate receptors leads to disruption of the normal responses to the light–dark cycle, with interference of *c-fos* induction. In addition, generation by the SCN of the normal rhythm of melatonin secretion by the pineal is upset, with alterations in the binding of melatonin to its receptors in the SCN. Thus the information demonstrates that, amongst the many changes caused by the parasite, there is extensive disruption of the complex events that control the SCN oscillatory activity and its circadian timing capacity (Bentivoglio *et al.*, 1994; Kristensson *et al.*, 1998; Kristensson and Bentivoglio, 1999).

Although brain parts where the blood–brain barrier is reduced may be especially susceptible, increases in capillary permeability similar to those that occur in most body tissues during trypanosomiasis also take place in the CNS. Chronic *T. b. brucei* infection in rats causes a generalized increase in permeability throughout the grey and white matter of most brain areas, as evidenced by influx of the fluorescent fluid-phase marker sulphorhodamine B (molecular weight 584). This is accompanied by a vasogenic oedema (Pentreath, 1999).

Astrocyte and microglial activation during the disease has been the subject of a number of laboratory model and *in vitro* studies. The responses of both cell types can take place within a few weeks, even in low-virulence parasite strains which produce chronic infections of several months. The activation of astrocytes occurs throughout the CNS, with a prevalence in the white matter, but the microglial response is most evident in periventricular and subpial regions, especially in the hypothalamus. Late disease stages are accompanied by a general invasion of macrophages (Adams *et al.*, 1986; Hunter *et al.*, 1992; Chianella *et al.*, 1999).

Much of the importance attached to glial cell activation lies in their induced capacity to synthesize and secrete a wide range of cytokines and mediator substances. Whilst such substances play key roles in coordinating protective responses and promoting the orderly repair of CNS tissue after injury, evidence also suggests that chronic exposure to high levels can lead to neurodegeneration. RNA transcripts for TNF-α, IL-1 and IL-4, but not IL-6, in astrocytes can occur within a few weeks in the chronic rodent model (Hunter *et al.*, 1992). Another study has demonstrated a chronic overexpression of mRNAs for TNF-α and IL-1_{β} commencing in the choroid plexus and circumventricular organs and then spreading throughout the brains of infected rats. The mRNAs for IFN-γ, IL-6 and inducible cyclooxygenase showed more restricted patterns of induction. In addition, there was a regionally selective apoptosis of scattered small cells and degeneration of nerve fibres and clusters of non-neuronal cells thought to be astrocytes, with the patterns of degeneration spatially related to the cytokine mRNA patterns (Quan *et al.*, 1999). This study is therefore one of the first to provide evidence of some degeneration within the CNS which may be directly associated with overproduction of cytokines. Several other potentially damaging, pro-inflammatory chemokines, including macrophage inhibitory proteins MIP-1α, MIP-2 and RANTES, are also produced by astrocytes and microglia, and later by macrophages and T cells in *T. b. brucei* infections in rats (Liu *et al.*, 1999).

Other works have analysed the levels of neurotransmitters, cytokines and mediators present in the brain tissue, choroids and CSF of infected rats and mice. Most of these do not provide evidence of cellular distribution. The CSF, in particular, acts as a large reservoir which can reflect production of substances in the parenchyma, but also that of the blood, especially if the choroid plexuses and blood–brain barrier have been damaged. It should also be noted that the composition of the CSF, in health, is not a particularly satisfactory environment for supporting the survival of *T. b. brucei* (Pentreath, 1999). However, the studies have indicated an increased turnover of several monoamine transmitters, including dopamine, noradrenaline and 5-hydroxytryptamine in several brain regions (Amole *et al.*, 1989). They have also further implicated IFN-γ, TNF-α, TGF-β, MIP-1, GM-CSF, prostaglandins ($PGF_{2\alpha}$, PGE_2 and especially PGD_2) and NO, amongst others, in the inflammatory pathogenesis which involves astrocytes, microglia, macrophages, activated T cells and other cell types present in the brains of the chronically infected rat. Some aspects of these substances are interesting because they may shed light on certain of the neurological features of the disease.

IFN-γ, which stimulates parasite growth and is released from lymphocytes by TLTF, is also expressed and released by TLTF from dorsal root ganglia neurons. This has led to studies of the possible role of IFN-γ in pain-related behaviour. Some IFN-γ receptors are localized on neurons in the spinal cord to where the sensory neurons project, and which also have the capacity to produce NO. Experiments with intrathecal injections of IFN-γ into rats and genetically modified mice with inactive IFN-γ receptors, together with manipulation of the NO synthetic pathway, have provided the interesting hypothesis that the deep pain sensations and hyperalgesia associated with sleeping sickness may be due in part to the parasite-induced release of IFN-γ in the spinal cord, which activates IFN-γ receptors to initiate NO production (Kristensson and Bentivoglio, 1999).

Overproduction of NO in the CNS has been implicated in a range of pathologies,

including the lesions associated with ischaemia, neuropathic pain-related disorders and also trypanosomiasis. In experimental infections of rats and mice, NO production in the sensorimotor cortex has been measured fairly precisely by pulse voltammetry. The levels of NO increase in parallel with disease development, thus raising the likelihood that this substance is involved in many components of CNS damage, including disturbances in the circadian rhythm (Buguet and Cespuglio, 1999).

The studies have been extended to *in vitro* systems. *T. b. brucei* causes expression of an inducible NO synthase (iNOS) in co-cultures of murine astrocytes and microglia. This is associated with NO release into the medium. IFN-γ strongly enhanced the NO production. The stimulation of iNOS activity required parasite contact with the cells and probably occurred at the transcriptional level (Girard *et al.*, 2000). Disrupted *T. b. brucei* products also activate production of prostaglandins, especially PGD_2 and PGE_2 by cultured mouse astrocytes. The production is amplified by bacterial endotoxin (Alafiatayo *et al.*, 1994). Moreover, it has been demonstrated that in organotypic slice cultures of rat CNS, *T. b. brucei* may penetrate the nervous tissue, actually occurring within the cytoplasm of astrocytes. It is not clear whether this is due to active penetration by the parasite, or the phagocytotic activity of the glial cells, or both. The presence of the parasites in the nervous tissue did not appear to be correlated with any significant alterations to the structure or electrophysiological properties of the slice tissue (Stoppini *et al.*, 2000).

Thus the pathogenesis in the CNS of animals and humans with chronic trypanosome infections has multiple underlying components. There are both generalized and more localized changes which may be associated with the different neurological manifestations. Some of the mechanisms and molecular interactions causing the changes are starting to be unravelled, and these in turn are progressing the understanding of neuroimmune mechanisms and the roles of glial cells. However, much of the neuropathogenesis remains a mystery.

Post-treatment reactive encephalopathy

The drug treatment of late-stage sleeping sickness in which the CNS has been invaded with parasites may be followed by a severe post-treatment reactive encephalopathy (PTRE) and be associated with a mortality rate as high as 50%. PTRE usually follows treatment with the drug melarsoprol (which crosses the blood–brain barrier) and has also been termed reactive arsenical encephalopathy. The neuropathological features of PTRE are very similar to those seen in uncomplicated CNS sleeping sickness, already described, namely an acute meningoencephalitis with perivascular cuffing and infiltration with macrophages, lymphocytes and plasma cells (Adams *et al.*, 1986). Activated astrocytes and microglial cells are also prominent. In its extreme form PTRE may show the features of acute haemorrhagic leucoencephalopathy. Despite its considerable importance, the pathogenesis of PTRE is not well understood, though a number of possible mechanisms has been suggested. The latter include sub-curative chemotherapy (for which there is some evidence based on molecular analyses of autopsy tissue), immune complex deposition in serum and CSF, a type of autoimmune mechanism, abnormal immune responses following chemotherapy which are directed against glial cell-attached antigens released from killed trypanosomes, and arsenical toxicity per se (Lambert *et al.*, 1981; Kennedy, 1999). It should be appreciated that these various possibilities are not mutually exclusive. The possible role of corticosteroid therapy in preventing PTRE is controversial (Atouguia and Kennedy, 2000).

Animal models of PTRE have been of considerable value in providing clues as to the aetiology of this condition. However, as already indicated, a degree of caution should be exercised in extrapolating the findings in animals to the human disease. A particularly useful mouse model mirrors many of the neuropathological features of human PTRE (reviewed in Kennedy, 1999). Intraperitoneal injection of mice with cloned stabilates of *T. b. brucei* leads to a chronic trypanosomal infection with establishment of

parasites within the CNS by day 21 following infection, and a meningoencephalitis that is very similar to human PTRE then develops in the late stage of infection. Administration of the drug diminazene aceturate, which clears the parasites from the extravascular compartments but not the CNS, exacerbates the severity of the meningoencephalitis. A similar reaction can be induced by treating the mice with sub-curative doses of drugs such as melarsoprol that cross the blood–brain barrier. The mice relapse with parasitaemia and eventually die.

The important role of the astrocyte and the secretion of a number of cytokines in the generation of PTRE in this model has been shown in several studies over the last decade (summarized by Kennedy, 1999). Activation of astrocytes occurs at days 14–21 after infection, which is before obvious CNS inflammatory lesions can be detected. Moreover, several cytokine transcripts are produced at the time of astrocyte activation, including TNF-α, IL-1, IL-4 and MIP-1. It is likely, but not established, that activated astrocytes secrete at least some of these cytokines. A number of autoantibodies to CNS antigens (myelin basic protein, galactocerebroside and gangliosides) have been detected in these animals and recent work has identified increases in acute-phase proteins such as serum amyloid P and haptoglobin in the CNS after induction of PTRE (Eckersall *et al*., 2002).

Experimental modulation of the meningoencephalitis seen in PTRE has been possible in several ways (Kennedy, 1999). The immunosuppressant drug azathioprine has been shown to prevent the development of PTRE but does not ameliorate the severity of an established PTRE. Somewhat surprisingly, the drug does not have a significant effect on the degree of astrocyte activation. The drug eflornithine (DFMO), which inhibits the enzyme ornithine decarboxylase, both prevents the development, and ameliorates the severity, of the meningoencephalitis associated with PTRE (Jennings *et al*., 1997) (Fig. 16.1). It also reduces the degree of astrocyte activation. Eflornithine's mode of action in producing these effects appears to be related, at least in part, to its effect on pathways of polyamine biosynthesis, and is unlikely to be mediated by immunosuppression. Recent work using a non-peptide Substance P (SP) antagonist has implicated an important role for this 11-amino-acid neuropeptide in the generation of the PTRE-associated inflammatory response. The SP antagonist RP-67,580, which binds specifically to NK-1 receptors, significantly ameliorated the severity of the meningoencephalitic response and also the amount of astrocyte activation in an established PTRE (Kennedy, 1999). There is no definite evidence, however, that this antagonist can significantly prevent the development of PTRE. The detailed mechanism of SP-induced meningoencephalitis in this mouse model has yet to be elucidated in detail. It is to be hoped that one or more of these disease-modifying agents, possibly as adjunct therapy, may eventually prove useful in treating human PTRE.

Substances Initiating the Pathogenesis

Important questions concern the nature of the substances that are responsible for triggering the multiple events in the pathogenesis of sleeping sickness. The intact, living parasites insinuate themselves into the extracellular spaces of most host tissues but these movements by the active trypanosome do not appear to cause the pathological responses. Instead, several other causative processes take place. These can be grouped into: (i) the breakdown products or remnants of dead or inactivated trypanosomes; (ii) the release of substances by the active, living forms; and (iii) substances produced within the host as a consequence of parasite presence, which may in turn contribute to the host's immune response. In practice, many components of these different processes may act synergistically and be extremely difficult to separate experimentally.

Dead trypanosome products include complex membrane components such as the variable antigenic coat, proteases (especially cysteine), free saturated fatty acids and phospholipases. These have been reviewed in detail by Tizard *et al.* (1978). Trypanosome VSG proteins are linked to the parasite membrane by a glycosylphosphatidylinositol

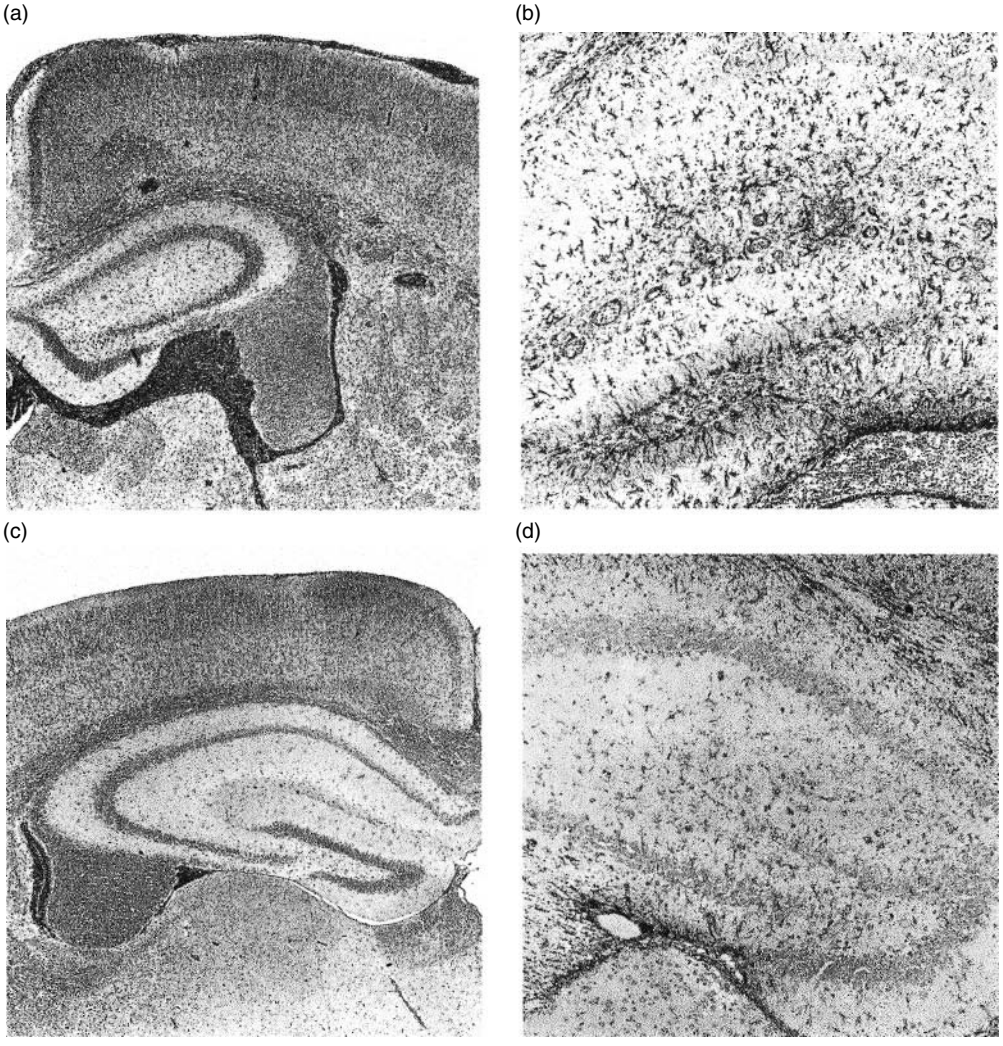

Fig. 16.1. Inflammatory responses in the mouse model of PTRE. Coronal sections through the hippocampal regions of trypanosome-infected mice after different treatment regimes. (a), (b) Sections from a mouse treated with diminazene aceturate at days 21 and 42 post infection. Note the severe meningitis, prominent perivascular cuffing, encephalitis, marked increase in cellularity and astrocyte activation as visualized by staining with haematoxylin-eosin (a) or for GFAP (b). (c), (d) Sections from a mouse treated with diminazene aceturate as above but followed 7 days later by treatment with eflornithine for 14 days. In contrast, eflornithine-treated animals show no encephalitis, minimal perivascular cuffing and meningitis, only very few inflammatory cells in the choroid tissue and a marked reduction in the degree of astrocyte activation. Sections stained with H and E (c) or for GFAP (d). Magnification: (a,c) × 40; (b,d) × 100. Reproduced, with permission, from Jennings *et al.*, *Neuropathology and Applied Neurobiology* (1997) 23, 225–234.

(GPI) anchor, and this may subsequently become inserted into host cell membranes, e.g. erythrocytes, which could sensitize the cells to complement-mediated lysis (Rifkin and Landsberger, 1990).

Substances released from the living parasite also include VSGs, phospholipases and fatty acids. The phospholipases in particular may be released in quite high concentrations and have the potential to cause quite

extensive damage to host cell membranes (Mellor, 1985). Studies with *T. b. brucei* have shown that the flagellar pocket may release proteins that affect the host, such as secretory filaments surrounded by a glycoprotein coat and the trypanokine TLTF described earlier. *T. b. gambiense* can metabolize tryptophan to tryptophol (indole-3-ethanol), which can induce sleep. *T. b. brucei* has further been demonstrated to produce the prostaglandins PGD_2, PGE_2 and $PGF_{2\alpha}$ (Kubata *et al.*, 2000). This is of special interest both from an evolutionary point of view and because PGD_2 is the most potent endogenous somnogen so far discovered, which is markedly elevated in the CSF of late-stage sleeping sickness patients (see earlier).

Other potential causative agents have non-parasite origins. An important class is the endotoxins of Gram-negative bacteria. In relation to this it has often been observed that many of the clinical and laboratory criteria are similar in sleeping sickness and endotoxaemic conditions. These include the polyclonal enhancement and subsequent immunosuppression, the complement and kinin activation, hypergammaglobulinaemia, and also the fever, headache and some aspects of the altered endogenous rhythms such as sleep. Small doses of endotoxin can aggravate or increase resistance in experimental animal infections, depending on the dosing regime, and it was hypothesized by Greenwood (1974) that many aspects of sleeping sickness pathology could be due to non-specific endotoxin-like substances (see also Pentreath, 1999). In accord with this, endotoxin levels become elevated in the serum of mice infected with *T. b. brucei* and in Gambian patients. In late-stage patients endotoxin levels were also elevated in the CSF, with the increases correlating with those in the serum, and in both compartments there were some large increases in antibodies against Gram-negative bacteria (Pentreath *et al.*, 1997; Pentreath, 1999). Damage to the intestine and the BBB are obvious underlying reasons for the endotoxin increases. It is also relevant that a number of features of the CNS pathogenesis in experimental sleeping sickness, including the patterns of cytokine expression, are very similar to those that follow peripheral administration of lipopolysaccharide (Quan *et al.*, 1999).

Thus many substances have the capacity to cause the changes that underlie sleeping sickness. Each of these induces its own form of pathology, but it is their combinations in the different parasite strains causing infection, together with the host's immune response, that will determine the pathogenesis in any particular individual.

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17 Pathogenesis of American Trypanosomiasis

Edison Reis Lopes and Edmundo Chapadeiro

Fundamental Pathological Processes

Depending on many factors and acting through direct and indirect mechanisms, *Trypanosoma cruzi* causes a set of alterations in diverse tissues and organs; these may be of a morphological, molecular or functional nature (lesions or pathological processes). The diversity of these processes and alterations gives rise to the morpho-functional pictures that characterize the various forms of Chagas disease (CD). Among the factors involved in the pathogenesis of CD, some are inherent to the parasite (strains, clonal selection, genetic and antigenic constitution, number of parasites inoculated, tissue tropism, attenuation of virulence, reinfection etc.) and others are related to the host (genetic constitution, sex, age, race, immune response and nutrition). As in other aggressive agents, the prejudicial effect of *T. cruzi* acts directly, inducing either molecular or morphological alterations, and indirectly through mechanisms that produce lesions when they are triggered to neutralize or eliminate the aggression. There are three fundamental pathological processes caused by *T. cruzi* in mammalian tissues: (i) inflammatory response (IR); (ii) cellular lesions; and (iii) alterations to the extracellular matrix. As in all pathological processes, these lesions are dynamic and therefore their clinical course leads to either chronic disease or cure. They are also sequential and, most frequently, simultaneous and interrelated. The fundamental pathological processes of CD may be observed in any tissue or organ but most frequently and seriously in the heart, gastrointestinal tract and central nervous system.

Inflammatory response

Both morphological and pathogenetic studies of the IR in human and experimental CD are almost totally based on chagasic myocarditis (Chagas, 1916; Lopes *et al.*, 1994; Milei, 1994; Marin-Neto *et al.*, 1999; Andrade, 2000). Only in the last few years have several works appeared on IR in the muscle layers of the oesophagus and large intestine (Adad *et al.*, 1991; Adad, 1996; Lemos, 1998). As far as can be ascertained, there have been no systematic studies of the IR to CD in other organs.

After inoculation, infectious forms of *T. cruzi* penetrate the cell interior (especially macrophages, fibroblasts, Schwann cells, smooth and striated myocytes) where they transform into amastigotes, which reproduce and create the parasitic nest (pseudocyst) (Fig. 17.1A,B). After a period of 3–5 days, the amastigotes transform into trypomastigotes. During the life cycle of *T. cruzi*, the pseudocyst ruptures the parasitized cell, releasing intact or

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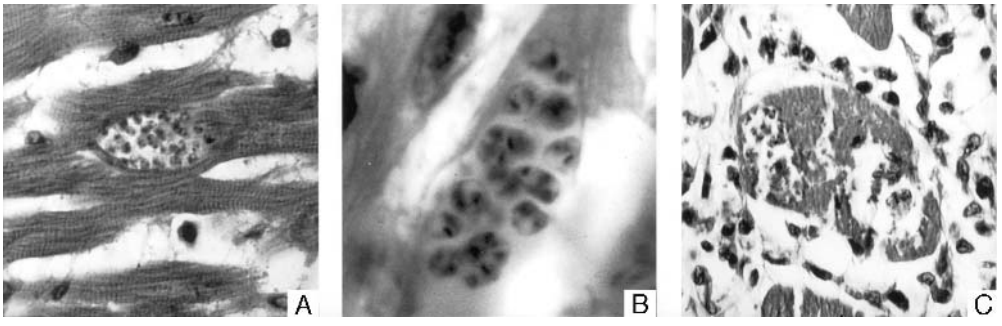

Fig. 17.1. (A) and (B) Amastigote forms of *T. cruzi* forming a nest inside cardiac myocytes. In (A) note the absence of inflammatory reaction around the myocytes and in (B) the kinetoplast can be easily recognized. (C) An infected and ruptured parasitized myocyte with inflammatory reaction around. HE stained sections, 400× (A and C) 1000× (B).

degenerate epimastigote, trypomastigote and amastigote forms of the parasite and remains of the host cell into the interstitium, to act as immunogens or chemical mediators and to trigger a focal IR (Fig. 17.1C). The number of inflammatory microfoci which form corresponds to the number of pseudocysts and cells that rupture; thus this may confer a diffuse aspect to the inflammation. In our experience, at least in humans, both focal (Fig. 17.2) and diffuse IR are observed during the two phases of infection in the myocardium and also in the

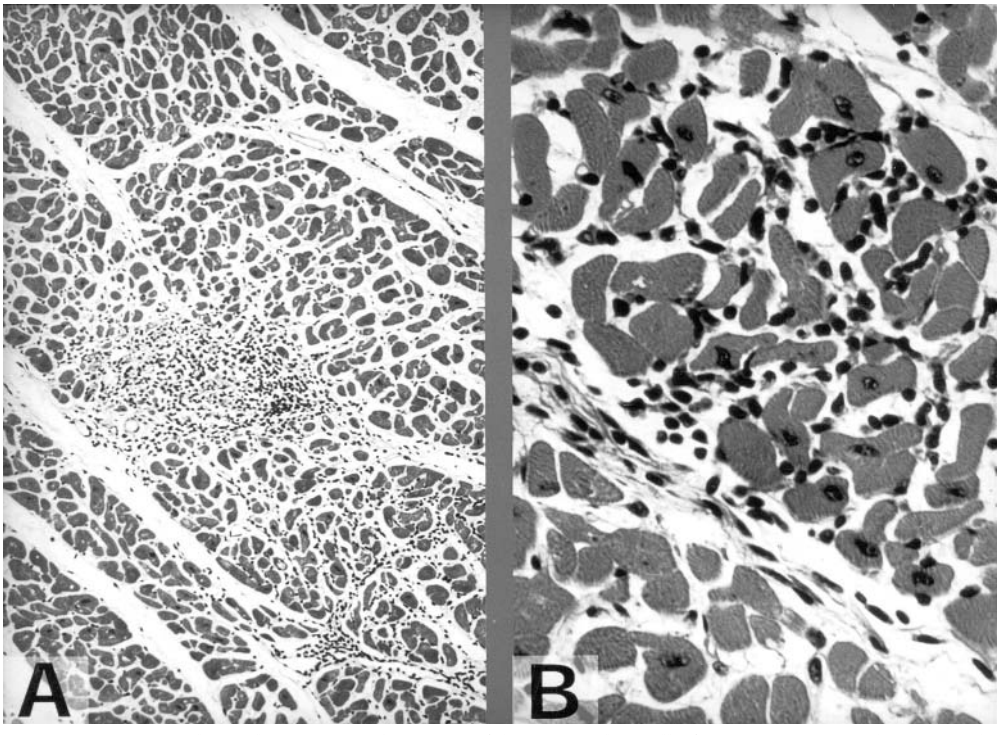

Fig. 17.2. Hematoxylin and eosin stained sections of the ventricular wall of an asymptomatic chagasic with a violent death. (A) Focal inflammatory response, 63×. (B) Detail of inflammatory focus. Note a predominantly mononuclear cell infiltrate closely associated with cardiac fibres, 400×.

muscle layers of the oesophagus and colon. However, the diffuse type of IR is undoubtedly observed with greater frequency in the myocardium than in other sites of infection.

A great number of studies have suggested that there is no single factor involved in the pathogenesis of the IR caused by CD. Analysis of the morphological characteristics of the IR allows some pathogenetic deductions. The frequency and intensity of the tissue parasitism during the initial period of the infection as well as its relationship with the inflammatory reaction and with other cellular alterations, justifies the importance of *T. cruzi* in triggering focal and diffuse lesions in the acute phase of trypanosomiasis. In the initial periods of inflammation the focal reaction comprises mononuclear cells. After a few days these associate with polymorphonuclear neutrophils and eosinophils, and with the course of the infection the exudate becomes predominantly composed of lymphocytes and macrophages. This change in the character of the infiltrate is associated, at least under experimental conditions, with the appearance of antibodies in the serum, *in situ* deposits of immunoglobulins and with hypocomplementaemia. While still in the acute phase of the infection in human cases, it is possible to observe granulomas in the myocardium. Further, *in vitro* studies have demonstrated that *T. cruzi*-sensitized lymphocytes exert a cytotoxic action against the cardiac muscle cells. It has also been ascertained that subcellular fractions of the parasite and myocardial cells have shared antigenic properties. This, together with other data, leads to the hypothesis that immune mechanisms, especially via hypersensitivity and autoimmunity, may play an important pathogenic role in the IR of CD (Kierszenbaum, 1986; Hagar and Rahimtoola, 1995; Kalil and Cunha-Neto, 1996; Languens *et al.*, 1999; Brodskyn and Barral-Netto, 2000). Argument against the autoimmunity hypothesis for the genesis of CD pathology, recently emphasized by Kierszenbaum (1999), is that immunosuppressants have been consistently reported to exarcerbate either human or experimental chagasic infection.

There is much yet to be clarified, such as when the immune reaction really begins whether this is the principal factor responsible for the serious inflammatory reactions seen in some chagasics, and if, besides the cellular immunity, the humoral also plays a role in pathogenesis of IR in chagasics. The role of the parasite in the pathogenesis of the IR has also not been clearly determined. Studies completed in the last few years suggest that negation or even minimization of the role of *T. cruzi* in the genesis of the inflammation in chronic chagasics may constitute a grave mistake. Analysis of the frequency, intensity and location of the parasite in myocardial fragments from chronic chagasic cardiopathy, using anti-*T. cruzi* polyclonal antibodies, has enabled detection of an association between the inflammatory infiltrate and the presence of *T. cruzi* antigen in 87% of the cases (Higuchi *et al.*, 1993). In support of these data, we have demonstrated (Jones *et al.*, 1993), using PCR on myocardial fragments from chronic chagasic cardiopathy, that *T. cruzi* or a portion of its genome was present in the foci of inflammation in each of the cases. Systematic searching for *T. cruzi* with the peroxidase–anti-peroxidase (PAP) technique, performed on chagasics with megaoesophagus (Adad *et al.*, 1991), enabled identification of amastigote forms of the parasite in 50% of the patients. The use of PCR to detect genomic fragments of *T. cruzi*, also performed on chronic chagasics with megaoesophagus, appeared to confirm the above results (Vago *et al.*, 1997). In addition, analysis of the parasitaemia, by haemoculture, in chronic chagasic patients showed positive results in 86% to 94% of the cases (Jörg and Baez, 1993; Luz *et al.*, 1993). Molecular biology techniques enable the detection of plasma antigens derived from *T. cruzi* in significant numbers of chagasics for whom conventional serological techniques have tested negative. Finally, there are data suggesting that frequent parasitism of the central vein of the adrenals by *T. cruzi*, has a direct relation with the number and extension of the foci of inflammation in chronic chagasic myocarditis (Teixeira *et al.*, 1993). This, together with other data, suggests that the parasites and/or their antigens play a

fundamental role in the IR of CD. This considerably reinforces the hypothesis that the parasite has continuing participation in the IR, not just in the acute phase but also in the chronic phase of CD, giving rise to relevant implications for the therapy of the infection.

In summary, the IR, constituting one of the basic lesions observed in CD, appears to be caused by a variety of factors, among which the role of the parasite and the cellular immune response act through hypersensitivity and autoimmunity mechanisms. It is possible that, due to the predominance of one or the other, the inflammation acquires the different aspects presented in the diverse phases and forms of CD.

Cellular lesions

Cellular lesions have a varied nature and intensity ranging from discrete degeneration to death. They occur in cells regardless of whether they are parasitized or not by *T. cruzi*, and are due to multiple mechanisms. The lesions of the smooth and cardiac muscle cells as well as the nerves derive special importance due to their consequences. As in other elementary lesions, various factors play a role in pathogenesis. In the acute phase of the disease, cellular lesions caused by direct action of *T. cruzi* are evident. The penetration of the parasite into the cell, which requires recognition of specific sites and activation of complement, already causes a cellular lesion. The mechanical action of *T. cruzi*, the direct cytolysis by acid hydrolase, the inhibition of mitochondrial oxidative phosphorylation, reduced myocardial adenylase cyclase and choline acetyl transferase depletion constitute other pathogenetic mechanisms arising from the direct effects of the parasite.

Cellular lesions in trypanosomiasis may also constitute one of the components of the degenerative and necrotic phenomena in the IR. For example, in the myocarditis of chronic chagasic cardiopathy, the infiltrate contains cytotoxic $CD8^+$ cells, a lesser number of $CD4^+$ cells, mixed with a few macrophages, which express tumour necrosis factor alpha (TNF-α) (Reis *et al.*, 1993b). There is also an increase in the expression of class I molecules of the principal histocompatibility complex (Reis *et al.*, 1993a). These data suggest the existence of a mechanism involving cytotoxicity mediated by cells in the lesions of the cardiac myofibres. The works cited and many others suggest the role of cytotoxic mechanisms in the pathogenesis of megas, as demonstrated by Lemos *et al.* (1998), in the inflammatory infiltrate of the large intestine of chronic chagasics with megas, pointing to the involvement of cytotoxic mechanisms in the genesis of the cellular lesions.

Abnormalities in the microcirculation may also lead to cellular lesions. A significant number of authors have suggested that abnormalities of the coronary microvasculature may at least contribute significantly to the myocytolysis frequently observed in CD.

As shown in the next section, abnormalities of the extracellular matrix also constitute mechanisms for the cellular lesion in CD.

Extracellular matrix

The most important alteration of the extracellular matrix (ECM) is the fibrosis characterized by the progressive deposits of fibronectin, laminin and collagen, with a consequent expansion and distension of the extracellular matrix. It occurs slowly but progressively and may be located in any organ, but the most important locations are the heart (especially the myocardium) and the gastrointestinal tract (oesophagus and colon).

In the heart, under experimental conditions, the fibrosis appears to commence within the first days of the infection. It is considered by some to be the principal factor responsible for the progressive loss of contractile activity of the myocardium in chronic chagasics. There is no other human myocarditis in which the fibrosis develops so intensely and with such singular characteristics as in chronic chagasic cardiopathy. Morphologically (Fig. 17.3), irregular areas of collagenous neoformation appear, which are modestly capillaried and only exceptionally take the characteristics of a granulation

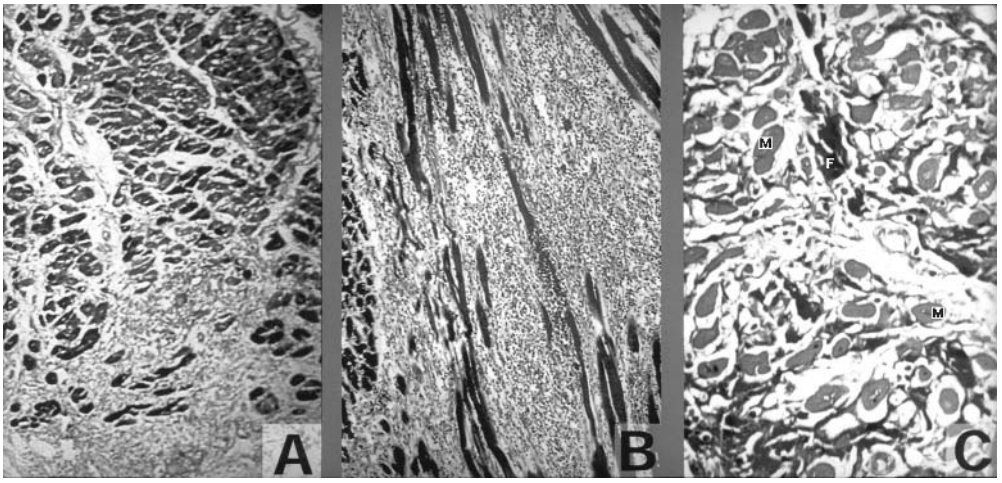

Fig. 17.3. Chronic fibrosing chagasic myocarditis. (A) Inflammatory infiltrate and extensive areas of collagenous neoformation (fibrosis), HE100×. (B) Irregular and extensive fibrosis which replaces areas of myocardium; note the hypotrophy and hyalinization of the remaining myocytes, HE100×. (C) interstitial fibrosis (F) depleted and surrounded myocytes (M). Masson's trichromic, 200×.

tissue. In the majority of cases, it presents as a fibrosis substituting the myocytes that have disappeared. The progressive loss of the myocytes in chronic chagasic myopathy is associated with a concomitant increase in cardiac fibrosis. The set of focal fibroses, which occur over a period of years, forms a final, extensive and serious picture, as observed especially in chagasics who die during or after manifestations of heart failure. Everything indicates that in the genesis of the fibrosis the inflammation, immune phenomena, vascular alterations and probably other factors may have an interrelated role or may act in isolation. Associating the fact that chronic chagasic myocarditis is, at least in part, an immuno-inflammation, the data indicate that the synthesis of collagen is markedly intensified in the immunocellular reactions. It is not hard to understand how immune phenomena act in the genesis of the fibrosis that accompanies CD. There are data suggesting that soluble factors, elaborated by the *T. cruzi*-infected mesenchymal cells, may stimulate a fibroblastic proliferation and the neoformation of collagen. As in many chronic inflammations, in chagasic myocarditis there is a tendency for healing by fibrosis.

More recent clinical and experimental evidence (Ramos and Rossi, 1999) suggested that alterations in the coronary microvasculature may also play an important role in the genesis of the fibrous myocardial process. Further studies are needed to confirm this hypothesis and may correlate chagasic cardiopathy with other cardiac diseases in which microvasculatory dysfunction damages the myocardial structure and function through varying mechanisms.

Hypotheses on regression of fibrosis in various organs and conditions indicate that understanding of fibrosis is also of great importance, due to the possibility of adopting prophylactic and therapeutic measures.

Other alterations of the interstitium occur in CD. It is known that *T. cruzi* produces a great variety of proteases, besides the collagenases which can degrade the proteins of the protean matrix. Experimental studies indicate that *T. cruzi*-infected endothelial cells may secrete an abnormal ECM. However, it is interesting that the addition of this abnormal matrix to non-infected cells also induces these to produce an abnormal ECM. This suggests that the infection of a small population of cardiac cells by *T. cruzi* may have ample effects on the transmission

of information and on metabolism of neighbouring cells. Finally, there is a reduction in the number and function of the intercellular junctions in cultures of myocytes from *T. cruzi*-infected rats, suggesting that cell-to-cell communication abnormalities may also contribute to the pathogenesis of the disease, especially in the mechanism of conduction disturbances and arrhythmia, so frequently seen in chagasic cardiopathy.

Phases of Chagas Disease

Acute phase

In its acute phase CD is characterized by the presence of *T. cruzi* in the blood, as demonstrated by direct processes, such as tests that use concentration or staining. The parasitaemia so revealed generally persists for 4–8 weeks and subsides spontaneously. In the majority of cases, this initial period of infection is not diagnosed, especially in adults. This is because the infection is asymptomatic or oligosymptomatic, or sometimes because the clinical picture is masked by important manifestations such as acute myocarditis or acute meningoencephalitis; thus the physician may overlook the aetiological origin of these manifestations. Both from a clinical and anatomopathological point of view, the most meaningful and important findings in acute Chagas disease (ACD) are located at the portal of entry by the parasite, in the heart or in the nervous system (autonomic and central).

Portal of entry

The penetration signs appear after an incubation period of approximately 7–10 days if the contamination is caused by infected triatomine bug faeces. When *T. cruzi* penetrates the conjunctiva, it gives rise to Romaña's sign; when the penetration is through the skin it forms the inoculation chagoma. The satellite lymph nodes are generally also damaged, which together with the conjunctival or cutaneous lesions form the ophthalmolymphonodal or cutaneolymphonodal complexes. Of the patients that develop ACD, nearly half have Romaña's sign and one-quarter the inoculation chagoma; the remainder present no portal of entry.

The majority of authors characterize the Romaña's sign, or ophthalmolymphonodal complex, by: (i) elastic or painless, unilateral bipalpebral oedema; (ii) rose-violet coloration of the palpebrae; (iii) congestion and oedema of the conjunctiva and neighbouring regions; (iv) satellite lymphadenitis (preauricular, submandibular and others) – the lymph node becoming enlarged and palpable but not adhering to the surface or deep layers; (v) palpebral and periorbital cellulitis, forming metastatic chagomas, sometimes with necrosis of the fatty tissue; and (vi) a large number of parasites, especially in the macrophages. The inoculation chagomas may appear in any part of the tegument but especially on the face and limbs. They consist of hard, rose-violet lesions with a furunculoid appearance and a discrete central oedema. Microscopical examination of the skin and hypodermis enables the detection of an acute focal inflammation that is rich in parasites. As a consequence of the propagation of the parasite by lymphatic means, the satellite lymphonodal reaction results in lymphonodal enlargement. The cutaneolymphonodal complex results from the association of this lymphonodal reaction with the inoculation chagoma.

Heart

Anatomopathological knowledge of ACD, in terms of the heart and other organs, is limited to studies of the few human cases with the serious symptomatic form that leads to death, and also to data obtained from animal models. More recently, and especially in Venezuela, studies have been performed using endomyocardial biopsies from acute human chagasics to determine the degree of myocarditis and to evaluate therapeutic results.

The fundamental pathological processes already referred to develop in the heart, causing epicarditis, myocarditis and parietal endocarditis. Therefore it constitutes a true pancarditis associated with lesions of the intracardiac autonomic nervous system (ICANS).

Macroscopically, there is an increase in the cardiac volume. The pericardial sac is distended and congestive and contains a greater quantity of clear and transparent liquid. The heart is flaccid and congestive, with a moderate increase in volume, especially due to the dilatation of the chambers. These characteristics basically result from the intense inflammation, which simultaneously damages the three cardiac tunica, especially the myocardium. The epicardium may present minuscule nodular whitish thickenings along the coronary branches, a finding that indicates a productive pericarditis. This lesion, termed moniliform or rosary epicarditis, is much more frequent in the chronic cardiopathy (see below). The endocardium generally has a normal aspect and thromboses may exceptionally occur. Another finding is the enlargement of the subepicardial lymph node situated between the aorta and pulmonary artery.

Microscopically, the fundamental lesion is confirmed as damaging the three cardiac tunica, resulting in a true pancarditis. Disseminated foci of myocarditis may be observed, which can sometimes acquire a diffuse aspect. The liquid exudate (oedema), which disassociates the cardiac fibres, associates with the cellular exudate consisting of mononuclear cells with macrophages, lymphocytes and their derivatives (blastocytes and plasma cells) and a variable quantity of granulocytes, neutrophils and eosinophils, as well as mast cells. These elements penetrate and line up in the endomysium or form accumulations, masking the fibre cells, disassociating them and separating them from the capillaries, extending also to the perimysium and from this to the adventitia of the vessels. This distribution of the exudate and its tendency to dissect the cardiac muscle is a unique characteristic of this myocarditis. There is intense tissue parasitism but there is no close correlation between its intensity and the phlogistic response. The myocytes present various types of degenerative lesions, hypertrophy and necrosis, better characterized under electron microscopy. The inflammation rarely acquires a granulomatous aspect; the granuloma is constituted of Langhans-type giant cells and surrounded by histiocytes and other mononuclear cells. This finding suggests a retarded type of immune response. These lesions occur both in the contractile myocardium and in the system for genesis and conduction of the cardiac stimulus. In the latter, the most important lesions are located in the atrial walls, in the sinusal nodule and in the intramyocardial portion of the right branch.

Experimental studies demonstrate that the fibrosis already begins in the initial phase of the disease and that, within 36 h after inoculation with *T. cruzi*, the proinflammatory cytokines are present in the heart together with high levels of nitric oxide in the cardiomyocytes.

Acute epicarditis can be observed with a varied intensity, at times focal and at others diffuse, which affects especially the fatty tissue (cellulitis) and the nerve structures (ganglia and fibres). Damage to these nerve structures by inflammation takes place either directly or by extension from the inflamed adipose tissue. As a consequence, ganglionitis, periganglionitis, neuritis and acute perineuritis appear, which may be accompanied by necrotic-degenerative lesions of their components together with a destruction and reduction in the number of neurones. According to some authors, the neuronal destruction in the acute phase seals the destiny of the chagasic patient. Neuronal parasitism is the exception; amastigote forms in the satellite cells are found more frequently.

These lesions of the ICANS display a very varied frequency and intensity; in certain cases they are striking, whilst in others very discrete. The parietal endocardium shows discrete focal or diffuse inflammation. Valvular damage does not occur.

The intrapericardial lymph node, situated between the aorta and pulmonary trunk, displays a picture of lymphocytic depletion; parasitism has not been reported.

In the acute phase of CD, there is a striking discrepancy between the severity of the myocarditis and the paucity of its clinical expression. Manifestations of acute carditis appear to result basically from the described myocardial lesions above. These, as well as the dilatation and insufficiency of the atrioventricular ostium, are the principal agents

responsible for the cardiomegaly, valvular disturbances and congestive heart failure. In the few cases that develop heart failure, this appears to be due to the intense and diffuse myocarditis. Lesions of the excitoconduction tissue, as already stated, show a close correlation with electrocardiographic data (which indicate a predominance of sinusal arrhythmia) and would suggest that the alterations in the acute phase are capable of leaving cicatricial sequelae in those individuals who progress to the chronic phase of the disease. According to some authors, the tachycardia, regardless of the fever presented in ACD, is an expression of the partial or total destruction of the heart's parasympathetic neurones. The epicarditis is responsible, at least partially, for the pericardial effusion, the formation of which is also caused by other factors such as those leading to the anasarca sometimes seen in acute chagasics.

Digestive tract

In the digestive system, the fundamental pathological processes (the basic characteristics of which have already been described) are predominantly found in the muscle layers and in the intramural nerve plexus of the digestive tube. There is focal myocytis with lesions of the muscle cells and interstitial components. In the intramural plexus, the inflammatory lesions have an irregular and unpredictable distribution with apparently normal ganglia alongside others that have been altered or completely destroyed. Tissue parasitism is frequent.

Nervous system

Besides the above-mentioned lesions in the autonomic nervous system, in patients with serious neurological manifestations there is multifocal meningoencephalitis, characterized by the nodular arrangement of the inflammatory exudate, comprised of mononuclear cells. *T. cruzi* amastigotes are frequently found close to the inflammatory foci, or in the glial cells of adjacent tissue. Chagasic meningoencephalitis is invariably associated with acute chagasic myocarditis, which is usually intense, and this association is responsible for the gravity of the picture and the mortality among these patients.

Besides such cases of serious involvement of the central nervous system (CNS), there are others with inflammatory lesions, although more sparsely distributed, with or without parasites and without clinical repercussions.

Other organs

Morphological lesions also occur in the skeletal muscles, liver, spleen etc. but these are discrete and of little practical importance.

Evolution

Over 90% of patients with clinical manifestations in the acute phase survive the initial infection. The fatality rate in untreated cases, as already stated in the natural history section, is low (2–10%) and is most often seen in infants and immunodepressed individuals who acquire the infection via blood transfusion or organ transplants. Meningoencephalitis and myocarditis, together with refractory cardiac insufficiency, constitute the principal causes of death.

Chronic phase

The natural history of human CD (Fig. 17.4) shows that in almost all cases the chronic phase follows the indeterminate form (IF), characterized by serological and parasitological confirmation of the infection, absence of signs and symptoms of the disease and normal conventional electrocardiographic and radiological examinations (heart, oesophagus and colon). This is a very practical operational definition, which has been approved by various committees of specialists. It is the most frequent form of human CD, affecting 50% of those infected in endemic areas. It has a very good prognosis in the medium to long term and may persist for life in 40–50% of such cases. Reports of spontaneous cure in individuals with the IF are rare and if this does take place it is only in exceptional cases. Likewise, death as a consequence of trypanosomiasis in this IF of human CD appears to be an exception or does not occur.

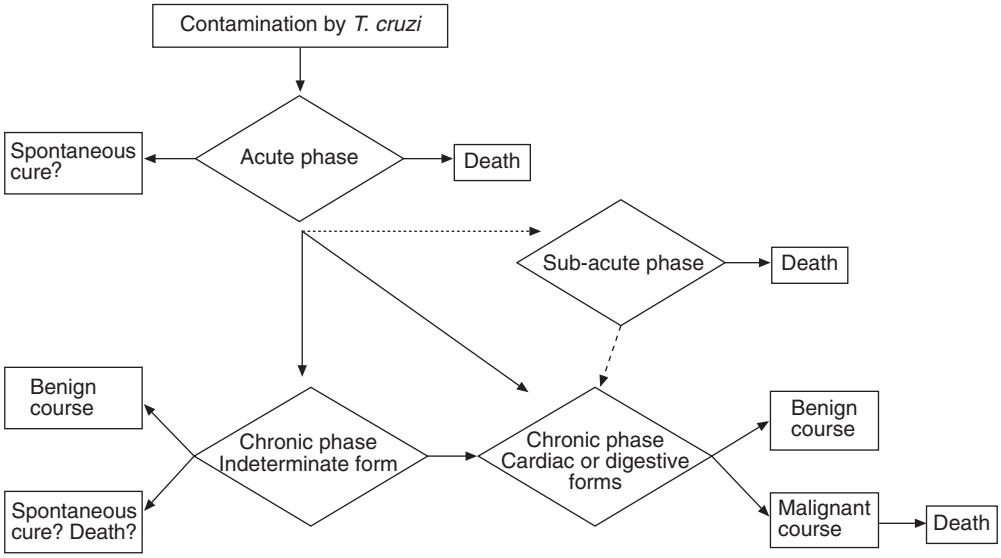

Fig. 17.4. Natural history of the acquired form of Chagas disease (modified from Dias, 1990).

The evolution from the IF to the determinate cardiac or digestive forms generally occurs, in an insidious manner, 10–20 years after the acute phase and at a rate of 2–3% of the cases per year. In the majority of cases, these forms of chronic CD assume a benign aspect or slow clinical course. However, a significant proportion of serious cases develop heart failure or arrhythmia and these cases have a very poor prognosis. In rare circumstances, there is a direct progression from the acute phase to one of the determinate phases of chronic human CD. In this case the picture is termed the subacute form. It generally affects young adults, who develop serious cardiopathy with refractory cardiac failure, though in the majority of cases they survive. There are considered to be two other forms of the chronic phase of human CD: one nervous and the other with acute exacerbation, as analysed below.

Indeterminate form

The following parameters characterize the IF form of CD: (i) positivity in serological and/or parasitological examinations; (ii) absence of symptoms and/or signs of the disease; (iii) normal conventional electrocardiograph; and (iv) heart, oesophagus and colon radiologically normal. The most sensitive examinations may detect alterations in patients with IF. Necropsy studies and *in vivo* investigations, using several sophisticated methods (vectorcardiograph, ventriculograph, ambulatory electrocardiographic monitoring etc.) to evaluate ventricular performance, myocardial perfusion, cardiac autonomic function, rhythm and findings from endomyocardial biopsies, demonstrate that virtually all chagasics in IF have at least some subtle degree of cardiac involvement (Marin-Neto *et al.*, 1999). It is possible that sophisticated tests may show the same for digestive and other alterations. Nevertheless, despite the artificiality of the concept, IF continues to be valid, especially for clinical evaluation and epidemiological studies, bearing in mind the prognostic indices that it offers. Patients with this form of CD lead a normal life and everything indicates that while it persists they may consider themselves to be free from the risk of sudden death. Chagasics may be considered to have the IF form whether they have been clinically and laboratorially characterized as having an acute phase (and the manifestations

have disappeared) or whether the acute phase was asymptomatic.

Information regarding the pathology of IF has been obtained at autopsy of individuals that died from other causes, including violent death and more recently by endomyocardial biopsies. Morphologically, the cardiac lesions are similar to those observed in the cardiac form of the disease (see below), however, they are markedly less in quantitative terms. There is discrete focal carditis (see Fig. 17.2) in 80% of the cases and a moderate to intense degree in 20%. The few cases in which the morphological behaviour of the conduction system has been studied have revealed either discrete inflammatory and/or fibrotic lesions or a complete absence of alterations. There have been no morphological studies in the literature regarding the ICANS in IF.

Ultrastructural studies in chagasic dogs with IF suggest that the focal inflammatory lesions are self-limiting, cyclical and with a balance between the formation of new inflammatory foci and the reabsorption of the oldest lesions. Again with canine models, it has been demonstrated that the cells that compose the inflammatory microfoci, after a certain life span, are removed by apoptosis, which occurs simultaneously with the degradation of the ECM excess. Based on these findings, some authors (Andrade *et al.*, 1997) have suggested that the lesions of the focal myocarditis of IF are subject to a self-limiting evolutive cycle, in which the appearance of new lesions is balanced by the disappearance of the oldest lesions – thus enabling prolonged survival of the host.

The finding of lytic antibodies and the clinical and epidemiological aspects in the IF of human CD suggests that the IF is an active form of trypanosomiasis.

Since there is generally a discrete focal carditis, it does not necessarily produce functional alterations, which explains the absence of signs and symptoms and the normal conventional electrocardiographs in the IF form. Likewise there is no increase in the cardiac area as shown by radiography of the thorax. On the other hand, the histopathological lesions explain the findings of the more sensitive non-invasive examinations.

Cardiac form

Chronic chagasic cardiopathy, the fundamental morphological basis of which is a progressive and fibrous chronic myocarditis, may be asymptomatic or present as a congestive syndrome and/or with alterations to the cardiac rhythm and conduction of the electrical stimulus. Thromboembolic manifestations due to cardiac parietal thrombosis are also relatively frequent. This explains why three syndromes are characteristic of the chronic chagasic cardiopathy: the arrhythmic, the cardiac failure and the thromboembolic syndromes.

Within the group labelled by clinicians as asymptomatic chronic chagasic cardiopathy, there are asymptomatic patients whose complaints are not attributable to cardiac insufficiency and/or arrhythmia. These chagasics are included in the cardiac form of chronic CD due to the electrocardiographic alterations, principally the disturbances in the conduction and ventricular repolarization and the extrasystoles. Under radiological examination, the cardiac area appears normal. In some cases the lack of symptoms is surprising, considering the seriousness of the signs found and anatomopathological lesions detected under microscopical examination. These chagasics may die suddenly (unexpected sudden death), especially in areas where CD is endemic.

In symptomatic chronic chagasic cardiopathy there may be a predominance of arrhythmia or cardiac failure, which explains why the symptoms most frequently presented are palpitation (related to the arrhythmia) and dyspnoea on effort (related to the myocardial insufficiency). Of the signs revealed by the objective cardiac examination, the arrhythmias and principally the extrasystoles are the most frequent, followed by systolic murmur in the mitral focus, hypophonesis of heart sounds and alteration of the second sound in the pulmonary focus.

Cardiac failure usually has a slow and progressive evolution. It is generally of a global type, with right failure predominating. Congestive phenomena of the systemic circulation predominate over those of the pulmonary region. Peripheral oedema, ascites, hepatomegaly and jugular ectasia are more marked than pulmonary congestion.

Thromboembolic manifestations due to cardiac parietal thrombosis are relatively frequent. Although brain embolism is by far the most common feature recognized clinically, on necropsy studies we can most frequently see presence of arterial thrombi or emboli in the lungs, kidneys and spleen and to a lesser extent in the brain.

After the manifestations of cardiac decompensation, the patient may or may not recover and then live with the compensated cardiac failure until succumbing to a further episode, as happens with half of the cases within 1 year after the initial decompensation. The patient may also die suddenly (expected sudden death) but this is sometimes the first manifestation of the disease (see section on sudden death, below).

PATHOLOGICAL ANATOMY In our experience, based principally on data from human necropsies, we consider that the morphological picture of the cardiac form of CD should be divided into two types: (i) those who die from or after a picture of cardiac failure (CF); and (ii) those who die suddenly in an unexpected manner.

Macroscopically, the shape of the heart may be unaltered when there are no signs of CF (Fig. 17.5, VD and SD), or may be globoid (Fig. 17.5, CF) when there is CF. In this condition, the apex of the heart is rounded and formed by both ventricles. Both in those chagasics who die during or after the CF manifestations and in those asymptomatic or oligosymptomatic individuals who unexpectedly suffer from sudden

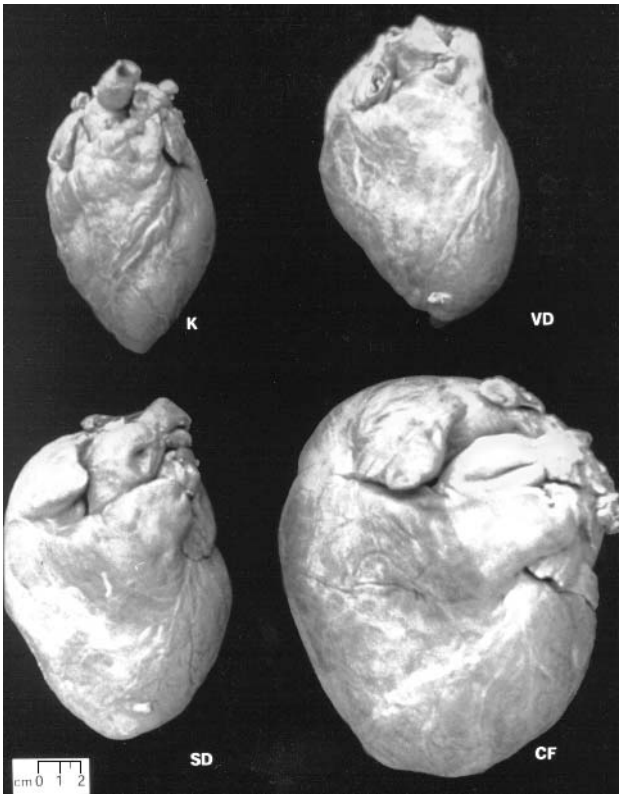

Fig. 17.5. K, Heart of healthy individual that had a violent death (weight: 320 g). VD, Heart of a seemingly healthy chagasic that suffered a violent death (weight: 310 g). SD, Heart of a chagasic with unexpected sudden death; note the discrete enlargement of the organ's volume (weight: 400 g). CF, Heart of a chagasic that died from a picture of cardiac failure (weight: 500 g). Enlarged globular heart with the apex formed by both ventricles. On the epicardial surface focal whitish thickenings can be seen.

death (SD), the heart shows an increased weight; on average this is 540 g in the former and 390 g in the latter group. The increase in weight is due especially to hypertrophy of the myocardium, though other factors, such as inflammation and muscular oedema, contribute towards it. In those who die during or after a picture of CF, the organ may reach large volumes (cardiomegaly). This results not only in muscle hypotrophy but also in the dilatation of the cardiac chambers. In general, if global cardiac dilatation is observed, there is usually a more marked dilatation of the right chambers. The pericardial sac is distended more or less intensely in accordance with the cardiac volume. In those individuals with CF, there is a greater quantity of liquid, generally straw-yellow or citron-yellow and limpid but sometimes cloudy, fibrinous or even serous-fibrinous-haemorrhagic; this occurs in those cases in which there are extensive atrial and auricular thromboses. The serological reactions realized with these liquids for diagnosing chagasic infection are highly sensitive and specific. This serological analysis has a high value in the *post mortem* diagnosis of chagasic infection.

The epicardium almost always shows a whitish thickening, in the form of plates, strips or streaks, disseminated also along the external and diaphragmatic surfaces (Fig. 17.5, SD and CF). The formation of nodules is frequently observed in the epicardium; these nodules are disseminated on the heart surface or along the coronary vessels (rosary-bead epicarditis; Fig. 17.6). Sometimes there is formation of villum plaque in the diaphragmatic surface of the left ventricle. Although not specific, in that they are found in other cardiopathies (rheumatic and hypertensive), these lesions are more frequent and intense in the chronic chagasic. Microscopically, all these epicardial lesions have the same nature: fibrous chronic inflammation of the subepicardial fatty tissue. Besides the thickening mentioned above, it is not uncommon for discrete fibrous epicarditis to occur in association with the described thromboses.

In approximately 75% of the chronic chagasics with SD and in 40% of those with CF, there is enlargment of the subepicardial lymph nodes situated in the intrapericardiac portion of the aorta and pulmonary artery (Fig. 17.7). In the former, the histological picture is generally one of a reactional state with a predominance of germinative centres and in the latter it is characterized by lymphocytic depletion. Parasites are not generally observed in these lymph nodes.

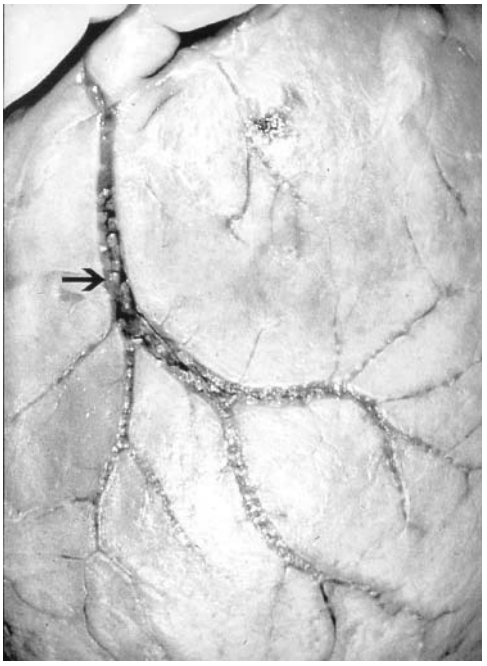

Fig. 17.6. Chagas heart disease; note the nodule formation along the coronary vessels (rosary-bead epicarditis).

The myocardium of chagasics with CF is flaccid and purplish-red, sometimes exhibiting punctuate petechial haemorrhages; in unexpected sudden death, it generally presents a normal aspect. In cases of congestive cardiac insufficiency (CCI), it frequently presents irregular whitish areas with a fibrous cicatricial (callused) aspect measuring a few millimetres. These are generally more extensive in the apical region.

In many cases, the apex of the left ventricle shows a peculiar lesion (Fig. 17.8) denominated vortical lesion (apical aneurysm, apical lesion, pseudoaneurysm of the apex) which is formed in the vortex.

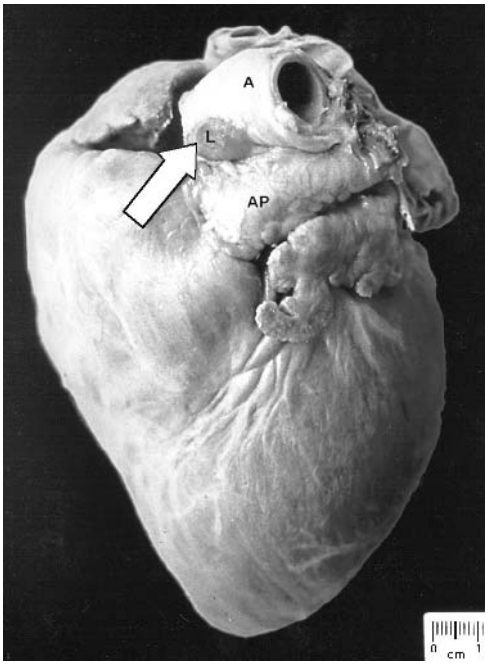

Fig. 17.7. Heart of chagasic patient with sudden death; note the enlargement of subepicardial lymph node (L) situated in the intrapericardiac portion of the aorta (A) and pulmonary (AP).

According to Almeida (1976) there are two types of apical lesion in the left ventricle of chronic chagasic patients: one (Fig. 17.8B,C) is basically formed by the separation of the muscle bundles that constitute the vortex, including the endocardium and thin layer of myocardium. In the other type of apical lesion (Fig. 17.8A) the muscle bundles become progressively thin without a clear separation, and with a dilatation in the apical region. The latter is more frequent and reaches larger diameters; it is also more frequently associated with thrombosis (Fig. 17.8D).

In our experience, vortical lesion occurs in 59.9% of chagasics with CF and in 56.7% of those who suffered unexpected sudden death. It is considered pathognomonic of chronic chagasic cardiopathy. Regarding its pathogenesis, opinions are divided among four principal hypotheses: (i) hypoxia; (ii) impairment of the conduction system; (iii) inflammation; and (iv) mechanical factors.

The histological picture of the cardiac form of CD is one of fibrous chronic myocarditis which remains active through the eclosion of repeated inflammatory foci; these tend to flow together, thereby conferring the zonal or diffuse aspect to the lesion in its advanced stages. The inflammatory foci are prevalently intrafascicular or perimysial and generally extend to the interfascicular conjunctiva or to the adventitia of the small- and medium-calibre veins; on the other hand, neighbouring foci tend towards a confluence. Microscopical studies using classic staining techniques indicate that, in human chronic chagasic myocarditis, the exudate predominantly comprises lymphocytes and macrophages together with a lesser number of eosinophils, plasma cells, neutrophils and mast cells. With a certain frequency, some authors report cases in which the eosinophilic infiltrate takes the major proportion, which has been interpreted as a sign of renewed acute inflammatory process. In the few cases of human chronic chagasic cardiopathy studied by immunohistochemistry, it has been observed that the predominant cells in the myocardiac inflammatory exudate were T lymphocytes, with few B lymphocytes and macrophages. Studies using human material have consistently demonstrated that the $CD8^+$ cells are two to three times more frequent than $CD4^+$ and also that the $CD8^+$ cells appear closer or adhered to the myofibres and express cytotoxic factor granzyme A. These elements of the exudation were localized in the endomysium, dissociating and separating the myocytes from each other and from the capillaries. Alongside the infiltrate, there is also oedema and venule–capillary congestion, sometimes accompanied by local leucocytosis; the oedema further contributes to the separation of the myocytes from each other and from the capillaries. Even in the initial stages of the process, under light microscopy the myocardial fibres show diverse alterations: hyalin change, oedema, hypotrophy and necrosis. These different types of cellular alterations represent, in part, not only the cause but also the effect of the CF itself. Besides these, another extremely interesting alteration is the invasion of some of the myocardial fibres which are normal or that have been altered by elements of the infiltrate.

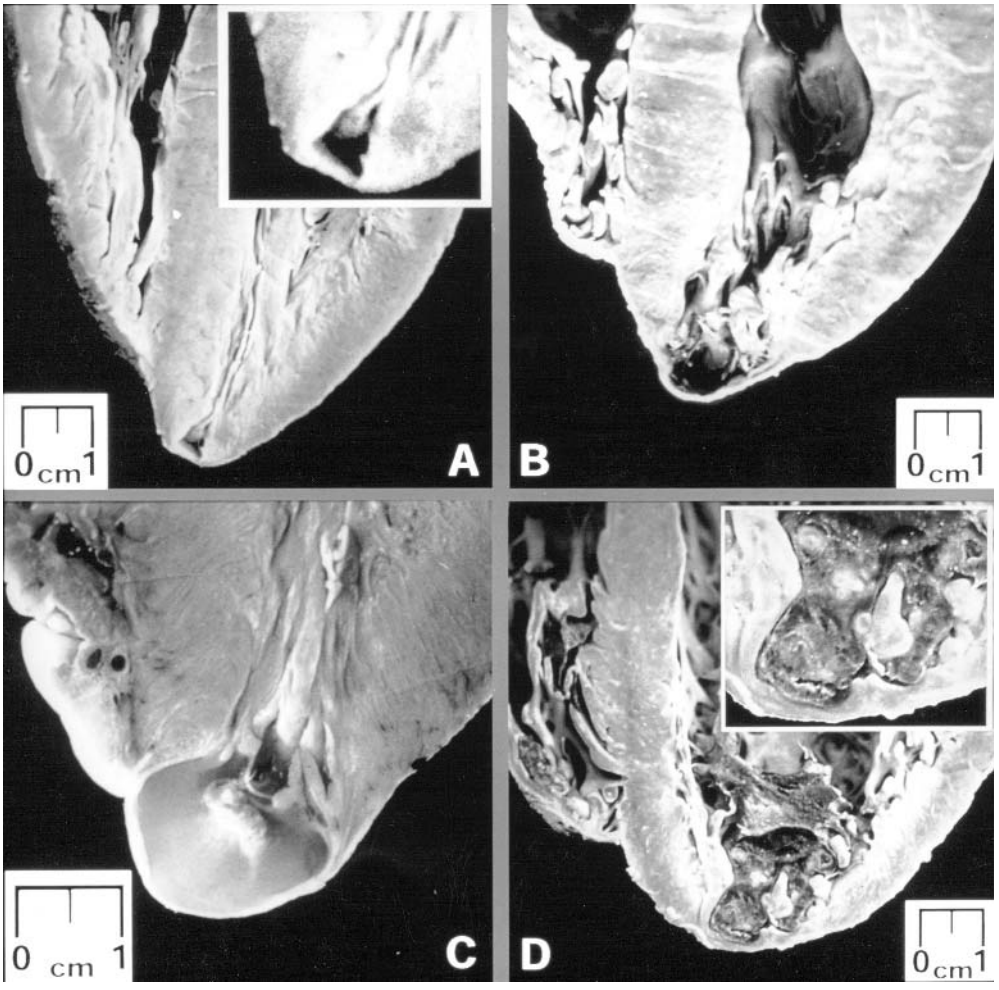

Fig. 17.8. Left ventricles of chagasic patients with characteristic vortical lesions (apical aneurysm). (A) An 11-year-old child with chronic chagasic infection and a violent death. In the apex is delimited circumscribed thinning of the myocardium, further evident in the inset; note the apposition of the endocardium to the epicardium. (B) and (C) Frontal sections of hearts from patients with chagasic heart disease; note the well-developed vortical lesions (apical aneurysm) in left ventricles. (D) Apical thrombosis in left vortical lesion. Inset is a higher magnification view of lesion and thrombus.

Electron microscopical analysis of myocardial fragments obtained in necropsies from chronic chagasic cardiopathy enables observation of the following lesions: (i) mitochondria – irregular distribution, volume increase, cristolysis, vacuolization, increase of intramitochondrial granules; (ii) myocytes – widening of the space between the myofibrillae, dissociation and disorganization of the myofibrillae, focal lysis, torsion and lateral dislocation; (iii) endoplasmic reticulum – cisternal enlargement, with rupture and blurring of the system; and (iv) sarcolemma – protusion, thickening and dehiscence of the intercalated discs. Studies carried out on ventricular endomycardial biopsy specimens showed also that basement membranes of capillaries, vascular smooth muscle cells and cardiac myocytes of patients with CD are markedly thickened, up to 20

times their normal thickness. Such alterations appear to represent non-specific manifestations of myocardial damage. More recently, deposits of a glycoprotein-like material were observed within the lumina of T tubules of myocytes and of a glycoprotein-like substance from the endoplasmic reticulum. This last finding was considered to be characteristic of chagasic myocarditis.

Endomyocardial biopsies from human chronic chagasics have also enabled histochemical analyses of the myocardium. A decrease has been observed in the concentration of all the enzymes investigated, together with an elevated concentration of phosphatases (lysosomic activity) and monoamine oxidase.

The degenerative processes, both those detected by the electron microscope and those perceptible under the light microscope, are of a varied intensity in the diverse myocardial areas. They affect the fibres, including those in the inflammatory foci or enclosed in the areas of fibrosis, but also occur in those fasciculi that are not directly affected by these two processes. The myocytes with the most lesions are not always related to the inflammatory foci, leading one to question which other factors, besides the inflammatory reaction, could be involved in the regressive alterations of the cells of the cardiac fibres in chagasic cardiopathy.

On examining the myocardium in chronic chagasic cardiopathy, the frequency with which one observes areas of mummification of the cardiac fibres, foci of vacuolation of myocytes with their progressive disappearance (myocytolysis), as well as other findings (e.g. microscopic infarctation), leads to the suspicion that the obstructive vascular lesions may also act in the pathogenesis of chronic chagasic myocarditis. The focal distribution of myocytolysis and interstitial reparative fibrosis, observed in chronic chagasic myocarditis, suggests that transitory ischaemic abnormalities occur in chagasic heart disease. Several authors have ascribed the responsibility for the 'ischaemic lesions' in chronic chagasic myocarditis to the pathological involvement of small coronary vessels. Other studies have not confirmed this hypothesis and at the most admit that these lesions merely contribute to the pathogenesis of the myocardial 'ischaemic lesions' – in those cases in which there is a concomitant chronic congestion and significant cardiomegaly in the cardiopathy (provided that one accepts that hearts weighing > 500 g present relative coronary insufficiency). More recently, evidence of a clinical and experimental nature has lent force to the idea that the transitory ischaemic disturbances occur at a microvascular level in chronic chagasic cardiopathy. In view of the data supporting this, it should be remembered that various independent clinical investigations using appropriate markers have reported perfusional myocardial defects (fixed, reversible or paradoxical) during effort and rest in chagasics with coronary arteries that appear normal under angiographic examination. This has been observed in the absence of other signs of myocardial damage. Thus, it can be concluded that whilst the role of these vascular alterations in the pathogenesis of the myocardial lesions appears to be important, it still requires greater clarification.

In the most advanced phase of the process, groups of myofibrils disappear totally amidst the cellular infiltrate and cellular remains are observed in the interior of the macrophages. *T. cruzi* amastigote forms are rarely seen in these foci. In other areas, the inflammatory foci are less destructive: the infiltrate diminishes or disappears and the basic structure of the sarcolemma and the endomysium remain intact.

In approximately 11% of the hearts of chagasics with CF and in 50% of those who die suddenly, the inflammation acquires a granulomatous type in which the Langhans-type giant cells or the foreign bodies are enclosed by histiocytes, between which are interposed varying quantities of lymphocytes, blastocytes, plasma cells, granulocytes and remains or segments of multinucleated cardiac fibres, similar to the Antischkow myocytes.

In parallel with the reduction in the inflammatory infiltrate, the fibrous conjunctiva substitutes the destroyed cardiac fibres

and separates and surrounds the other fibre cells, which become hypotrophic and may disappear. In consequence, the neoformed conjunctiva partially or totally ruptures the muscle cells and entire muscle fasciculi. This fibrosis may also affect and involve the perimysium and unite with the fibre of adjacent fasciculi, which become not only interrupted but also fixed one to another by the neoformed sclerotic conjunctiva. The majority of the cardiac fibre cells not affected by the degenerative–necrotic processes become hypertrophied and the intensity of this hypertrophy appears to be directly linked to the fibrosis. Experimental data from mouse models indicate the participation of type I and III collagens together with an increase in type IV collagen and fibronectin. Experimental studies also suggest that the myocardial fibrosis may regress after specific therapy. The demonstration of the greater presence of type IV collagen has a special significance when one considers the ultrastructural finding of thickening of the basement membrane of the capillary vessels, vascular smooth muscle fibre and cardiac myocytes up to 20 times their normal thickness in cases of chronic chagasic myocarditis, as mentioned above.

The final myocardial fibrosis of those with chronic chagasic cardiopathy who die from congestive cardiac insufficiency appears to represent the sum of all the partial focal fibroses that occur over the years. In general, all the evolutive phases of the process can be found in the myocardium of those with chronic chagasic cardiopathy, from the small inflammatory foci (constituted of mononuclear conglomerates) up to the final fibrosis.

The lesions of the endocardium consist of parietal endocarditis, thrombosis and fibroelastic proliferation. The endocarditis is generally far more discrete and more circumscript than the alterations of the remaining tunica. The inflammatory infiltrate is similar to that described in the other tunica. The fibrosis in general is more discrete, presenting in the form of whitish thickening of the endocardium of the left ventricle and papillary muscle. Valvular lesion is not found.

Thrombosis is common and more frequent in those with CF (approximately 75% of the cases) than in those who die from unexpected sudden death. In those chagasics with CCI, the most frequent location is the right atrium followed by the left ventricle. In those who die suddenly, there is a greater incidence in the latter chamber. The thrombosis of the endocardium is principally due to the inflammatory lesion of the tunica and/or to congestion in the dilated chambers; other factors (e.g. atrial fibrillation may also contribute to the formation of the thrombi). Fragments of the thrombi may be dislodged to constitute emboli, with those from the left ventricle and right atrium affecting the general circulation and lungs, respectively. The thrombi intimately adhered to the endocardium frequently suffer total or partial organization (cicatrization), making the subjacent endocardium very thick fibrohyalin, which commonly suffers calcifications. Irrespective of the presence of thrombi in the cardiac apex, the endocardium may also appear thickened due to the fibroelastic proliferation which seems to result from the response of this region of the endocardium to the shock from the blood wave.

The excitoconductor system is harmed basically by the same lesions that occur in the functional musculature. Besides these, the existence of intense dilatation and tortuosity of the lymphatic and capillary veins, infiltration by the adipose tissue and fibrosis of the intima and media layers of the small arteries and arterioles suggests a complex and multi-factorial pathogenesis of the lesions of the excitoconductor system. These lesions combine with each other in different proportions from case to case. There is a close correlation between the lesions and electrocardiographic alterations. The blockage of the right branch of the atrioventricular bundle is related to the specific pathological process singular to the right branch in the disease, which occurs more than any other anatomic disposition. In cases of sudden death, mastocytosis in the sinoatrial nodulus has been described.

More common, and of an arguable interpretation, are the lesions of the intracardiac autonomic system (ICANS). This system, comprising ganglia and fibres distributed in

the fatty tissue of the epicardium of the atria and the interatrial septum, is also affected by the inflammatory process with the same characteristics as those already described, causing chronic ganglionitis, periganglionitis and neuritis (Fig. 17.9). The lesion commences in the acute phase and contributes to the neuronal destruction, which continues into the chronic phase of the infection. The destroyed neurones are substituted by the proliferation of the satellite cells, which leads to the formation of nodular structure (satellitosis); the remaining neurones show diverse alterations: retraction of the pericardium, tigrolysis, hyperchromasia etc.

CHRONIC CHAGASIC CARDIOPATHY AND SUDDEN DEATH Chagas and Villela (1922) affirmed that they knew of no other pathology which caused SD in such a high percentage as CD. Later studies confirmed this statement and Luigi Bogliolo, an important research worker in tropical pathology in Brazil, affirmed (personal communication, 1978) that, in percentage terms, chagasic cardiopathy is responsible for more SD than ischaemic cardiopathy. The SD in CD, in practically all cases, is cardiac and as in other cardiopathies it is useful to distinguish between two types: expected (SED) and unexpected (SUD). Two important aspects should be highlighted: firstly, to emphasize that the division between SED and SUD contributes to the study of SD in individuals with CD, facilitating the observation of risk factors and the identification of those patients needing preventive care; and secondly, the aspect that is of a medico-legal nature. For this branch of medical and legal practice, the unforeseeable aspect is a fundamental characteristic for diagnosing SD. Consequently the coroners do not include SED in the sudden deaths.

Although many studies in Brazil and abroad refer to the high prevalence of SD in CD, few present numerical data, especially regarding SUD. Studies carried out in an endemic area by Lopes and Chapadeiro during 1980 indicated that CD was responsible for 24.1% of the SUD in the city of Uberaba. A similar study carried out 10 years later showed that the frequency of this type of SD had fallen to 6.8%. Data gathered in a field study suggested that SUD occurs in approximately 37.5% of the chagasic patients living in endemic areas. In hospitalized patients it was observed that SED occurs in approximately 38% of uncompensated chronic chagasic patients. The analysis of these and other data con-

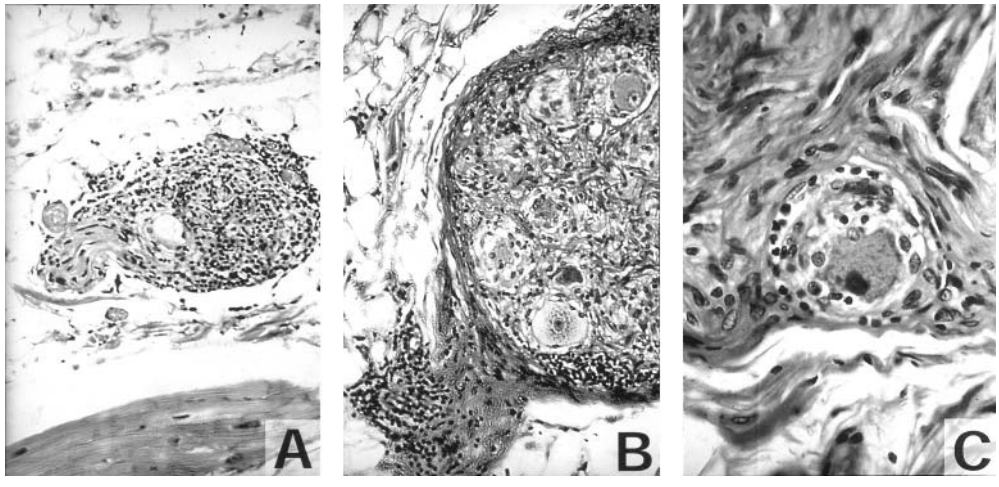

Fig. 17.9. Lesions in the atrial subepicardial ganglia in chronic chagasic cardiopathy. (A) and (B) Inflammatory reaction in the epicardial adipose tissue (cellulitis) that extends to the ganglia (ganglionitis) and to the nerve (neuritis). HE stained sections, A: 100×, B: 200×. (C) The field illustrates a damaged neurone with surrounding mononuclear cells. HE stained section, 400×.

firms the findings of the World Health Organization showing that SD (including the unexpected and expected forms) is the principal cause of death among those suffering from chronic chagasic cardiopathy.

SUD is the type of death that meets all the classical criteria of SD. In fact it occurs more or less rapidly, in a few seconds, hours or even days, but in an unpredictable manner, and without any apparent cause, in hitherto healthy individuals or those who presented only slight disorders, at least in appearance to those around them. As this type of SD occurs in patients without any apparent sign of clinical disease, it attracts attention and causes fear and constraint. The majority of chagasics with SUD are male, aged on average 38 years. In 80% of the cases there is an associated family history.

It should be highlighted that the SUD of a chagasic does not imply that the patient was asymptomatic. We performed a retrospective investigation based on data obtained from family members and friends together with the circumstances of the deaths of 35 chagasics who died of SED/SUD. This enabled us to verify that 35.2%, while still alive, did not present any symptoms or suspicion of cardiopathy. This group comprises those chagasic patients who present SD as the first manifestation of the disease. In 57.1% of the cases, we managed to detect a history of dizziness (42.8%), dyspnoea during physical activity (48%), loss of consciousness (34.2%) and palpitations (31.4%). Dysphagia and constipation were present in 38% of the cases. Death was brought on by physical effort or emotion and rarely during sleep. In half of the cases, the lethal event was instantaneous; in the remainder, it was preceded by premonitory symptoms. For ECGs carried out between 1 and 5 years before death, 95% of these cases showed abnormalities. As mentioned previously, SD in indeterminate forms of CD is an exception and SD in chagasics is cardiac SD, with non-cardiac causes being exceptional (e.g. encephalic vascular accident).

MORPHOLOGICAL BASIS OF CLINICAL MANIFESTATION IN CHRONIC CHAGASIC CARDIOPATHY As already described in the natural history of CD, after remaining asymptomatic for several years a certain number of chagasics develop an evident symptomatology, characterized by manifestations of myocardial insufficiency or by the predominance of arrhythmias. On the other hand, as also mentioned, sudden death may sometimes be the first manifestation of chronic CD or occur after a picture of cardiac insufficiency and/or arrhythmias.

Analysis of the morphological findings suggests that at least three anatomical factors may be involved in the genesis of cardiac failure and arrhythmias in chronic chagasics: (i) progressive reduction of the myocardial muscle mass; (ii) interruption of fibres and fasiculi of the myocardium; and (iii) lesions of the sympathetic and parasympathetic systems.

The reduction of the myocardial muscle mass results from the inflammation, degeneration or destruction of the cardiac fibres. The reduction of the muscle mass is continuous and progressive and the interruption of the fibres and fasiculi causes the fixing of the bundles to each other and to the interfascicular connective septa. Besides promoting the disarrangement of the electrophysiological syncytium, these lesions impair the movements of a given number of fibres during the cardiac contraction and constitute accessory factors to the hypotrophy of myocytes. Furthermore, they reduce the efficiency of myocytes and force the myocardial fibres to pull an additional mass of inelastic and non-contractile connective tissue.

Another factor is the destruction of sympathetic and parasympathetic systems. Taking into account the early, intense and largely predominant parasympathetic denervation in CD, a neurogenic theory has been proposed: a long-lasting autonomic imbalance would lead to a catecholamine-induced cardiomyopathy (Köberle, 1968; Oliveira, 1985).

This last hypothesis has been widely questioned. Anatomopathological studies and clinical investigations have demonstrated that the parasympathetic denervation is also found in other cardiac pathologies (rheumatic, dilated cardiomyopathy, endomyocardiofibrosis etc.) but in

general with much less expression than that seen in CD. Clinical studies in chagasics have documented intense levels of depression of the cardiac autonomic control, which is predominantly but not exclusively parasympathetic. There are other studies demonstrating that the frequency and intensity of the neuronal destruction, besides being extremely variable at an individual level, does not correlate with any myocardial dysfunction parameter. However, the methodology employed in the above-mentioned clinical investigations does not enable the rejection of the hypothesis that eventual autonomic nervous disturbances occur at the ventricular myocardial level. A more appropriate insight into this aspect was obtained using scintigraphy to investigate the evolution of myocardial sympathetic nerve terminals (Simões 1999). Segmental areas of sympathetic impairment were detected in a high proportion of patients even in the indeterminate form of CD. This appears to be the first evidence of disturbances in the autonomic nervous system at a ventricular level in chronic chagasic cardiopathy (Marin-Neto *et al.*, 1999).

The predominance of the congestive manifestations of dysfunctions in the right chambers observed in some chagasics is, according to some researchers, due to precocity of the right ventricular lesions and the singular anatomical and functional characteristics of this ventricle. However, there has been no conclusive demonstration regarding the precocity of the right ventricular lesions in relation to those of the left ventricle.

The inflammation of the myocardium also appears to play an important role in the genesis of the arrhythmias and may produce foci able to unleash re-entry mechanisms. Some of the arrhythmias depend, at least in part, particularly on the fibrosis – for example, as occurs with auricular fibrillation.

The harm to the excitoconductor system of the heart caused by the inflammatory process or the fibrosis could explain the different types of blockage. The lack of morphological alterations does not necessarily signify an absence of functional alterations, which may be dependent on chemical and pharmacological mediators. Controversy surrounds the role of the lesions of the autonomic nervous system and cardiac paraganglia, and the role of immunobiological factors of and discharges from the central nervous system in the pathogenesis of the arrhythmias.

With reference to SD, a set of data shows that in both the expected and unexpected forms the death is preceded by fibrillation, constituting terminal arrhythmia. Among the factors that interact to cause the ventricular fibrillation, we highlight the anatomical substrate, the trigger elements and the facilitating factors. Foci of inflammation, areas of fibrosis, ventricular dilatation and vortical lesion favour the occurrence of re-entry, which constitutes the principal electrophysiological mechanism involved in the chain of events leading to ventricular tachyarrhythmias in chronic chagasic cardiopathy. However, the presence of anatomical substrate alone is not capable of originating the tachyarrhythmia. The participation of trigger elements, in this case represented by the ventricular extrasystoles, is also usually necessary. Thus the propagation of these premature impulses through zones of unidirectional block and conduction disturbances, resulting from the myocardial structural alterations, are capable of causing the re-entry process. Completing the model of arrhythmic SD is the role of the facilitating factors, which on interacting with the substrate and trigger elements may cause electrical instability in the system and cause fatal arrhythmias, such as ventricular fibrillation. The acute haemodynamic deterioration, hypoxaemia, acidosis electrolytic disorders and lesions of the autonomic nervous system (intra- and extracardiac) are examples of factors that can destabilize the arrhythmogenic substrate. Amongst these, the behaviour of the autonomic nervous system merits special attention.

Digestive form

The digestive form is represented by alterations to the digestive secretion, mobility and absorption, and in the more serious cases by the megas of the digestive tract.

In Brazil, Bolivia, Argentina, Chile and Peru a varying percentage of chronic chagasics manifest functional disturbances, especially of the oesophagus (chagasic oesophagopathy) and colon (chagasic colonopathy), which may or may not be followed by the dilatation of these viscera ('megas'). In other geographical areas, such as Venezuela and Panama, where chronic chagasic cardiopathy occurs, the digestive forms of CD are not observed, suggesting the existence of regional differences.

The occurrence of the digestive manifestations, in the chronic phase of CD, in endemic areas is varied; it is estimated that 15–35% of chronic chagasics present digestive impairment. Data from field studies undoubtedly best express the prevalence of such manifestations. According to these, megaoesophagus (MO) and megacolon (MC) are by far the most frequent, whilst 'megas' occur more rarely in other segments of the digestive tract (megaduodenum, biliary megavesica) and in other hollow viscera (megabladder, megabronchus etc.). The diverse digestive manifestations may exist concomitantly in the same patient, and in 50% of the cases are associated with chronic chagasic cardiopathy.

From the anatomopathological point of view, various Brazilian authors have focused on the frequency of the different types of 'megas' in necropsies, almost all being 'megas' of the digestive tract. Table 17.1 summarizes the results of these studies. The majority of clinical works show that MO is the most frequent digestive visceromegaly, whilst in the greater part of the studies based on necroscopic findings there is a predominance of MC. Various reasons may explain these differences, such as the facts that: (i) the clinical diagnosis of MO is performed more frequently than for MC, since dysphagia (the principal manifestation of MO) is generally conferred a greater diagnostic value than constipation, which is the primordial complaint in MC; (ii) in the majority of the clinical studies and especially those carried out in the field, X-ray is used to aid the diagnosis of MO but is not applied for MC; and (iii) for the clinical diagnosis of MO, radiological criteria are frequently employed and these sometimes consider cases of oesophagopathy in organs with a normal calibre as MO level 1. The pathologist would not label such cases as MO.

'MEGAS' The 'megas' (from the Greek *mega*, meaning large) are permanent and diffuse dilatations of the hollow viscera or canals, with an increase in the muscle mass, which may or may not be accompanied by elongation of the wall. These dilatations are not caused by mechanical obstruction and their functional anatomical substrate is a lesion of the intramural autonomic nervous system (IMANS). This definition applies as much to the chagasic 'megas' as those of other aetiologies, and not solely to the digestive tract but also to other hollow structures (megaloureter, bronchiectasis, etc.).

Pathological anatomy The hollow viscera or canals with 'megas' are permanently expanded and without mechanical obstacles (Figs 17.10 and 17.11). Specifically for the oesophagus, dilatation is considered to be present when the diameter of the fixed organ exceeds 25 mm. The increase in muscle thickness and alterations in the mucosa (leucoplasy, ulcerations etc.), secondary to the stasis, confirm the permanent character of the dilatation. In certain cases the wall may be normal or even appear thinned, since the dilatation masks the increase in muscle; however, morphometric studies have shown an increase in muscle mass in all cases of MC. Compared with that seen in MO, the end portion of the organ may present a normal or reduced diameter.

Microscopically, the most characteristic and constant lesions are those of the muscle and IMANS (Fig. 17.12). These are evident in the myenteric plexus and identical to those seen in the intracardiac autonomic nervous system as described above. There is inflammation of the ganglia (ganglionitis and periganglionitis) and nerves (neuritis and perineuritis) together with intense degenerative phenomena of the neurones, which can lead to their complete destruction. For this reason, there is a varying degree of neuronal depopulation: in some ganglia the destruction is total; in others the

Table 17.1. Prevalence of megas in necropsies, according to various Brazilian anatomopathologists.

						Percentage of megas									
				Chagasics		Megacolon		Megaoesophagus		Megacolon + megaoesophagus		Others		Total	
Author	Year	Location	No. Necropsies	No.	%	Chagasics	Necropsies	Chagasics	Necropsies	Chagasics	Necropsies	Chagasics	Necropsies	Chagasics	Necropsies
Koberle	1962	Rib. Preto	–	250	–	27.6	–	24.8	–	–	–	10.8	–	63.2	–
Chapadeiro *et al.*	1964	Uberaba	318	133	41.8	7.5	3.1	7.5	3.1	1.5	0.6	0.8	0.2	17.3	7.3
Koberle	1968	Rib. Preto	–	500	–	11.8	–	8.4	–	8.4	–	10.8	–	39.4	–
Barbosa *et al.*	1966	Belo Hte.	15,000	875	5.8	9.8	0.57	2.8	0.16	3.9	0.23	–	–	16.6	0.9
Andrade *et al.*	1967	Salvador	1,600	–	–	–	0.9	–	0.43	–	0.9	–	0.06	–	2.4
Lopes *et al.*	1998	Uberaba	4,690	1,708	36.4	8.3	3.02	4.1	1.51	2.8	1.02	0.7	0.2	15.9	5.8

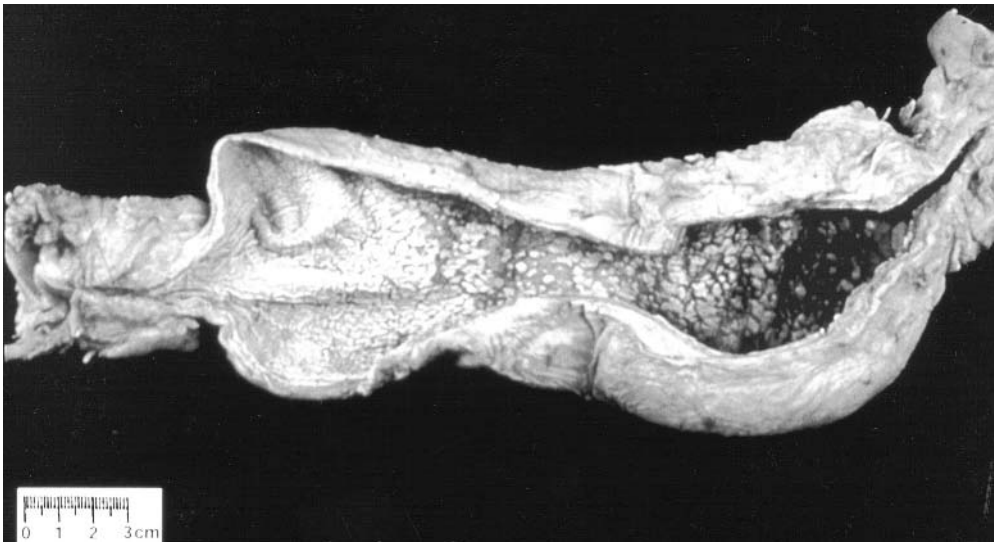

Fig. 17.10. Chagasic megaoesophagus. Elongation and dilatation of the organ with thickening of the wall and secondary alterations in the mucosa (leucoplasia, ulcerations etc.).

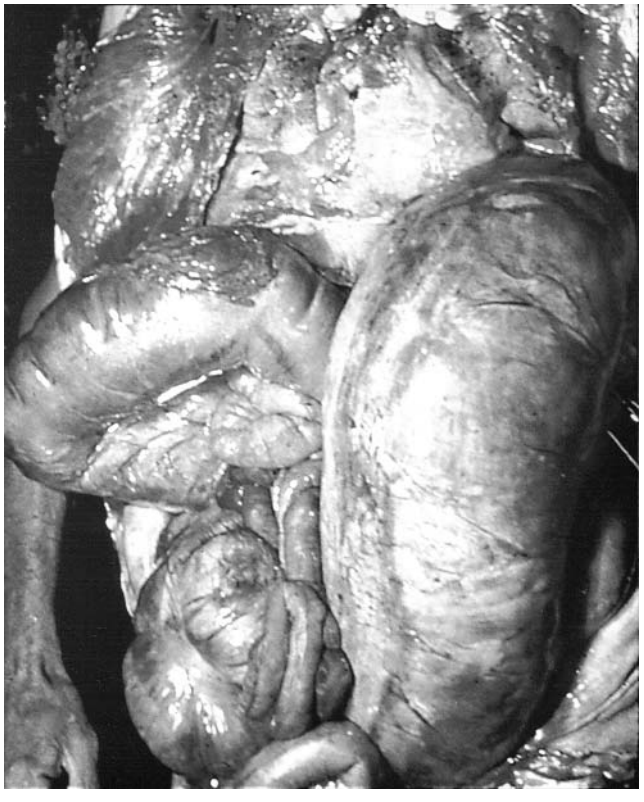

Fig. 17.11. Chagasic megacolon. Marked enlargement and elongation of the large intestine are seen, from the descending colon, and including the transverse colon.

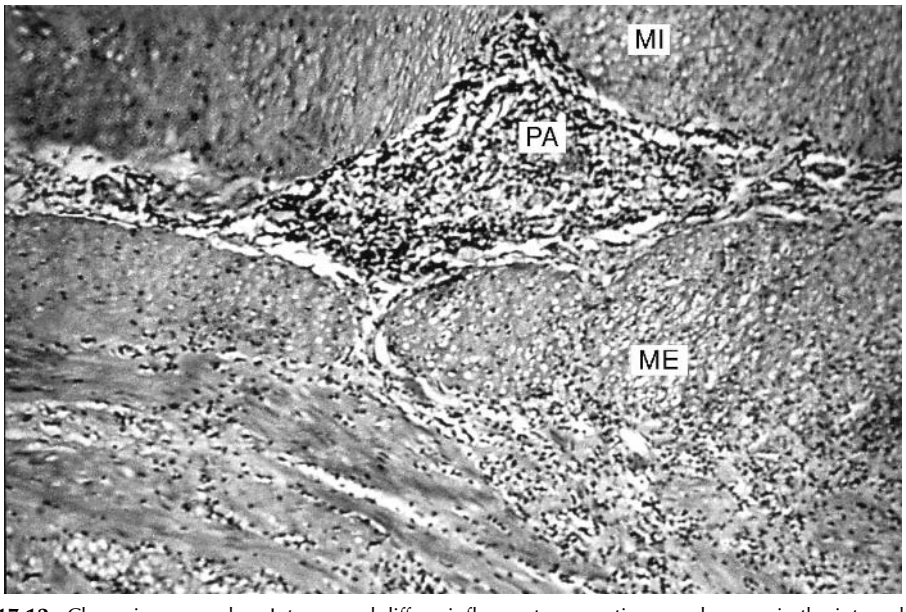

Fig. 17.12. Chagasic megacolon. Intense and diffuse inflammatory reaction may be seen in the internal (MI) and external (ME) muscular layers and in the Auerbach plexus (PA). HE stained section, 200×.

aspect is normal. In the muscle itself, foci of myositis are observed, of varying intensity, with degeneration and necrosis of the myocytes and focal or diffuse intramuscular fibrosis, related directly or indirectly with foci of the myositis. Histological analysis of the mucosa shows lesions arising from the stasis or reflux (acanthosis, inflammation etc.). Amastigote forms of *T. cruzi*, forming nests, are observed, leading to the conclusion that parasitism does not appear to be as rare as once believed. The remaining findings confirm the macroscopic alterations.

Histochemical analysis of the oesophagus and colon (Adad, 1996; Lemos *et al.*, 1998) of chagasics with 'megas' showed infiltrates with a predominance of $TCD3^+$ lymphocytes and $CD68^+$ cells with macrophage morphology. Quantitative analysis of the T lymphocyte (LT) subpopulations has shown a greater number of $CD4^+$ in relation to the number of $CD8^+$ cells (in contrast to that observed in chronic chagasic myocarditis). The presence of TIA-1 cells suggests that cytotoxic mechanisms play a role in the pathogenesis of the 'megas'. Evaluation of the mononuclear cell phenotype in the peripheral blood of chagasics at an advanced stage of the disease and with 'megas' has demonstrated a significant decrease in the absolute number of $TCD3^+$ and $BCD19^+$ lymphocytes. An inversion of the CD4:CD8 ratio has also been observed, due to the reduction in the absolute number of $LTCD4^+$ in the blood, associated with the progression of the disease. Decrease in the absolute number of $TCD4^+$ has also been detected, especially in the group of cells coexpressing CD28. These data suggest that the reduction in the $TCD4^+$ and $C28^+$ cells may be associated with the development of the chagasic 'mega'.

Ultrastructural studies of the oesophagus and colon have confirmed the light microscope observations and added others, prominent amongst which are the quantitative and qualitative alterations of the vesicular component (dense granular and agranular vesiculae) of the neurones of the myenteric plexi, which represent the morphological substrate of various pharmacologically active substances. To date, nine types of morphologically diverse neurones have been distinguished and no fewer than 11 active substances, which are considered to be direct or indirect neurotransmitters. Amine precursor uptake and decarboxylation (APUD) sys-

tem cells of the gastrointestinal mucosa also produce many of these; some decrease and others increase in CD. All these data suggest that the autonomic nervous system (ANS), including the non-cholinergic and non-adrenergic, may have an active participation in the pathogenesis of the chagasic 'megas'.

Systematic studies (Adad *et al.*, 1991; Adad, 1996) have also shown that the lesions – especially of the muscle itself and intramural autonomic nervous system (IANS) – occur in MO and MC as well as in the non-ectastic oesophagus and colon; however, these are more intense and significant in the 'megas'. The same studies have confirmed that there is no MO without marked denervation of the IANS and showed the existence of non-ectasic oesophagopathy in patients with aganglionosis or intense hypoganglionosis of the myenteric plexus. In MC, the denervation does not always reach the intensity observed in MO; however, in general, in the non-ectasic colon the denervation is < 50%.

MO may arise at any age, with cases reported as young as 2 and up to 86 years old. The majority of cases are observed in the 29–40 age range and with a greater incidence in males than in females. Symptoms of MO usually present after the chronic phase and only in exceptional cases are these registered soon after the acute phase. In general, the oesophagopathy antecedes the cardiopathy in the natural history of human CD.

The main symptom of oesophagopathy, especially in MO, is dysphagia with odynophagia, followed by other symptoms such as epigastric or retrosternal pain, regurgitation, pyrosis, hiccuping, coughing and sialosis. The MO is frequently accompanied by hypertrophy of the salivary glands (mainly parotid) and malnutrition. Radiological examination is the most important method for the diagnosis of MO, which presents with various aspects, ranging from the slightest motor impairment to major dolichomegaoesophagus.

Whilst MO does not cure spontaneously, it may remain stationary. Its course is intermittent, with periods of dysphagia interspersed with others of improvement. Besides the oesophagitis, which is a frequent complication (principally after certain surgical treatments), hyperplastic and dysplastic modifications (acanthosis, scaly metaplasia etc.) of the epithelium of the mucous membrane appear. It is probable that these lesions predispose to oesophageal cancer, which occurs most frequently in this group of patients.

MC is observed mostly in adults between 30 and 60 years of age and principally affects males. Its diagnosis is reached later than MO, since obstipation, its most characteristic albeit inconstant symptom, is commonly found in other conditions, such as simple functional disturbances. The obstipation begins insidiously, requiring constant use of laxatives. Besides this, meteorism, difficulty in defecating, abdominal pain, halitosis, anorexia and asthenia can be observed. The frequent association with MO aggravates the malnutrition. Spontaneous cure of MC has not been reported. Those with this visceromegaly may die of cachexia, due to the chronic intestinal ectasia that it causes. The three most serious complications of MC are: (i) intestinal obstruction; (ii) torsion or volvulus; and (iii) perfuration. The most common is acute intestinal obstruction secondary to either formation of large faecaloma (resulting from the chronic faecal stasis) or to torsion of the segment. The perforation may be secondary to the ischaemic alterations or a consequence of ulcers of the mucous colon (decubitus ulcers), which corrode the wall. These ulcers frequently result from the pressure exerted by the faecaloma against the intestinal wall but may be secondary to ischaemic problems. At autopsy, the most frequent complications observed are: (i) decubitus ulcer without perforation or peritonitis (28.6% of the cases with complications); (ii) decubitus ulcers with perforation and peritonitis (40%); (iii) ulcers without perforation but with peritonitis (5.7%); and (iv) volvulus (25.7%). Cancer of the colon does not appear to occur with great frequency among chagasics with MC.

The morphological basis of clinical manifestations in chagasic 'megas' The lesions of the nervous plexus cause hypersensitivity of the digestive tract organs, which, in the presence of cholinergic substances, exhibit

an exaggerated motor response (Cannon's law), demonstrated in chagasics for both the oesophagus and the colon. According to some authors, when the neuronal destruction has affected 50% of the neurones of the myenteric plexus of the oesophagus, it disorganizes all the organ's motor activity, and if this destruction reaches 90% it causes progressive dilatation. In the colon, the dilatation begins when 55% of the myenteric neurones have been destroyed. However, recent studies have shown cases of non-ectasic oesophagus in chronic chagasics with marked or total denervation of the myentric plexus. It is necessary to establish conclusively the cause-and-effect relationship between denervation and the 'megas'. This would clarify questions such as whether the lesions of the IANS occur uniformly along the length of the entire viscera, the occurrence of eventual sphincteral lesions (as suggested by physiopathological studies but not proven morphologically) and the role of the alterations of the intrinsic reflex, which together with the extrinsic reflex (which involves the autonomic enervation) controls oesophagogastrointestinal movements. As examples of the multiple and obscure aspects of the physiopathology of the digestive manifestations seen in chronic chagasics, one can cite the dyskinesia and stasis that occur in the MO and MC. It is still unknown whether the stasis results from the dyskinesia or alterations of the sphincter. It appears that the motor discoordination depends on various factors and more complex mechanisms, such as: (i) alteration of the cholinergic and adrenergic enervation; (ii) quantitative and qualitative alterations of the non-adrenergic and vesicular components (disordered synthesis of the P substance etc.); and (iii) alterations of the APUD system and of the paraganglial chemoreceptor system, both of which are intimately related to the intrinsic peristaltic reflexes of the digestive tract.

Nervous form

There is doubt over the existence of the nervous form (NF) of CD. From the anatomopathological point of view, systematic studies indicate that the morphological basis is lacking, at least at the light microscope level, for this so-called NF of human CD. On the other hand, some isolated studies have demonstrated qualitative and quantitative lesions of certain structures, such as the dorsal nucleus of vagus nerve, hypoglossus and anterior hypothalamus in chronic chagasics, even in the absence of cardiac insufficiency. The presence of focal inflammatory lesions and parasitism in the brain of some chronic chagasics at autopsy is also unquestionable.

In order to clarify as convincingly as possible the existence or not of a morphological substrate for NF in CD, anatomoclinical correlation studies appear indispensable in chagasics with: (i) motor sensitive polyneuropathy (the morphological alterations of which may be situated in the spinal medulla); (ii) alterations of the metabolism of carbohydrates and lipids; and (iii) evident laboratory and clinical signals of impairment of the central nervous system. A clear conclusion regarding this matter may only be reached after such an analysis.

Forms with acute exacerbation (reactivation)

When not interrupted by sudden death, the course of the chronic phase of CD is habitually slow or stationary. In 1911, Carlos Chagas wrote that, in some chronic chagasic cardiopathy, symptoms arise that are similar to those observed in the acute or subacute phase, leading to an unfavourable prognosis. Chagas denominated this clinical form 'chronic chagasic cardiopathy with acute exacerbation'. During the last few decades, various events such as the use of cytostatic agents and immunosuppressors, the progress in organ transplantation and, especially, the advent of AIDS have established conditions for reactivation of infection by *T. cruzi*. This prompted some to revive the old concept of acute exacerbations of CD, proposed by Chagas in 1911 but discarded by him in 1916. The Tenth Annual Conference of Applied Research into Chagas Disease, held in Uberaba (Brazil) in 1994, recommended the use of reactivation to denominate this form of CD.

The most serious and frequent manifestation of this form of CD is a peculiar focal necrotizing chagasic meningoencephalitis. This has been described in approximately 80% of the fatal cases. This type of focal necrotic meningoencephalitis was only described, until now, in immunodeficient chagasics. Another frequent finding in this group of patients is an acute type of myocarditis with intense parasitism. In over half of immunodepressed chagasics with AIDS, *T. cruzi* is detected in circulating blood (Rocha *et al.*, 1994, 2000).

Other manifestations

Parasitism and inflammations have been described in various other organs of chronic chagasics, such as the adrenals and skeletal muscles, but their significance has not been clarified. The frequent detection of parasites in the wall of the central vein of the suprarenals in chronic chagasics appears relevant to a better understanding of the pathogenesis of the natural history of CD. Placental lesions, independent or whether or not there is congenital transmission of the infection, have been described (see next section). In other organs, important lesions occur due to the thromboembolic phenomena or cardiac insufficiency.

Congenital Chagas Disease

Chagas disease may cause abortion, premature birth or intrauterine growth retardation as well as stillbirth and neonatal death (Bittencourt, 1995, 2000). According to the literature, prevalence varies not only from country to country but also between the regions within each country. In Santa Cruz de la Sierra, Bolivia, CD has been reported in 14.8% of neonates from mothers selected at random, whilst in Bahia the figure is 10.5% and in other areas of Brazil, such as the Triângulo Mineiro region (Minas Gerais State) and in the municipality of Ribeirão Preto (São Paulo State), the prevalence is very low to non-existent.

T. cruzi parasitizes the Hofbauer cells in the villous stroma, after having actively passed through the chorium. According to some authors, the placenta must present prior lesions or functional alterations that facilitate penetration by the parasite. On the other hand, the possibility must be considered that a more virulent or infectious *T. cruzi* strain may be involved – a fact that would explain the great geographical variability in the prevalence of this form of CD. From the Hofbauer cells, *T. cruzi* reaches the fetal circulation and may parasitize other cells of the organism, though it is most frequently detected in the myocardium, intestines, brain, skin, skeletal musculature and in the cells of the mononuclear phagocytic system. Macroscopically, the placenta has an increased weight and volume, is pallid, with voluminous cotyledons, succulent and, in general, whitish. The fetal side has a milky and congestive aspect. The villous layer is pallid and friable with extensive compact areas, substituting the normal spongy aspect. The picture is similar to that seen in syphilis and in haemolytic disease of the neonate. Microscopically, in some cases there is disorganization of placental parenchyma and the lesions are abundant, disseminated and show granulomatous villitis and perivillitis associated with necrosis and heavy parasitism of the Hofbauer cells. The inflammatory reaction shows predominance of mononuclear cells, often associated with neutrophils. In 50% of these placentas the inflammation is also observed in the chorionic plate. In the placentas of newborns with asymptomatic or less severe infection, focal villitis and perivillitis are seen without parasites or with rare parasites. In the umbilical cord and in the extraplacental membranes, amastigote forms of *T. cruzi* may be seen. According to some, the finding of amastigotes in the villous stroma constitutes evidence of congenital transmission, but others do not agree with this conclusion.

In fatal cases, the principal alterations observed at autopsy of the fetus are: (i) hydrocephalus, maceration and dead fetus within the uterus (similar findings are seen in syphilis and erythroblastosis foetalis); (ii) hydrothorax, ascites, hepatosplenomegaly and micropolyadenopathy; (iii) pneumonitis (seen in about 25% of the autopsied cases of

CD); and (iv) presence of parasitized giant cells with a single and hyperchromatic nucleus. In these cases the most frequent causes of death are carditis, meningoencephalitis or intercurrent infection.

It is important to highlight that the transmission of *T. cruzi* from mother to child does not occur exclusively by transplacental means. Another method of transmission, albeit very rare, is through maternal milk.

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18 Pathogenesis of Animal Trypanosomiasis

Katherine Taylor and Edith M.-L. Authié

Clinical and Pathological Features

Knowledge of the clinical and pathological responses to trypanosomiasis infections in domestic animals is derived from more than 100 years of observation of the disease in the field. This knowledge has been supplemented by studies of cattle, goats, sheep, buffalo, horses, pigs and camels infected experimentally with *Trypanosoma brucei brucei, T. congolense, T. vivax, T. evansi, T. equiperdum* and *T. suis*. Many of the clinical and pathological manifestations of trypanosomiasis are common to domestic animals, irrespective of the species of trypanosome involved. None the less, the range and severity of the pathological effects are influenced by a variety of factors, which are discussed below and are summarized in Table 18.1.

Distinct pathological changes may be caused by the different livestock-infective trypanosome species. Pathology in tissues is associated with the relative ability of the parasites to invade extravascular spaces and organs. For example, whereas *T. congolense* remains confined to the vascular system, trypanosomes of the *Trypanozoon* group (*T. b. brucei, T. evansi* and *T. equiperdum*) are distributed in both the circulation and in the tissues. *T. vivax*, although primarily a vascular parasite, has also been found in extravascular locations. Furthermore, there is remarkable intraspecies variation in the pathogenicity of different parasite stocks, especially stocks isolated from distinct geographical regions. Some East African isolates of *T. vivax* may cause an acute haemorrhagic disease in cattle, in contrast to a milder non-haemorrhagic disease that results from infection with most West African *T. vivax* isolates.

A number of host factors also contribute to determining the severity of disease. For example, the wildlife of Africa is generally more resistant than domestic ruminants and often serves as a reservoir for human- and livestock-infective trypanosomes. In contrast, exotic imported ruminants (e.g. improved dairy cattle) are more severely affected than local genotypes, which exhibit a range of breed and individual susceptibility. The physiological status of the host, as well as nutritional and environmental factors, also play important roles in modulating the severity of trypanosomiasis.

This section concerns salivarian trypanosomes that are infective and pathogenic for domestic animals. *Trypanosoma theilerii*, a mechanically transmitted trypanosome that is widespread in cattle around the world but does not cause clinical disease by itself, will not be considered further. Pathogenic trypanosomes are responsible for at least three pathological entities that have been given the vernacular names nagana (African trypanosomiasis), surra and dourine.

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Table 18.1. Host range and pathogenicity[a] of African trypanosomes of domestic animals.

			Degree of severity[b]								
Subgenus and species	Transmission[c]	Disease	Cattle	Goats	Sheep	Water buffalo	Horses	Donkeys	Camels	Pigs	Dogs
Trypanozoon											
T. b. brucei	Tsetse	Nagana	Mild	Mod	Mod	–	Severe	Severe	Severe	Mild	Severe
T. evansi	Mech	Surra	Mod	Mild	Mild	Severe	Severe	Mild	Severe	Mild	Severe
T. equiperdum	Venereal	Dourine	–	–	–	–	Severe	Mod	–	–	–
Nannomonas											
T. congolense	Tsetse	Nagana	Severe	Mod	Mod	–	Mod	Mod	Severe	Mild	Mod
T. simiae	Tsetse/Mech	Nagana	–	Mild	Mild	–	–	–	–	Severe	–
Duttonella											
T. vivax	Tsetse/Mech	Nagana[e]	Severe	Mod	Mod	–	Mod	Mild	Mod	–	–
Pycnomonas											
T. suis[d]	Tsetse	Nagana	–	–	–	–	–	–	–	Severe	–

[a]Under field conditions, but may be modified by many factors.
[b]Degree of severity: Mild = mildly pathogenic; Mod = moderately pathogenic; Severe = Severely pathogenic, may cause acute disease.
[c]Mech = mechanical.
[d]Rare.
[e]Also causes trypanosomosis in South America, where the term nagana is not used.

African trypanosomiasis (nagana) and South American *T. vivax*

Nagana is a group of diseases of ruminants, camels, equines, swine and carnivores in Africa caused by tsetse-transmitted *T. congolense, T. vivax, T. simiae, T. b. brucei* and *T. suis*.

In South America, trypanosomiasis due to infection with *T. vivax* is predominantly a disease of cattle but sheep, goats, horses and water buffalo can also be infected. *T. vivax* infection appears to be endemic throughout much of South America but clinical disease is usually associated with either the introduction of infected 'carrier' animals into a new area or as a result of stress-induced breakdown of carrier status. The pathogenicity of South American stocks of *T. vivax* is very similar to that of West African *T. vivax.* In some South American countries a wasting syndrome known as *secadera, huequera* or *caucho hueco* is blamed on *T. vivax*. It is assumed that, in South America, *T. vivax* is spread by mechanical transmission by various biting insects.

General signs and lesions

The prepatent period, i.e. the time from intradermal inoculation of metacyclic forms to the detection of parasitaemia, is usually 1–3 weeks, depending on the virulence of the infecting trypanosome, the infective dose and the immune status of the host. Experimental infection with African trypanosomes typically follows three successive stages: acute, stabilization and chronic. Under natural challenge, however, the scenario may be more complex.

The early acute phase of the disease is characterized by the continuous presence of trypanosomes in the blood at detectable concentrations (10^3–10^8/ml). Fever is highest at the first peak of parasitaemia and fluctuates thereafter with parasitaemia waves. With the onset of parasitaemia, anaemia develops. Anaemia is the most prominent feature of animal trypanosomiasis and may be observed as pallor of the mucous membranes. The virulence of the infecting parasite population and the age, nutritional status and breed of the host influence the severity of anaemia. Lymph nodes and spleen are enlarged, with greater reactivity of the lymph nodes draining the inoculation site. Weakness, lethargy and loss of condition are patent; abortion and reduced milk production are common. Calves born to infected cows are often small and weak, resulting in high rates of neonatal mortality. In the terminal stages of the disease, animals become extremely weak and are often unable to rise. Death of the infected animal may occur in the first few weeks or months of infection as a result of this acute disease.

In some cases, the clinical condition stabilizes after 6–8 weeks and a slow recovery process begins. More frequently the animals enter into the chronic phase.

In contrast to the acute phase of infection, anaemia in the chronic phase is not strictly associated with the presence of parasites in the blood. Animals may be intermittently parasitaemic or aparasitaemic in this period. Lymph nodes and spleen frequently return to normal size and, in some cases, eventually atrophy and sclerose. Stunting, wasting and infertility are characteristics of cattle suffering from 'chronic trypanosomiasis syndrome'. Regardless of their weakened condition, animals continue to eat. As Murray and Dexter (1988) pointed out in their excellent review of the topic, 'Animals that eat but do not produce are the scourge of the savanna lands of Africa.' The chronic phase may last for months or years and is most often terminated by death.

There is no pathognomonic lesion in trypanosome-infected animals. The chancre, a local skin inflammatory lesion, may develop within a few days at the site where parasites are inoculated. The lesion appears as a swelling, several centimetres in diameter, that is warm and painful to the animal. The host and its immune status, the parasite, the rate of parasite multiplication and the inoculating dose may all influence chancre development and cellular composition of the lesion. None the less, congestion, oedema, neutrophils, lymphocytes, macrophages and mast cells are generally characteristic. The chancre is not prominent under natural challenge conditions, nor is it required for the establishment of a patent infection.

General lesions are congestive, inflammatory and degenerative, and sometimes haemorrhagic. They may affect various organs: heart, central nervous system (CNS), eyes, testes, ovaries and the pituitary gland. Congestive heart failure is an important cause of death in chronic cases and is related to the combined effects of prolonged anaemia, myocardial damage and increased vascular permeability.

Bovine trypanosomiasis

T. congolense, *T. vivax* and, to a lesser extent, *T. b. brucei* are the major pathogenic species in African cattle (reviewed by Morrison *et al.*, 1981). Mechanically transmitted *T. vivax* infects cattle in South America. The pathogenicity of the three species is variable. Despite being more likely to invade other tissues, such as the CNS, *T. b. brucei* is generally less pathogenic than *T. congolense* and *T. vivax*. A notable exception from this was recorded from western Kenya and Uganda, where *T. b. brucei* and *T. b. rhodesiense* were found in the CNS of native cattle and were associated with significant mortality (Wellde *et al.*, 1989). Whereas *T. vivax* is responsible for relatively high parasitaemias (up to 10^8 trypanosomes/ml of blood), parasitaemia during *T. congolense* and *T. b. brucei* infections is generally lower and in the chronic stages often remains below the threshold of detection using standard techniques. Mixed infections that involve two or three parasite species are frequent in areas of medium to high tsetse challenge.

All modes of disease evolution may occur in cattle, from the hyperacute syndrome leading to death in 3–4 weeks, to a chronic disease lasting for years. Nevertheless, the most common form of disease in endemic areas is chronic.

The early acute phase is characterized by a continuous drop of the haematocrit value (packed red cell volume, PCV), haemoglobin concentration and red blood cell numbers. The PCV may fall from a normal value of 30% or above to 15% after 2–3 months of infection with *T. congolense* or *T. vivax*. The degree of anaemia is correlated with the loss of productive performance of trypanosome-infected livestock. Trypanosomiasis generally induces a more severe anaemia than the other common causes of anaemia in endemic areas, such as anaplasmosis, babesiosis, tick infestations, helminth infections and malnutrition. In addition, differential diagnosis is not particularly difficult if epidemiology is taken into account. Many researchers agree that anaemia may be one of the major contributors to death in trypanosome-infected cattle.

In cattle that do not die from acute disease, the PCV stabilizes after 6–8 weeks and may progressively regain normal levels within a few months, while parasitaemia becomes intermittent. Clinical recovery may occur in the most resistant individuals infected with parasites of moderate virulence and kept under favourable environmental conditions. However, in cattle of susceptible breeds, the PCV may fluctuate at low levels for months or years. Chronically infected cattle develop cachexia; their productivity (milk, growth and draught) is impaired and reproductive performances are diminished. The chronic phase may lead to death, to self-cure, or to a persistent carrier state. Cattle can die with or without signs of acute disease.

Super-infections with diverse trypanosome stocks and species often occur under high tsetse challenge and can overwhelm the resistance of the host. In addition, trypanosome-infected animals are more susceptible to infection with other pathogens; thus the additive deleterious effects of concurrent infections may result in the death of an otherwise resistant host. Exposure to harsh environmental or nutritional conditions, or a particular physiological status (exhaustion, lactation and parturition), also negatively affects survival of chronically infected cattle.

The effect of trypanocidal treatment on infected cattle depends on the stage of infection. In the early phase, treatment usually results in rapid clinical and haematological recovery. In contrast, during the longstanding chronic diseases, despite clearance of parasites by drug therapy, clinical recovery may be very slow.

Haemorrhagic *T. vivax* stocks have been isolated from East Africa. These stocks cause a hyperacute disease, characterized by high parasitaemia, severe anaemia and haemorrhages, which have been related to intravascular disseminated coagulation (DIC). Cattle may die within 2 weeks or, under favourable conditions, rapidly self-cure after 2 months.

Small ruminants

Although susceptible to the same parasite species as those infecting cattle, local breeds of sheep and goats generally exhibit fewer clinical manifestations of trypanosomiasis. None the less, trypanosome infection can have significant effects on the health and productivity of small ruminants kept under moderate to high risk (Osaer *et al.*, 1999). Even the most resistant breeds suffer reduced PCV levels, depressed weight gains, longer parturition intervals and higher neonatal mortality. As with other hosts, the negative effects of trypanosomiasis are exacerbated by helminth infections and malnutrition.

Camels

T. vivax infection of camels is most often a result of the mechanical transmission of the parasites by biting flies and is usually chronic. Tsetse-transmitted infection rarely occurs in camels but *T. congolense* and *T. simiae* cause an acute, potentially lethal disease. *T. b. brucei* is responsible for a syndrome similar to surra.

Horses

T. b. brucei is highly virulent in horses, causing an acute or subacute disease leading to death in 2 weeks to 3 months. Most characteristic is the development of subcutaneous oedema on the ventral abdominal wall, thorax and limbs, development of ocular signs (keratoconjunctivitis) and neurological disorders leading to ataxia and paralysis. Infections with *T. vivax* or *T. congolense* are usually chronic and may cure spontaneously in local breeds.

Donkeys

While donkeys may also suffer from acute disease due to infection with *T. b. brucei*, they are less susceptible than horses to other trypanosome species. *T. vivax* infection, in particular, appears to be asymptomatic.

Carnivores

Carnivores are refractory to infection with *T. vivax*. The most frequent pathogenic trypanosome species in dogs and cats is *T. b. brucei*, which is responsible for an acute disease with high parasitaemia and fever, oedema, keratitis (inflammation of the cornea) and, characteristically, a neurological syndrome that resembles rabies and is terminated by death within a few weeks. Dogs are also susceptible to *T. congolense*, which may cause a chronic disease with ocular signs (keratitis, uveitis and blepharoconjunctivitis) and oedema (head, thorax and forelimbs). Less frequently, this parasite causes an acute syndrome in imported dogs, with fever, prostration, severe anaemia and death in 2–3 weeks.

Swine

Domestic swine are refractory to *T. vivax* and moderately susceptible to *T. congolense* and *T. b. brucei* infections, which mainly affect reproductive performance (abortions). *T. simiae* is responsible for periodic outbreaks of a severe, acute or hyperacute disease that may kill pigs in only 2 days to 4 weeks of high parasitaemia, with no specific signs. *Trypanosoma godfreyi* infects the same hosts as *T. simiae*, but is less pathogenic and only causes a subacute disease in piglets. *T. suis* is a rarely reported swine-specific trypanosome that severely affects piglets in the form of a subacute disease leading to death in 2–3 months, while adults develop a chronic and mild form.

Surra

Surra is a disease caused by *T. evansi* which is widely distributed in Asia, South America

and Africa. While the name surra is most commonly used, the disease affecting horses in South America has local names, such as *mal de caderas*, or *tristeza*. *T. evansi* is mechanically transmitted by bloodsucking insects, mainly of the genera *Tabanus* and *Stomoxys*. In South and Central America, *T. evansi* can also be transmitted by vampire bats (*Desmodus rotundus*), which act as both reservoirs and vectors. A wide variety of domestic animals is affected, including equines, camels, cattle, water buffalo (*Bubalis bubalis*), small ruminants, carnivores and pigs. The disease is most severe in camels, horses, water buffalo and dogs; cattle are only moderately affected and infections of pigs are usually asymptomatic. *T. evansi* infection is one of the most frequent diseases of camels in northern Africa, where it causes severe economic losses.

The disease can be acute in young animals and pregnant females, which die within a few weeks, but the usual form in endemic areas is a chronic one that lasts for years and leads to cachexia and death. Some individuals that survive chronic infection for years may eventually self-cure. Clinical signs of surra are intermittent fever, anaemia, emaciation, oedema, conjunctivitis and lacrymation, enlargement of the lymph nodes and spleen, impaired motor function and abortions. Neurological signs may be seen in the late stages. In camels, *T. evansi* infection is sometimes complicated by pulmonary infections. In horses, the African and Asian forms of the disease are severe and resemble that caused by *T. b. brucei*; oedema and impaired motor function are usual. The South American form (*mal de caderas*) is less severe and causes a chronic syndrome. Donkeys undergo chronic or asymptomatic infection and may act as reservoirs. Dogs in South America and Asia develop an acute disease with ocular, vascular and neurological signs, also similar to *T. brucei* infection in Africa. Cattle are often asymptomatic carriers in South America, whereas they develop chronic forms of the disease in Asia. Water buffalo develop acute or subacute infections that lead to death within a few weeks in 5–15% of the cases.

Dourine

Dourine is a venereally transmitted disease caused by *T. equiperdum* that exclusively affects equines and has a wide geographical distribution (reviewed by Barrowmann *et al.*, 1994). The disease is endemic in North Africa, the Middle East, Eastern Europe, South America and Indonesia. Despite being closely related to *T. evansi*, the two species localize in different tissues of the host and exhibit different pathogenicity. *T. evansi* and other 'African' trypanosomes are essentially blood parasites that cause systemic infections; *T. equiperdum* is rarely detected in the bloodstream and normally localizes in the capillaries of the mucous membranes of the urogenital tract. The parasites present in the seminal fluid and on the mucous membranes are responsible for direct contamination during coitus.

Horses are very susceptible to *T. equiperdum* and usually die at the end of a chronic disease that may last for 1–2 years. Occasionally, acute forms that lead to death in 2–3 months are seen in thoroughbred horses. Donkeys and mules, despite being susceptible to infection, develop a mild syndrome or remain asymptomatic.

The incubation period in horses ranges from 1 week up to 6 months. There are typically three clinical phases. The early signs consist of oedema of the genital area and genital organs, which may spread to the ventral abdominal wall. Petechiae and ulceration of the genital mucosae are seen at this stage. The second phase begins 30–40 days after infection and is characterized by urticarial plaques on the skin and enlargement of the lymph nodes. This coincides with the appearance of general signs, namely fever, anaemia, weight loss and possibly abortions. Motor functions may be impaired and neurological disorders are frequent. The plaques last for a few days only and are characteristically round, swollen and exudative. Later in infection, the neurological signs worsen, leading to a paralytic syndrome. Polyneuritis and neuronal degeneration are the main microscopic lesions. Animals that die from dourine are usually severely emaciated and anaemic.

Infection Process and Pathogenesis

Cattle, sheep and goats infected either naturally or experimentally with *T. congolense*, *T. vivax* or *T. b. brucei* represent the most well-studied natural host–African trypanosome relationships, due possibly to the significant economic losses imposed by these infections in sub-Saharan African. Consequently, the following text refers to evidence derived mainly from those host–parasite interactions.

African trypanosomes are transmitted to large and small ruminants following cyclical development in the tsetse fly vector or mechanical transmission on the mouthparts of biting flies, depending on the species of trypanosome. Trypanosomes undergo a period of multiplication in the skin. From the dermis and subcutis the rapidly dividing parasites enter the afferent lymphatics, are transported through the regional lymph node, and via the efferent lymph to the thoracic lymph duct and finally the blood. Once in the blood the parasites have access to most other major organs of the body. Valli and Forsberg (1979), Masake and Morrison (1981), Omeke and Ugwu (1991), Anosa *et al.* (1992, 1997a,b) and Mwangi *et al.* (1991, 1996) gave descriptions of histological changes in the major organs of trypanosome-infected livestock. The following sections are arranged to follow the course of the infection and the subsequent development of the immune response and pathogenesis through the host.

Skin and afferent lymphatics

Tsetse-transmitted and mechanically transmitted trypanosomes gain entry through the skin barrier of the vertebrate host as an infected tsetse fly or a biting fly takes a blood meal. Trypanosomes deposited in the dermis undergo a period of extensive multiplication. While there are no macroscopic lesions within the first few days, significant histological changes, characterized by extensive oedema and changes in mast cells indicative of degranulation, are occurring in the skin.

The chancre may be observed at the site where the tsetse bites the host within 4–10 days (in some cases up to 2–3 weeks) and precedes the detection of trypanosomes in the blood using standard techniques by at least 4–6 days. The chancre appears as a hot and painful swelling, several centimetres in diameter. Parasite multiplication and the inoculating dose may influence chancre development and cellular composition of the lesion but congestion, oedema, neutrophils, lymphocytes, macrophages and mast cells are generally characteristic. The inflammatory nature of the chancre and the timing of the response suggest that, during primary infections, the host's innate (non-adaptive) immune response is triggered by the parasite.

Metacyclic and bloodstream-form trypanosomes are covered by a variable surface glycoprotein (VSG) coat. The conformational structure of the VSG coat determines the parasite's serologically defined variable antigenic type (VAT). Populations of trypanosomes with the same repertoire of VATs represent a serodeme. Although the VAT of the metacyclic trypanosome population that is transmitted by the tsetse fly vector is heterogeneous, the range of metacyclic VATs is limited – estimated at fewer than 20.

Immunity to reinfection with parasites expressing the same metacyclic VATs is associated with reduced chancre development. Thus the control of parasites in the skin of animals undergoing secondary infections appears to be mediated by an adaptive VSG-specific immune response and a limited inflammatory response (Akol and Murray, 1985). Immunization with VSG or with intact or irradiated trypanosomes can protect against infection with parasites expressing the homologous VAT or VAT repertoire. Thus the significance of metacyclic VAT-specific immunity to animals in the field is related to the number of parasite serodemes circulating in the population and the frequency of exposure to specific serodemes. Animals exposed repeatedly to the same serodeme can develop metacyclic VAT-specific immunity.

In contrast, the number of bloodstream VATs for any given serodeme of *T. b. brucei* or *T. congolense* is virtually limitless – in the range of several hundred to 1000; consequently,

immunity to the complete repertoire of bloodstream VATs is unlikely. In contrast, *T. evansi* and *T. vivax* are thought to have far more limited VSG repertoires than *T. congolense* and *T. b. brucei*. Evidence from the field suggests that serodeme-specific immunity to bloodstream VATs of *T. vivax* does occur.

After a period of multiplication in the skin, trypanosomes begin to appear in extremely large numbers in the afferent lymph that drains from the site of challenge. During this time the cellularity of the afferent lymph increases significantly and is characterized by $CD4^+$ and $CD8^+$ lymphocytes, and an increased proportion of lymphoblasts and B cells.

Draining lymph node and efferent lymphatics

Trypanosomes enter the lymph node draining the site of tsetse fly challenge. The lymph node is profoundly enlarged, due mainly to cellular proliferation in B-cell areas and migration of leucocytes from the chancre. Histologically, enlarged lymph nodes show marked expansion of lymphoid follicles and germinal centres in the cortex and in the medulla, where the B-cell and macrophage populations are expanded significantly. The paracortical areas (T-cell areas) are relatively reduced but contain numerous macrophages. Medullary and cortical sinuses are markedly distended with macrophages, lymphocytes and lymphoblasts, while the medullary cords contain numerous lymphocytes and plasma cells.

Despite the early activation of the draining lymph node, by day 30 after infection the lymph nodes contain fewer B-cell follicles. The paracortical areas are still sparsely populated with T cells and high numbers of $CD8^+$ cells are present in the medulla. As the disease becomes chronic, the lymphoid follicles become depleted.

Parasites enter the efferent lymphatics approximately 1–2 days after being detected in the afferent lymphatics. Cellular output is increased greatly and coincides with chancre development and the onset of patent parasitaemia. Similar to the efferent lymph, the proportion of lymphoblasts is increased and B cells predominate.

Blood

The timing of the appearance of parasites in the skin, lymph nodes and lymphatics suggest that trypanosomes enter the peripheral blood within 5–6 days of infection. None the less, probably due to their dilution in a large blood volume, parasites are not detected until the second week of infection. The first peak of parasitaemia usually occurs within the first 2–3 weeks of infection. The kinetics and degree of parasitaemia are highly variable, influenced by both the virulence of the parasite and the innate and acquired resistance of the host (see section on immune responses, below).

The first peak of parasitaemia also coincides with the appearance of trypanosome-specific antibodies in the blood. Titres usually peak 3–4 weeks after infection. Very high quantities of serum IgM typically characterize trypanosome-infected animals. The mechanisms that mediate the massive increase in B cells and IgM are not known.

Blood chemistry changes of trypanosome-infected animals are not well documented. The most comprehensive studies were done in *T. congolense*-infected sheep (Katunguka-Rwakishaya *et al.*, 1992, 1996). These animals had lowered cholesterol and lipid concentrations, leading to a reduction in total serum lipids. Plasma levels of zinc, copper, calcium, magnesium and inorganic phosphate were not changed. Similarly, total protein and iron were unchanged. These and earlier studies consistently demonstrated decreased serum albumin and elevated globulin. Hypoglycaemia is associated with periods of very high parasitaemia and again just before death. No consistent pattern of change in the levels of fibrinogen or fibrin degradation products has been detected.

Changes in the cellular and complement components of the blood are discussed in the section on systemic pathology, below.

Spleen and liver

The spleen and the liver are particularly important for their capacity to filter pathogens from the blood, as well as remove

old or damaged erythrocytes. Splenomegaly is a feature of the 'acute' or parasitaemic phase of infection and is mainly the result of red cell and lymphocyte sequestration and an expanded macrophage population.

In the acute phase of infection there are marked changes in both the white and red pulp of the spleen. The first change detected is the expansion of lymphoblasts within the pariarteriolar sheath of the spleen. This is followed by the development of secondary lymphoid follicles with prominent germinal centres, an important site of antibody production in infected animals. At the same time, the red pulp also expands due to a marked increase of macrophages, lymphocytes and plasma cells in the sinuses. Erythrophagocytosis and haemosiderosis may be prominent.

The liver is a major site of erythrocyte and parasite clearance. Kupffer cells phagocytose parasites that are bound by host antibodies. The liver may be enlarged and congested. Kupffer cell hyperplasia and periportal mononuclear cell infiltration are common. In animals with severe anaemia, mild centrolobular necrosis has been reported.

Bone marrow

Trypanosome infection is associated with pancytopenia: reduced red cells, white cells and platelets (see later). All the cellular elements of blood arise from haematopoietic stem cells in the bone marrow. Thus inappropriate bone marrow responses are believed to mediate some aspects of this pathology.

During the preparasitaemic phase of infection, macrophages, lymphocytes and plasma cell numbers increase in the bone marrow of *T. congolense*-infected cattle (Anosa *et al.*, 1997a,b). The acute phase corresponds with the onset of anaemia, leucopenia, and thrombocytopenia in the peripheral blood, and is characterized by a decrease in the percentage of erythroid cells and an increase in granulocytic and lymphoid cells. While anaemia, leucopenia and thrombocytopenia persist in the peripheral blood in the chronic phase, erythroid cells increase and granulocytic and lymphoid cells decrease in the bone marrow. Activated macrophages phagocytose mature cells of the erythroid and granulocytic series and thrombocytes but lymphocytes and more immature cells of these lines are seldom engulfed. During the chronic phase large quantities of haemosiderin are present in macrophages.

Events in the bone marrow of cattle infected with a haemorrhagic strain of *T. vivax* are essentially similar to those described for the acute phase of infection with *T. congolense*. One significant difference may be that trypanosomes are able to invade the bone marrow of *T. vivax*-infected calves, allowing their direct contact with haematopoietic cells (Anosa *et al.*, 1992).

Heart

The heart is consistently damaged in animals infected with *T. congolense*, *T. b. brucei* and *T. vivax*. The lesions are distinct for the three species (Murray *et al.*, 1980). In animals infected by *T. b. brucei* and *T. vivax*, marked cellular infiltrates are found in perivascular and interstitial locations and are composed of lymphoid cells, plasma cells, macrophages and occasional eosinophils. Extravascular parasites have been observed. In contrast, in *T. congolense*-infected cattle, parasites have only been seen in the circulation and the interstitial cellular infiltrate is usually scanty and consists of small lymphocytes and the occasional macrophage and plasma cell. Perivascular and interstitial oedema is common to all three species of trypanosome and is found particularly in terminal cases.

The cardiac microvasculature is frequently distended with cells and the vessel walls are occasionally swollen and vacuolated, but never necrotic. The perivascular oedema possibly reflects increased permeability. In all three infections there is extensive degeneration of the heart fibres. The mechanisms that contribute to this damage probably include anoxia caused by the prolonged anaemia; immune-mediated pathology has also been implicated.

Cattle living under natural range conditions may be required to forage up to 27 km in a day, despite a PCV of less than 20% (Murray *et al.*, 1980). A red-cell deficit of this order must restrict the ability of cattle to forage over these long distances and the effort to achieve this may to lead to cardiac decompensation.

Endocrine and reproductive organs

The endocrine system plays a central role in the regulation of important body functions such as growth, differentiation, reproduction, maintenance of the internal environment and adaptation to changes in the external environment. End-organ dysfunction during infection may be due to an effect along the hypothalamic–pituitary axis or as a result of direct injury to the organ. Again the ability of *T. b. brucei* to invade tissues and the confinement of *T. congolense* to the vasculature are factors that influence the types of lesions inflicted by these parasites.

Extravascular localization of *T. b. brucei* in pituitaries of infected sheep is associated with focal coagulative necrosis and interstitial mononuclear infiltration. *Trypanosoma congolense* were found in the microvasculature of the pituitaries of cattle, which was highly distended with trypanosomes, erythrocytes, macrophages, cellular debris and pituitary cell secretory granules (Abebe *et al.*, 1993). The authors demonstrated an inability of the pituitary to respond to stimulation from corticotropin-releasing factor (CRF). Reduced plasma thyroxine concentration in infected animals appears to be due to pituitary dysfunction, as the thyroid itself showed no pathology. In contrast, hypothalamic, and not pituitary, dysfunction was suggested in female goats experimentally infected with *T. congolense*, in which the production of gonadotropin-releasing hormone (GnRH) was compromised (Ng'wena *et al.*, 1997). End-organ pathology was demonstrated in infected animals that displayed enlarged adrenal glands, which were characterized by inflammatory changes (Ogwu *et al.*, 1992).

Reproductive disorders frequently occur in animals infected with trypanosomes (reviewed by Ikede *et al.*, 1988). Inflammation of the testicles occurs in male animals infected with *T. b. brucei*. Parasites localize in the scrotal skin and hydrocoel fluid and also invade the tunica vaginalis, testis, epididymis and spermatic cord. These lesions lead to degeneration of the seminiferous tubules, aspermatogenesis and aspermia and in severe cases bulls become infertile. Decline in semen quality and quantity and spermatozoal abnormalities were observed in goats infected with *T. evansi*. The most severely affected became totally aspermic and their testicles atrophied (Ngeranwa *et al.*, 1991).

Males infected with *T. vivax* and *T. congolense* also suffer from infertility but the mechanisms appear different. Inflammatory changes in the genital organs are usually mild or absent but there is progressive and marked testicular degeneration that can lead to atrophy (Sekoni *et al.*, 1990). The cause is believed to be prolonged fever, thrombosis of spermatic blood vessels and the general wasting of body organs. Bulls infected with *T. vivax* and *T. congolense* may have semen of poor quality: ejaculates of low volume, low sperm concentration and increased percentage of abnormal sperm. Some of these effects may be due to lack of pituitary stimulation, as noted above for other endocrine organs.

Abortion, long intervals between calving, irregular oestrous cycles, poor milk production and infertility are common in chronically infected cows and ewes. Although abortions are often reported from cows infected with trypanosomes, parasites are rarely detected in the fetus. Persistent corpus luteum, a condition normally associated with endometritis, occurs in *T. congolense*-infected cattle and goats. Cystic ovaries and endometritis have been reported from cattle infected with *T. vivax*.

It is not fully understood whether or what types of damage to the endocrine and reproductive organs are reversible.

Central nervous system

CNS involvement in humans infected with *T. b. rhodesiense* or *T. b. gambiense* is responsible for the clinical manifestation of sleeping

sickness. Although this syndrome is not frequently observed in livestock infected with trypanosomes, severe meningoencephalitis due to *T. b. rhodesiense* and *T. b. brucei* infection has been documented in cattle and goats (Morrison *et al.*, 1983; Wellde *et al.*, 1989). The authors described the pathological changes in the brains of infected cattle as similar to those described for fatal cases of human trypanosomiasis. There was extensive infiltration into the meninges and perivascularly throughout the brain and spinal cord of cells composed predominantly of lymphocytes, plasma cells and macrophages. Severe meningoencephalitis was also observed in pigs that were infected with *T. b. brucei* and trypanosomes were isolated from the brains of these animals (Otesile *et al.*, 1991). *T. vivax* was reported from the CNS of infected goats (Whitelaw *et al.*, 1988). Although *T. congolense* alone has never been detected in the CNS of infected livestock, CNS involvement was reported in cattle concurrently infected with *T. congolense* and *T. b. brucei* (Masake *et al.*, 1984). *T. evansi* was isolated from the cerebrospinal fluid, cerebrum, cerebellum and pons of cattle in Thailand that died displaying distinct neurological signs (Tuntasuvan *et al.*, 1997).

Immune Responses

Induction of adaptive immune responses

Adaptive immune responses are not initiated in the skin but occur in the peripheral lymph node directly downstream of the site of infection. T cells play a central role in determining the character of the immune response. $CD4^+$, $CD8^+$ and $\gamma\delta$ T cells isolated from the draining lymph node of trypanosome-infected cattle 10 days after infection are blasting and dividing. Despite this general activation, only the $CD4^+$ T cells proliferate in response to trypanosome antigens (Taylor *et al.*, personal observation). No parasite-specific T-cell responses can be detected from lymphocytes derived from the non-draining lymph nodes at any time during infection, suggesting that normal recirculation of parasite antigen-specific T cells does not occur (Lutje *et al.*, 1995). $CD8^+$ T cells do not appear to play a significant role in the outcome of infection, as cattle depleted of these cells experience the same parasitaemia and anaemia as immunologically intact animals (Sileghem and Naessens, 1995).

B-cell maturation and differentiation to parasite-specific antibody-secreting cells is both T cell-independent and T cell-dependent and occurs in the major lymphoid organs. T cell-independent B-cell responses result from B cells activated by type 1 antigens (B-cell mitogens), independent of the B-cell receptor antigen specificity, or by type 2 antigens, which have highly repetitive structures that activate by cross-linking the antigen-specific B-cell receptor. Early hypotheses suggested that trypanosomes acted as type 1 T-independent antigens and thus were able to signal polyclonal activation of the host's B cells. This theory, in part, explained the massive expansion of B cells and high titres of IgM associated with trypanosome infection. The theory has not been resolved and experimental evidence exists both to support and refute it. On the other hand, the repetitive nature of the VSG surface coat with its carbohydrate moieties may act as a type 2 antigen. Significantly, a high frequency of the B cells identified in the blood of *T. congolense*-infected cattle express the CD5 antigen on their surface (Naessens and Williams, 1992). In other diseases, $CD5^+$ B cells are associated with antibody responses driven by type 2 antigens in which they produce low-affinity polyspecific IgM. Trypanosome-infected animals also produce polyspecific IgM.

Antibody responses to variant antigens and parasite clearance

The extracellular nature of the trypanosome and current experimental evidence suggest that parasite killing is a function of the antibody, complement and phagocytic responses. Despite antibody:complement-mediated lysis of trypanosomes being easily demonstrated *in vitro*, the significance of extracellular lysis *in vivo* is probably limited. Two observations support this contention. Firstly, radiolabelled

parasites incubated in immune serum and inoculated into normal goats were removed from circulation within minutes. Fifty per cent of the activity was recovered in the liver and the lungs (Whitelaw *et al.*, 1989). Secondly, terminal complement proteins (C5–C8) are necessary for extracellular lysis of pathogens. Despite early complement factors being decreased (see section on hypocomplementaemia, below), no change in the terminal complement proteins has been associated with trypanosomiasis.

The nature of the surface of the trypanosome and its extracellular niche predict that antibodies specific for the surface-exposed epitopes, which by definition are VAT specific, will play a major role in parasite clearance. It should be emphasized that VAT-specific epitopes are conformational. If the integrity of the surface coat is perturbed, conformational epitopes may be altered and 'buried' VSG epitopes exposed. These buried epitopes may be either conformational or linear.

The successive waves of parasitaemia that characterize infection can be explained by VAT-specific antibody-mediated parasite clearance. The host mounts a humoral immune response specific to the dominant VAT and eliminates those parasites that express it. The small percentage of the population that express a different VAT avoid antibody-mediated destruction and give rise to successive waves of parasitaemia. In bovine trypanosomiasis, cattle immunized with irradiated trypanosomes or VSG are successfully protected against challenge with homologous (expressing the same VSG), but not heterologous, trypanosomes. This strongly argues for the importance of VAT-specific response to parasite control.

The ability of antibody to fix complement and bind to FcγR on phagocytic cells is dependent on the antibody isotype. *T. congolense*-infected cattle produce IgM, IgG_1 and IgG_2 antibodies specific for the surface-exposed conformational VSG epitopes (Williams *et al.*, 1996). In contrast, antibodies specific for the 'buried' VSG epitopes consist of IgM and IgG_1 but not IgG_2. Although both IgM and IgG are able to fix complement, IgM is believed to be more efficient. Activation of phagocytosis by IgG immune complexes requires binding of both FcγR and complement receptor-1 on the surface of the phagocytic cells; IgM immune complexes require complement receptor-1 and the presence of C5a. These isotype-dependent requirements for activation of the phagocytic system represent a means by which distinct isotype profiles in infected animals could result in different abilities to clear parasites or immune complexes.

Antibody responses to invariant antigens

Non-VSG or invariant antigens are shared by various stocks and, in some instances, by different species of trypanosomes. In the last 15 years, it has been demonstrated that parasite molecules other than the VSG, especially transporters and receptors for nutrients, are exposed to the host environment. Most of these molecules are located in the flagellar pocket of the parasite, where interactions with specific substrates and antibodies are permitted by the absence of a glycoprotein coat. Moreover, internal antigens may be secreted in the plasma or released by dead trypanosomes and thus interact with important biological systems (components of the immune, complement and coagulation systems). The pathogenicity of the released molecules (see section on phagocytic cells, below) and the efficacy of their specific or non-specific neutralization by the host may contribute to determining the severity and pathological features of the disease.

Humoral responses to non-variant antigens have been examined in cattle experimentally infected with *T. b. brucei* (Shapiro and Murray, 1982) and *T. congolense* (Authié *et al.*, 1993). Few data are available regarding other species of parasites and hosts. Trypanosome-infected cattle mainly respond to non-variant antigens with IgM and IgG_1. IgM are transient and directed to very few antigens, while IgG_1 persist longer and have a wider range of specificities. The kinetics of responses to non-variant antigens, and the spectrum of antigens recognized, appear to differ depending on the genetic background of the host (see next section).

Immune suppression – B cell, T cell and monocyte effector functions

Immune suppression is a well-recognized and well-studied characteristic of trypanosomiasis in livestock, humans and mice. Trypanosome infection of livestock has been shown to result in: (i) a reduced capacity to mount a primary humoral response to non-trypanosome antigens; (ii) an inappropriate antibody response to trypanosome antigens; (iii) depressed T-cell proliferation to mitogen and trypanosome antigens; (iv) reduced cytokine production, most notably interleukin-2; (v) phenotypic changes in monocyte effector functions; and (vi) an inferior response to vaccination(s). The end result is that the immune-compromised host may be less able to control the infecting trypanosome population, control other concurrent diseases or respond normally to vaccination regimes.

Trypanosome-specific antibody responses of susceptible and tolerant breeds of cattle differ. Susceptible cattle produce less IgG_1 against specific trypanosome invariant antigens (Authié *et al.*, 1993) and the buried VSG epitopes (Taylor *et al.*, 1996a) than tolerant cattle, suggesting that isotype-switching of antibodies to specific trypanosome antigens is blocked or altered in susceptible animals during infection. In addition, vaccination of trypanosome-infected cattle may result in depressed or delayed antibody responses against the immunizing agent.

The factors affecting B-cell growth and differentiation are complex and it is not presently understood at what level responses are altered in susceptible animals. Cytokines produced by helper T cells influence the isotype of antibody that is produced. T-helper type-1 cells produce interleukin-2 (IL-2) and interferon-γ (IFN-γ) and induce an antibody response dominated by IgG_2, whereas T-helper type-2 responses are characterized by IL-4 production and drive production of IgG_1. The observation that transcription of IL-4 is depressed in *T. congolense*-infected trypanosusceptible Boran cattle but increased in similarly infected trypanotolerant N'Dama cattle suggests a role for IL-4 in the superior IgG_1 response of trypanotolerant cattle (Mertens *et al.*, 1999).

Characterization of parasite-specific T-cell responses has been hampered because specific T-cell activity can only be detected intermittently and at very low levels in the peripheral blood. More recently, attention has focused on the lymph node draining the site of infection where the inductive immune events are expected to occur (Lutje *et al.*, 1995). In this compartment it was shown that T-cell mitogen-induced proliferation, IL-2 production and IL-2 receptor expression are depressed during the first weeks of infection in cattle (reviewed by Taylor, 1998). T-cell responses specific for both variant and invariant antigens are present in the draining lymph node at 10–14 days after infection and are characterized by proliferation and IFN-γ, but not IL-2, production. However, by 1 month after infection these responses are nearly absent from the draining lymph node. The precise mechanisms responsible for the loss of trypanosome-specific responses from the lymph node are not known. It is known, however, that responses are not restored *in vitro* by: (i) the addition of IL-2; (ii) blocking prostaglandins or nitric oxide (Taylor *et al.*, 1996b); or (iii) blocking IL-10 or IFN-γ (Taylor *et al.*, unpublished data).

A paradox of trypanosomiasis is related to macrophage function. Despite increased phagocytic activity, increased production of monocyte effector molecules such as prostaglandins, tumour necrosis factor-α (TNF-α) and nitric oxide has not been documented in trypanosome-infected cattle. In fact, the monocytes of cattle infected by *T. congolense* and *T. vivax* have a blunted response to activation with IFN-γ (Taylor *et al.*, 1996b, 1998, unpublished data). Further, transcription of the proinflammatory cytokines TNF-α and IL-1β is not elevated in lymphocytes derived from the peripheral blood (Mertens *et al.*, 1999), lymph node or spleen (B. Mertens, Nairobi, 2000, personal communication) of *T. congolense*-infected cattle. In contrast, Suliman *et al.* (1999) reported increased transcription of these factors in the kidneys and bone marrow of infected cattle (see section on dyserythropoiesis, below). The production of acute-

phase proteins is also an indicator of an inflammatory response. These proteins were not elevated significantly in cattle experimentally infected with *T. congolense* or *T. vivax* (Taylor and Eckersall, unpublished data).

It is not currently known whether these changes in monocyte function represent a form of immune suppression or merely the differentiation of monocytes to a distinct phenotype. Neither is it known whether these specific phenotypic changes influence the ability of the host to respond to infection. Just as inflammatory mediators have the potential to combat invading pathogens, they also have the potential to mediate pathology (reviewed by Taylor, 1998).

The transcription of IL-10 mRNA is increased in mononuclear cells derived from the peripheral blood, lymph node and spleen of trypanosome-infected cattle. IL-10 is a pleuripotent cytokine that suppresses macrophage effector and co-stimulatory functions. Its up-regulation during infection might, therefore, be related to the apparent failure of monocytes and macrophages of trypanosome-infected cattle to produce inflammatory products. Bovine IL-10 also has the ability to suppress the proliferation of antigen-specific T-cell clones and down-regulate the expression of IL-2 and IFN-γ. These suppressive activities are consistent with observations from trypanosome-infected animals.

Systemic Pathology

Leucopenia

Total leucocytes may drop 30–50% in trypanosome-infected animals. The initial decrease is due mainly to an absolute decrease in T cells ($CD4^+$, $CD8^+$ and γδ), eosinophils and neutrophils (granulocytes). In contrast, monocytes may be increased transiently at this stage. The initial leucopenia is followed by a leucocyte response that is characterized by increased numbers of B cells (two- to threefold). During weeks 4–6 of infection, the number of T cells may recover to pre-infection values but monocyte counts often become depressed. In some infections, granulocytopenia may persist as a result of continued phagocytosis of mature cells and their precursors in the bone marrow accompanied by phagocytosis of mature cells in the spleen, liver and haemolymph nodes.

Thrombocytopenia

Reduced numbers of circulating platelets is a prominent feature of animal trypanosomiasis (reviewed by Davis, 1982). The severity and persistence of thrombocytopenia appears to be related to the onset, intensity and prevalence of parasitaemia. It has been hypothesized that initially platelets are damaged by trypanosome products, whereas later in infection removal of platelets is immune-mediated. Trypanosome antigen: antibody complexes or autoantibodies bound to platelets could mediate their clearance by a hyperactivated mononuclear phagocytic system. This is supported by the demonstration of platelet-specific autoantibodies in the plasma of cattle infected with haemorrhagic stocks of *T. vivax* (Assoku and Gardiner, 1989).

Anaemia

The mechanisms that induce, mediate and sustain anaemia are not clearly defined but it is widely agreed that different mechanisms are involved in the control of anaemia during the acute and chronic phases of disease. While erythrocyte destruction is associated with both the acute and chronic phase, ineffective erythropoiesis may play a more important role in the chronic phase.

Anaemia is often categorized based on changes in the size and haemoglobin content of erythrocytes. While many variations have been reported in trypanosome-infected livestock, the most common type of anaemia during the acute phase appears to be normocytic normochromic, i.e. size and haemoglobin content are within normal ranges, a feature of haemolytic anaemias. After 1 month of infection, macrocytes and

abnormally shaped erythrocytes appear in the bloodstream. Macrocytosis increases until 2–3 months after infection and thereafter progressively recedes.

Erythrocyte destruction

Current evidence suggests that erythrocyte destruction is a result of phagocytosis by an activated and expanded mononuclear phagocytic system. The half-life of erythrocytes from infected animals is reduced significantly. Erythrophagocytosis is mainly extravascular, occurring in organs such as spleen, liver, lymph nodes, haemal nodes, lungs and bone marrow. With the exception of haemorrhagic strains of *T. vivax*, there is no evidence that abnormal bleeding contributes to anaemia. Neither is there evidence for significant intravascular haemolysis during infection with livestock-infective trypanosome species.

Why erythrocytes are targeted for phagocytosis is not known but possibly involves: (i) immunological mechanisms such as host autoantibodies or trypanosome antigen:antibody:complement complexes bound to the surface of erythrocytes: (ii) trypanosome enzymes mediating erythrocyte damage; (iii) complement activation; (iv) fever; (v) an overreactive mononuclear phagocytic system; and (vi) variation in erythrocyte size and irregularities in shape.

When erythrocytes are phagocytosed by macrophages they are broken down intracellularly. The haemoglobin is reduced to iron and globin and both are reutilized. The iron is stored mainly as ferritin and haemosiderin in macrophages in the lymph nodes, spleen, haemolymph nodes, bone marrow, liver, lung and kidney of infected animals.

Dyserythropoiesis

During the chronic phase of anaemia, erythrocyte synthesis is not sufficient to compensate for erythrocyte destruction, which suggests impairment of bone marrow function. However, it bears noting that evidence from some studies does not support the theory of dyserythropoiesis. The disagreement may result from the fact that, although infected animals make an erythropoietic response, the response is insufficient to offset erythrocyte destruction. It is not known whether the response is impaired or whether the maximum response of the bone marrow is unable to keep pace with erythrocyte removal.

An elevated plasma-iron turnover rate, increased rate of utilization of radiolabelled iron in erythrocytes and elevated reticulocyte counts are all evidence for increased erythropoiesis during the chronic stage of infection. In contrast, other workers found iron reutilization impaired and implicated blockage of reticuloendothelial iron release as a cause of anaemia. Large deposits of haemosiderin may account for the unavailability of iron. Measurement of plasma and red-cell volume show that the lower PCVs of the infected cattle are due to reductions in red-cell volume and not to haemodilution.

Erythroid clonogenic assays demonstrated an erythropoietic response by *T. congolense*-infected calves between 35 and 70 days after infection that was characterized by increases of both the early and late erythroid progenitor cells (Andrianarivo *et al.*, 1995), However, the magnitude of the response was not associated with the severity of anaemia.

Erythropoiesis is under the control of the hormone erythropoietin (Epo). Produced in the kidneys, Epo stimulates erythroid progenitors by binding to cell-surface Epo receptors (EpoR). Despite elevated transcription of Epo and EpoR mRNA in *T. congolense*-infected cattle (Suliman *et al.*, 1999), suggesting a bone-marrow erythropoietic response, the level of Epo expression does not correlate with the severity of anaemia. None the less, transcription of the EpoR is significantly higher from the bone marrow of cattle that are able to control anaemia (trypanotolerant N'Dama – *Bos taurus*) compared with cattle that do not (trypanosusceptible Boran – *Bos indicus*). Notably, transcripts of some inflammatory cytokines, which are negative regulators of erythropoiesis, are expressed at lower levels in the kidneys and bone marrow of cattle that control anaemia (N'Dama) compared with cattle that do not (Boran).

Disseminated intravascular coagulation (DIC)

DIC results from the intravascular activation of the clotting system leading to the deposition of fibrin thrombi in the microvasculature. DIC and various degrees of consumption coagulopathy have been described in trypanosome-infected animals. Thrombocytopenia, which precedes the other coagulation abnormalities in trypanosome-infected cattle, may be related to the development of DIC. The accelerated destruction of platelets is thought to result in the release of a phospholipid (platelet factor 3) that triggers the coagulation cascade. Increased coagulation could contribute to the haemolytic anaemia of infected animals.

The massive bleeding that occurs in some acute *T. vivax* infections in cattle is consistent with DIC. In less acute trypanosome infections, haemorrhagic spots in the skin and mucous membranes are regularly observed at necropsy and are also consistent with coagulation abnormalities. Murray and Dexter (1988) concluded that DIC is most likely to play a significant role in those infections with *T. vivax* that are acute and characterized by high parasitaemia.

Hypocomplementaemia

Low complement haemolytic activity has been reported in cattle infected with *T. congolense*, *T. vivax* or *T. b. brucei*, in sheep infected with *T. congolense*, and in camels infected with *T. evansi*. In cattle, the kinetics of haemolytic complement activity parallel those of anaemia, with a decrease that is detectable at or soon after detection of parasitaemia. There is a continuous decrease of complement activity during the first weeks of infection and values as low as 10–20% of the normal values may be reached. A very low complement activity with no tendency to recover portends a bad prognosis in an infected animal. The decrease in complement activity in trypanosomiasis is attributed to a drop in plasma concentrations of complement factors C3 and B, indicative of activation via the alternative pathway. Trypanotolerant cattle, as opposed to fully susceptible breeds, also experience hypocomplementaemia upon infection, but appear to have an ability to maintain or restore complement levels after the initial, acute phase of the disease (Authié and Pobel, 1990; Doko *et al.*, 1997).

It has long been assumed that intravascular consumption of complement factors is triggered in trypanosome-infected animals by immune complexes, either in circulation or bound to the parasite surface coat. However, the precocity of complement decrease during infection and the rapid restoration of complement activity after an early trypanocidal treatment suggest that complement depletion does not result only from specific antibody responses and immune complex formation.

The apparent negative correlation between parasitaemia and complement levels, together with *in vitro* studies demonstrating complement activation by parasites in the absence of antibodies, suggests that live trypanosomes or their products may directly affect complement levels in the host. Bloodstream trypomastigotes evade complement lysis by high-affinity binding of complement factor C3b to their surface coat, followed by inhibition of the formation of C3 convertases – the key enzymes of the complement cascade – thereby restricting complement activation (Devine *et al.*, 1986). Thus, live bloodstream forms of *T. b. brucei*, *T. b. gambiense* and *T. congolense* 'activate' complement in their normal hosts, i.e. they consume complement factors, though they are not lysed in non-immune serum. Restriction of complement activation at the trypanosome surface appears to be mediated by the VSG. Paradoxically, intact VSG may both bind C3b and protect bloodstream forms against complement-mediated lysis. Further, soluble extracts of *T. congolense* and *T. b. brucei*, as well as purified VSG from *T. b. brucei* (Musoke and Barbet, 1977), were shown to activate the complement activation pathways directly, resulting in complement consumption. Binding of C3 to host cells such as erythrocytes has also been reported in bovine trypanosomiasis (Kobayashi *et al.*, 1976), but the extent of this phenomenon is unknown. The respective roles of these

processes in the consumption of complement components in trypanosome-infected animals are not known and remain hypothetical at this stage.

In vitro, complement activation is triggered at the parasite cell surface in the presence of variant-specific antibodies, leading to trypanolysis. Although the role of complement-mediated lysis in parasite clearance is probably limited *in vivo*, C3 binding to the parasite surface contributes to opsonization and facilitates phagocytosis. Thus complement depletion might limit the efficiency of parasite clearance.

Other host functions may also be adversely affected by depletion of complement factors. For example, solubilization and elimination of circulating immune complexes requires C3; severe C3 deficiency in chronic infection could lead to tissue deposition of insoluble complexes and subsequent inflammatory lesions. The anaphylatoxins C3a and C5a that result from the cleavage of C3 and C5, respectively, may enhance this process. Further, C3b has the dual capacity to bind antigens covalently and to interact with specific receptors at the surface of lymphocytes and follicular dendritic cells, stimulating antigen-specific B-cell activation and promoting long-term B-cell memory. Thus the early and dramatic C3 depletion in trypanosome-infected animals may contribute to determining immunological defects such as the impairment of isotype switching mechanisms.

Host Factors Contributing to Pathology

The genetic contribution is examined in Chapter 25.

Autoantibodies and polyspecific antibodies

Parasite-specific, host-specific and polyspecific antibodies have been detected on the surface of erythrocytes of trypanosome-infected animals. The presence of antibodies and complement on the surface of erythrocytes mediates destruction of these cells and thus may contribute to the anaemia experienced by infected animals. Erythrocyte autoantibodies may result from the exposure of normally hidden epitopes on the surface of the erythrocyte by the action of trypanosome proteases (see next section). These normally hidden epitopes are not recognized as 'self' and therefore elicit an antibody response. This possibility is supported by the observation that cattle infected with a haemorrhagic isolate of *T. vivax* produce autoantibodies that do not cross-react with parasite antigens and, together with C3, bind to the surface of erythrocytes and platelets from both normal and infected cattle (Assoku and Gardiner, 1989).

Alternatively, the expansion of autoreactive antibodies during infection might occur if trypanosomes share common antigens with host erythrocytes. The existence of antibodies that recognize human, murine and bovine antigens as well as VSG eptiopes has been demonstrated (Muller *et al.*, 1996). In addition, spectrin-like proteins are present in *T. b. brucei* (Schneider *et al.*, 1988). Spectrin is a highly conserved structural protein that is also found in red blood cell membranes. Antibodies that cross-react with *T. b. brucei* VSG and sheep erythrocytes have been reported in *T. b. brucei*-infected cattle. In addition, antibodies that recognize sheep and chicken erythrocytes increase in cattle infected by *T. vivax* and *T. congolense*. However, it is important to note that the production of erythrocyte-specific autoantibodies has not been observed in *T. congolense*-infected cattle or in cattle infected with non-haemorrhagic isolates of *T. vivax*.

The contribution of the immune response to anaemia was also suggested by the adherence of trypanosome antigen:antibody:complement complexes to the surface of erythrocytes. This passive sensitization of erythrocytes would predispose their removal by phagocytic cells (Kobayashi *et al.*, 1976).

Phagocytic cells

Macrophages, neutrophils and eosinophils are able to destroy opsonized trypanosomes and remove circulating immune complexes. None the less, increased phagocytosis of leucocytes, platelets and erythrocytes in

animals infected by *T. congolense* and *T. vivax* suggests a generalized activation of the mononuclear phagocytic system that is detrimental to the host. Phagocytosis of these cells is thought to lead to anaemia, leucopenia and thrombocytopenia and is positively correlated with high parasitaemia.

Nutrition

Nutrition is one of the most important factors in determining the outcome of infection with trypanosomiasis for animals kept under range conditions. Adequate nutrition undoubtedly enhances the ability of infected animals to withstand the adverse effects of infection by promoting body weight gains and moderating the severity of the pathophysiological changes associated with trypanosomiasis (reviewed by Holmes *et al.*, 2000). The degree of anaemia and growth retardation experienced by trypanosome-infected livestock is influenced by both protein and energy intakes (Katunguka-Rwakishaya *et al.*, 1997, 1999). In addition, the metabolic rate of infected animals may be increased, resulting in a greater maintenance energy requirement for trypanosome-infected animals (Verstegen *et al.*, 1991). Yet, in the often harsh environments of sub-Saharan Africa, animals that are already weakened by disease may be required to walk long distances just to find suboptimal grazing and water.

Parasite Factors that Contribute to Pathology

Cysteine proteinases are the most abundant and best characterized among proteinases of Trypanosomatids (reviewed in Coombs and Mottram, 1997). There is increasing evidence from genetic manipulations and the use of chemical inhibitors that these enzymes are necessary to parasite survival and also play major roles in pathogenesis, particularly in modulating the host immune responses (reviewed in Mottram *et al.*, 1998). Proteases released in the bloodstream are normally inhibited by natural plasma inhibitors, which abrogate potentially deleterious effects. *In vitro* studies demonstrate, however, that interactions between trypanosome proteases and their natural inhibitors do not necessarily result in complete enzyme inhibition and, in some instances, release biologically active molecules that may contribute to pathology (Del Nery *et al.*, 1997). In African trypanosomes, proteases have been described in *T. brucei* sp. and *T. congolense*. Congopain, the major cysteine protease of *T. congolense*, has been fully characterized (Mbawa *et al.*, 1992; Chagas *et al.*, 1997). Congopain is present as a circulating antigen in infected cattle (reviewed in Authié, 1994) and cysteine protease activity is present in the plasma of *T. congolense*-infected cattle (Authié *et al.*, unpublished observation). Although congopain degrades a variety of host proteins at physiological pH, its possible roles as a pathogenic factor remain to be investigated.

The bloodstream forms of *T. vivax* express sialidase activity (Engstler *et al.*, 1995). Sialidases hydrolyse sialic acid, an important component of the erythrocyte surface membrane. Its removal from the erythrocyte surface is normally an age-dependent process that occurs naturally and leads to removal of aged cells through phagocytosis. Decreased erythrocyte-surface sialic acid in cattle infected with *T. vivax* has been described (Esievo *et al.*, 1986). While this provides a hypothetical explanation for the accelerated removal of erythrocytes during infection with *T. vivax*, sialidase and trans-sialidase activity are restricted to the insect forms of the other livestock-infective trypanosomes.

Phospholipase A2 was isolated from *T. congolense*. The enzyme possessed both haemolytic and anti-coagulating properties (Nok *et al.*, 1993).

Other parasite factors have been shown to have possible immunomodulatory effects. Soluble extracts from *T. b. brucei, T. evansi* and *T. congolense* activate murine macrophages to produce TNF-α; the activity was associated with the glycosylphosphatidylinositol (GPI) anchor of the VSG (Magez *et al.*, 1998). A trypanosome factor named T lymphocyte triggering factor (TLTF) induces murine $CD8^+$ T cells to secrete IFN-γ (Olsson *et al.*, 1991).

While these trypanosome-derived factors have demonstrated activity on artificial substrates or murine leucocytes, their ability to modulate responses of livestock or induce pathology *in vivo* has not been determined, though it would clearly be advantageous for the host to produce antibodies that neutralize these potentially immunoregulatory and pathogenic parasite factors. Isotype-dependent processes could effect their neutralization through direct binding of specific antibodies or by mechanisms involved in the clearance of antigen:antibody complexes such as complement activation or binding to phagocytic cells.

New Immunological Approaches

The discovery that live trypanosomes expose proteins other than VSG to the host immune system has renewed the hope that an anti-parasite vaccine may be designed. Initial studies have used crude or fractionated preparations of flagellar pocket proteins. Immunization trials using *T. b. rhodesiense* fractions isolated from the flagellar pocket indicated that a degree of protection may be achieved in rodents (McLaughlin, 1987). Immunization of target species with defined flagellar pocket antigens therefore appears as a very promising avenue, in a long-term research effort that involves prior identification, characterization and purification of protective antigens.

An alternative approach, based on an 'anti-disease' rather than an anti-parasite strategy, was proposed following observations that 'trypanotolerant' African taurine cattle, which have a natural ability both to control trypanosome infection and to limit the associated pathology, develop prominent antibody responses to a *T. congolense* protease (congopain) (reviewed in Authié, 1994). Congopain is potentially pathogenic and IgG antibody elicited during infection in trypanotolerant cattle inhibits congopain activity (Authié *et al.*, unpublished observation). This suggests that antibody-mediated inhibition of congopain may be one of the mechanisms that contribute to bovine trypanotolerance.

While most efforts focused on designing chemical inhibitors of trypanosomal proteases, the possibility of inhibiting these enzymes through appropriate immune responses has only been considered recently. Antibody-mediated inhibition of proteases and, more generally, immunological neutralization of pathogenic factors appear, however, as valid strategies in African trypanosomiasis. The localization of the parasites – extracellular, intravascular – and the recurrent release of parasite components in the bloodstream create appropriate conditions for efficient neutralization of pathogenic factors. Thus, ongoing and future studies aim at determining whether a degree of resistance to trypanosomiasis – or enhancement of resistance in trypanotolerant breeds – may be achieved through immunization against proteases and other potentially pathogenic molecules of trypanosomes. The practical goals of this non-conventional approach are not to prevent trypanosome infection but to improve livestock productivity and reduce drug usage in enzootic areas.

Rodent Models

Mice and rats are susceptible to infection with *T. congolense*, *T. b. brucei* and *T. evansi*. Most *T. vivax* isolates do not establish in rodents but a good number can be adapted by repeated short passages. Many of the published studies are based on rodents infected with *T. b. rhodesiense* and *T. b. gambiense* as model systems for studying human African trypanosomiasis. Although these subspecies are closely related to *T. b. brucei* and *T. evansi*, when thinking about models for trypanosomiasis in livestock it is probably prudent to limit consideration to models that utilize the major livestock-infective species.

Other factors that should be borne in mind when extrapolating from rodent models of African trypanosomiasis to livestock are as follows.

- Rodents are usually inoculated with a high dose of bloodstream-form trypanosomes into the peritoneal cavity; thus there is no equivalent tissue phase of development in the skin.

- Peak parasitaemia in infected rodents may be several logs higher per millilitre of blood than in most infected livestock.
- Immunological responses of inbred strains of mice are more polarized than those of outbred livestock.
- Production of inflammatory mediators such as nitric oxide, prostaglandins, TNF-α and acute-phase reactants is increased in rodent models of trypanosomiasis, but not in cattle.
- Immune suppression is more severe in trypanosome-infected rodents compared with livestock.
- Trypanosomiasis in rodents always leads to death within several weeks to several months, which is not the case in cattle.

Despite these differences, rodent models provide an economical alternative for research on trypanosomiasis and may be particularly relevant for studying direct effects of specific antibody on parasite clearance.

Conclusions

Trypanosomiasis is the most economically important disease of livestock in sub-Saharan Africa and its distribution and economic importance are expanding in other regions of the world. The worldwide impact of this disease complex on the agricultural systems of nations struggling to overcome the effects of poverty and hunger is enormous. Improved methods of control are needed desperately. Throughout the history of modern medical and veterinary medicine, the prevention of disease by vaccination has proved to be the most effective and economical approach to disease control. Thus the development of vaccines that protect against trypanosomiasis should be a global priority. Understanding the pathogenesis of trypanosomiasis in its natural hosts is a crucial first step towards developing immune-based control methods. This research is best carried out in endemic regions on natural hosts. It requires sophisticated laboratory facilities and long-term financial commitment. It is now well accepted that vaccine development for complex pathogens cannot occur in the absence of fundamental knowledge of the parasite's biology, the host's responses and the epidemiology of the disease. Although the investment required is high, the cost of ignoring the problem is higher. Moreover, it is those who have no voice in the boardrooms, where decisions that govern research investment are made, who suffer the burden.

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PART 5.
DISEASE IMPACT

19 Medical Significance of American Trypanosomiasis

Gabriel A. Schmunis

Introduction

From the north of Mexico to the south of Argentina and Chile, there are between 10 to 12 million people infected with the haemoflagellate *Trypanosoma cruzi* (Schmunis, 1999a); this is 6 to 8 million cases fewer than those reported in the 1980s (WHO, 1990, 1991). As an enzootic disease, American trypanosomiasis is more widely distributed than human infections. It extends approximately from latitude 42° N in northern California and Maryland, to latitude 43° S in southern parts of Argentina and Chile.

Human infection usually occurs through contact of the skin or mucosa with contaminated faeces or urine from triatomine bugs infected with *T. cruzi*. It mostly happens in poor rural or periurban areas of South and Central American countries where housing conditions favour intimate contact between infected bugs and the human host. A few cases have been found in French Guiana, Guyana and Surinam but vectorial transmission has not been reported from Cuba and the Dominican Republic. Vectorial transmission is extremely unusual in the USA, due to the better living conditions of the population and the silvatic characteristics of the local species of bugs. Only four autocthonous cases have been reported (Ochs *et al.*, 1996).

Although there are a few cases of human infection in which a previously positive serology becomes negative without treatment (possibly indicating spontaneous cure), most untreated infected individuals remain infected throughout their lives. *T. cruzi* may be detected in the blood of at least 50% of seropositive individuals. Thus, it is not uncommon for *T. cruzi* to be transferred to an uninfected person by blood transfusion. It is estimated that up to 20% of those who receive an infected unit of donor blood will become infected (WHO, 1991). Transfusion of blood or blood products is considered to be the second most common way of acquiring the infection (Dias and Brener, 1984; WHO, 1991; Dias, 1992).

Another means of acquiring infection is through the placenta, which occurs in 1.6–11.7% of newborns from *T. cruzi*-infected mothers. This percentage increased to 18.5% when underweight newborns from chagasic mothers were selected for study (Bittencourt *et al.*, 1985; Arcavi *et al.*, 1993; Zaidenberg and Segovia, 1993; Freilij *et al.*, 1994; Streiger *et al.*, 1995; Schmunis, 1999a).

Morbidity of *T. cruzi* Infection in the General Population

Estimates based on the prevalence of the infection vary in South America from 20%

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of the population in Bolivia to 1.06% in Chile and 1.2% in Uruguay. Between these extremes lie Argentina with 7.0% of the population, Brazil with 1.21%, Colombia with 4.02% and Venezuela with 4.4% of the population (Fig. 19.1). In Central America, 20% of the rural population of El Salvador is infected, as is 9.8% of the population in Guatemala, 7.4% in Honduras and 1.82% in Nicaragua. Similar prevalence has been found in some areas of Mexico (WHO, 1990, 1991; Schmunis, 1999a) (Fig. 19.2).

The potential problem of *T. cruzi* infection in non-endemic countries has been attributed to migration from Latin American countries to the USA and Europe. It has been estimated that there are between 100,000 (Skolnick, 1989) to 370,000 individuals chronically infected with *T. cruzi* living in the USA, 75,000 of them with chronic myocardiopathy (Milei *et al.*, 1992). This number is probably an underestimation, since the USA 2000 census indicated that more than 30 million were hispanic, and a significant number of them

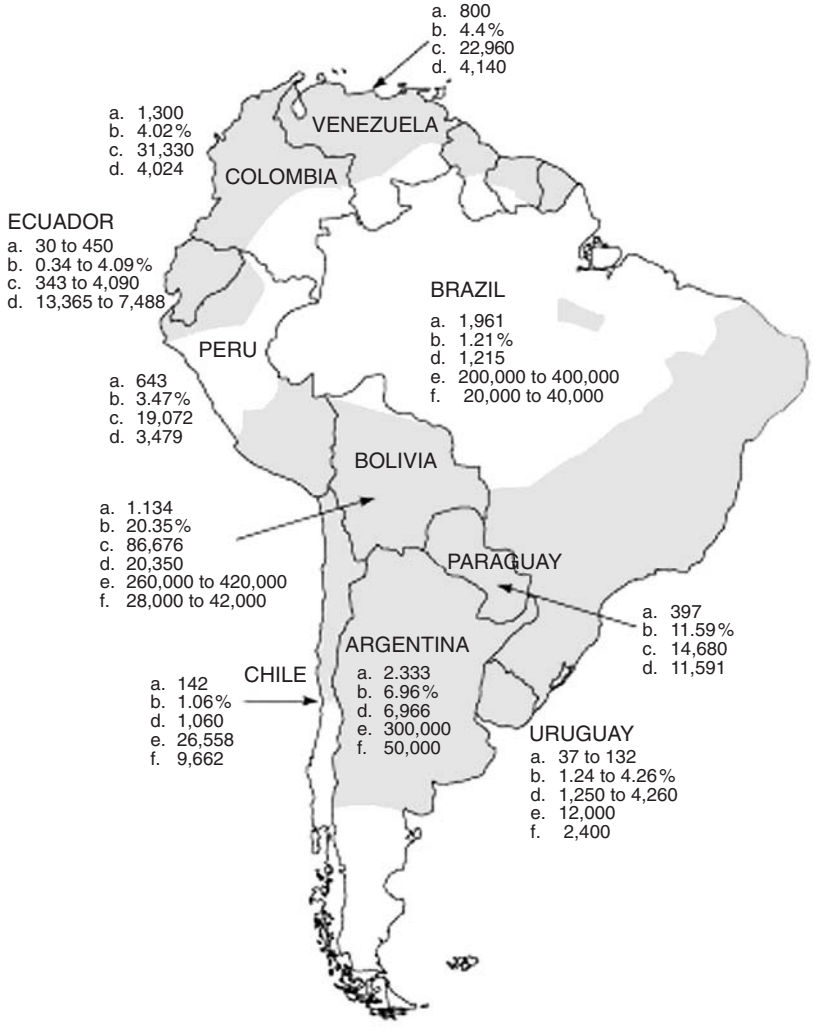

Fig. 19.1. Endemic area for human *Trypanosoma cruzi* infection and Chagas disease in South America (shaded area): (a) number of infected individuals in thousands; (b) percentage in the total population; (c) annual incidence; (d) infection rate per 100,000 population; (e) number of individuals with symptoms; (f) number of individuals with severe symptoms.

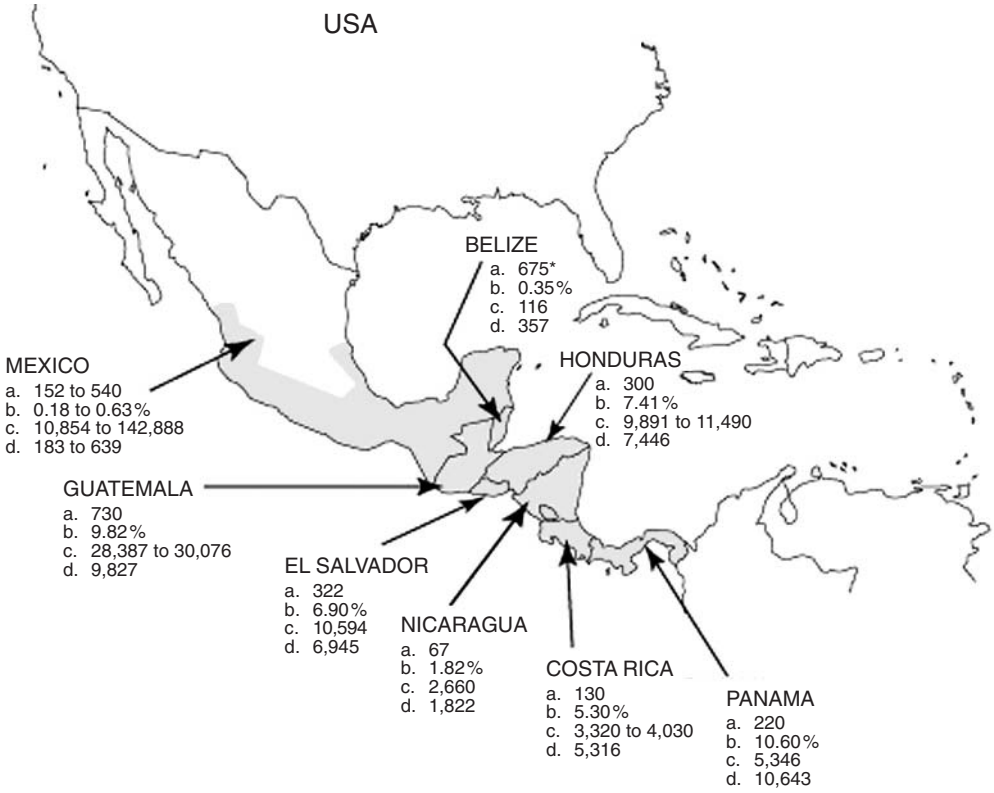

Fig. 19.2. Endemic area for human *Trypanosoma cruzi* infection and Chagas disease in Central America (shaded area): (a) number of infected individuals in thousands; (b) percentage in the total population; (c) annual incidence; (d) infection rate per 100,000 population; *Total number.

from *T. cruzi*-endemic countries. The potential of finding *T. cruzi*-infected individuals also exists in Europe, Australia and Japan where, in the late 1980s, 250,000, 80,000 and 200,000 immigrants from Latin America were living, respectively (Schmunis, 1999b). It was not a complete surprise when a case of congenital *T. cruzi* infection was detected in Sweden (Pehrson *et al.*, 1981).

In South America, estimated incidence rates varied from 86,676 cases per year in Bolivia to 31,330 cases in Colombia, 22,960 in Venezuela, 13,365 in Ecuador, 14,680 in Paraguay and 19,072 cases in Peru (Fig. 19.1). In Central America, the highest incidence was in Guatemala with 30,076 cases per year, followed by Honduras with 11,490, El Salvador with 10,594, Panama with 5346, Costa Rica with 4320 and Nicaragua with 2660 cases (Hayes and Schofield, 1990; Schofield and Dujardin, 1997) (Fig. 19.2). Incidence estimates for congenital Chagas disease per country are shown in Fig. 19.3. Considering that at least 3% of children born to chagasic mothers may become infected, at least 15,000 cases of congenital infection are expected annually in the Americas (Schmunis, 1999a).

Morbidity via Blood Banks

Transfusional infection is a significant public health problem. Even if the majority of countries in Latin America had laws, regulations and guidelines making blood-donor screening mandatory for infectious diseases, including *T. cruzi* (El Salvador does not have laws on the subject), very often law enforcement is lax or

even non-existent. Although serology for *T. cruzi* is mandatory in most countries, in Chile and Mexico serology in blood banks is only mandatory in endemic areas (Guzman Bracho *et al.*, 1998). Even then, screening in Mexico is not done routinely in all endemic states. In places like Santa Cruz, Bolivia, where the prevalence of *T. cruzi* antibodies in the population is above 50%, there is a strong possibility of acquiring *T. cruzi* infection through a blood transfusion if donor screening is not carried out (Carrasco *et al.*, 1990).

The real incidence of *T. cruzi* acquired through blood is unknown, because most cases are either not apparent or *T. cruzi* is not recognized as the aetiological agent. Danger may come not only from whole blood but also from packed red cells, platelets, white cells, fresh frozen plasma and cryoprecipitate. On the other hand, the use of lyophilized products seems to be safe.

The risk of receiving infected blood is proportional to the prevalence of the infection in the donor population and to the number of transfusions performed. Therefore, polytransfused individuals, such as haemophiliacs, patients with other haematological disorders, or those undergoing dialysis, are at greater risk. In Argentina, 50% of haemophiliacs became infected after receiving 30 or more transfusions each from a blood bank with a 2% prevalence of positive serology for *T. cruzi*. In another study, in Chile, it was found that 15% of individuals who had multiple transfusions, had positive serology for *T. cruzi*, whilst the general population was 2% positive. Polytransfused individuals from a blood bank with 2% positive serology for *T. cruzi* were 8.7 times more likely to be positive than individuals who did not receive transfusions. On the other hand, the theoretical risk of transmission for individuals receiving only one transfusion may vary from 12% to 20% (WHO, 1991).

Information available from countries prior to 1993/94 has some limitations. In 1992, with the exception of Chile, countries lacked a nationwide information system on the number of donors and the number of screened donors, both of which are needed to calculate screening coverage. If that information had been available, coupled with a knowledge of the prevalence of the infection, it would have been possible to calculate the risk of acquiring *T. cruzi* infection through a transfusion, and the potential number of cases transmitted by blood (Schmunis *et al.*, 1998). Despite the limitations, blood-bank data were still extremely useful not only in establishing the prevalence of *T. cruzi* antibodies in blood donors but also by providing information on the prevalence of the infection in a given geographical area. This was especially the case in countries where knowledge of the status of human infection was lacking and control measures for vectorial transmission of *T. cruzi* were not implemented. Such data also indicate the need to implement control strategies for prevention of transfusional transmission.

A report analysing national data on the coverage of blood-donor screening in four countries in Central America (El Salvador, Guatemala, Honduras and Nicaragua) and seven countries in South America (Bolivia, Chile, Colombia, Ecuador, Paraguay, Peru and Venezuela) estimated the incidence of transfusional transmission of *T. cruzi* in those countries in 1993–1994. Estimates were based on results of screening activities as reported by these countries (Schmunis *et al.*, 1998). Only Venezuela and Honduras screened 100% of donors for *T. cruzi*. Prevalence rates in donors ranged from 0.20% in Ecuador and 0.24% in Nicaragua to 4.5% in Paraguay and 14.8% in Bolivia. In 1993 Costa Rica, Peru and Mexico had not yet introduced routine screening for *T. cruzi* in blood banks (Table 19.1). Information from Argentina indicated that screening coverage for *T. cruzi* was not complete; in Brazil, screening was routinely done in blood banks from the public sector but no information was available from the private sector.

Potential infectivity estimates for blood supply are shown in Table 19.1. The probability of receiving a potentially infected unit of blood in each country varied from a maximum of 1096/10,000 transfusions in Bolivia to 10.48 in Nicaragua or around 13/10,000 transfusions in Honduras and Venezuela, where screening coverage was 100%. The probability of getting an infection through

Table 19.1. *Trypanosoma cruzi*: coverage of screening of blood donors, prevalence, risk of receiving an infected transfusion or of acquiring an infection, number of blood units discarded and of cases prevented by screening (1993) (from Schmunis *et al.*, 1998; Schmunis, 1999b).

Country	Number of donors	Prevalence ($\times 10^3$)	Coverage (% donors screened)	Risk[a]		Total no. infected units	No. cases produced	No. discarded units/No. cases prevented
				P(R)	P(I)			
Bolivia	37,948	147.90	29.4	1096.38	219.28	4,160	832	1,650/330
Chile	217,312	12.00	76.7	11[e]	2[e]	55[e]	11[e]	2,000/400
Colombia	352,316	12.00	1.4	124.24	24.85	4,375	875	59/12
Costa Rica	50,692	–	0	–	–	–	–	–/–
Ecuador	98,473	2.00	51.0	10.29	2.06	100	20	1,004/201
El Salvador	48,048	14.70	42.5	88.75	17.75	425	85	300/60
Guatemala	45,426	14.00	75.0	36.75	7.35	165	33	417/95
Honduras	27,885	12.40	100.0	13.02	2.60	35	7[f]	346/69
Nicaragua	46,001	2.40	58.4	10.48	2.10	50	10	64/13
Paraguay	32,893	45.00	86.8	62.37	12.47	205	41	1,603/321
Peru[c]	52,909	23.60[d]	0	247.80	49.56	1,965	393	–/–
Venezuela	204,316	13.20	100.0	13.86	2.77	285	57[f]	2,697/539

[a]All data from 1993, except for Ecuador and Paraguay which were for 1994.
[b]P(R), probability of receiving an infected unit; P(I), probability of a transfusion-acquired *T. cruzi* infection.
[c]Lima only.
[d]Prevalence based on data for a limited number of donors.
[e]The prevalence rate used for calculations was that found in the nonendemic area (1/1000) where *T. cruzi* serology was not mandatory.
[f]Residual infection in spite of 100% coverage of screening, because of lack of sensitivity of diagnostic test.
–, information not available.

an infected unit was 219/10,000 in Bolivia, 24/10,000 in Colombia, 17/10,000 in El Salvador, and between 2 and 49/10,000 for the nine other countries (Table 19.1).

Infectivity risks, defined as the likelihood of being infected when receiving an infected transfusion unit, were assumed to be 20% for *T. cruzi* (WHO, 1991). Based on these estimates, the absolute number of infections induced by transfusion was 832 in Bolivia and 875 in Colombia. In all the other countries this number varied from seven in Honduras to 85 in El Salvador. The infection/donation ratio for each country indicated that one *T. cruzi* infection might have been transmitted for every 46 donations in Bolivia to 4924 donations in Ecuador (Schmunis *et al.*, 1998). Even in those countries with 100% screening coverage, the potential of receiving an infected transfusion still exists. Residual infectivity is due to the lack of absolute sensitivity of the reagents available for diagnosis (Schmunis *et al.*, 1998).

In countries such as Costa Rica, Mexico and Peru where nationwide official data were not available, speculation regarding the status of *T. cruzi* in blood donors was considered to be a danger to the blood supply. Surveys in blood donors in Costa Rica from 1983 to 1985 indicated the presence of donors with positive serology for *T. cruzi* (Schmunis, *et al.*, 1998), suggesting that risks may well exist. Data from a survey among donors in Lima indicated a prevalence of 2.36% (Schmunis *et al.*, 1998). If these figures demonstrate the actual prevalence in that city, the number of *T. cruzi*-contaminated units transfused in 1993 would have been 1965 whilst the number of individuals infected through blood transfusion could have been 393 (Table 19.1). On the other hand, if blood had not been screened at all in the 11 countries that reported *T. cruzi* prevalence, the number of infected units transfused could have amounted to several thousand.

Screening coverage in seven countries of the Americas improved between 1993 and 1997. Figures 19.3 and 19.4 show the number of donors, screening coverage and prevalence of the infection in South and Central American countries in 1997. In Central America, except for Guatemala, screening of donors for *T. cruzi* prevented the transfusion of 1365 units and the potential infection of 277 individuals in 1996. In the same year, in seven countries of South America, screening prevented the transfusion of 36,017 infected units and 7201 potential cases of transfusional infection (Schmunis, 1999b). The 'bottom line' is that two countries (Honduras and Venezuela), screened 100% of donors in 1993, whilst five countries (Argentina, El Salvador, Paraguay, Uruguay and Venezuela) did the same in 1997 (Schmunis *et al.*, 2001).

Mexico does not officially report the nationwide coverage of screening for *T. cruzi* or its prevalence, and it is believed that blood donors may be infected with *T. cruzi*. Screening is not done routinely for *T. cruzi* (Guzman Bracho *et al.*, 1998; Dumonteil, 1999). It has recently been estimated that of the 850,000 annual donors, 12,750 potentially infected with *T. cruzi* (1.5%) were not rejected as a result of lack of screening (Guzman Bracho *et al.*, 1998). Thus, out of 1912 recipients, 15% of those receiving an infected unit have a high risk of acquiring a transfusion-transmitted *T. cruzi* infection (Guzman Bracho *et al.*, 1998). Another estimate considered that the overall prevalence of *T. cruzi* infection in blood donors was similar to that found in the overall population: 0.20% instead of 1.5% (Guzman Bracho *et al.*, 1998). With this in mind it is possible that up to 1700 recipients may have received infected units and some 340 individuals may have been infected. These calculations were based on the fact that every recipient received one whole unit of blood. As every unit of blood is split, the risk of receiving a contaminated unit containing *T. cruzi* is much higher (Guzman Bracho *et al.*, 1998).

In Brazil, the screening coverage for infectious diseases for the 1,645,559 units collected by the public sector was 100% in 1997. However, no official nationwide information was available on donors from the private sector.

Morbidity in the Newborn

Congenital infection may occur in all endemic countries but is diagnosed only in a

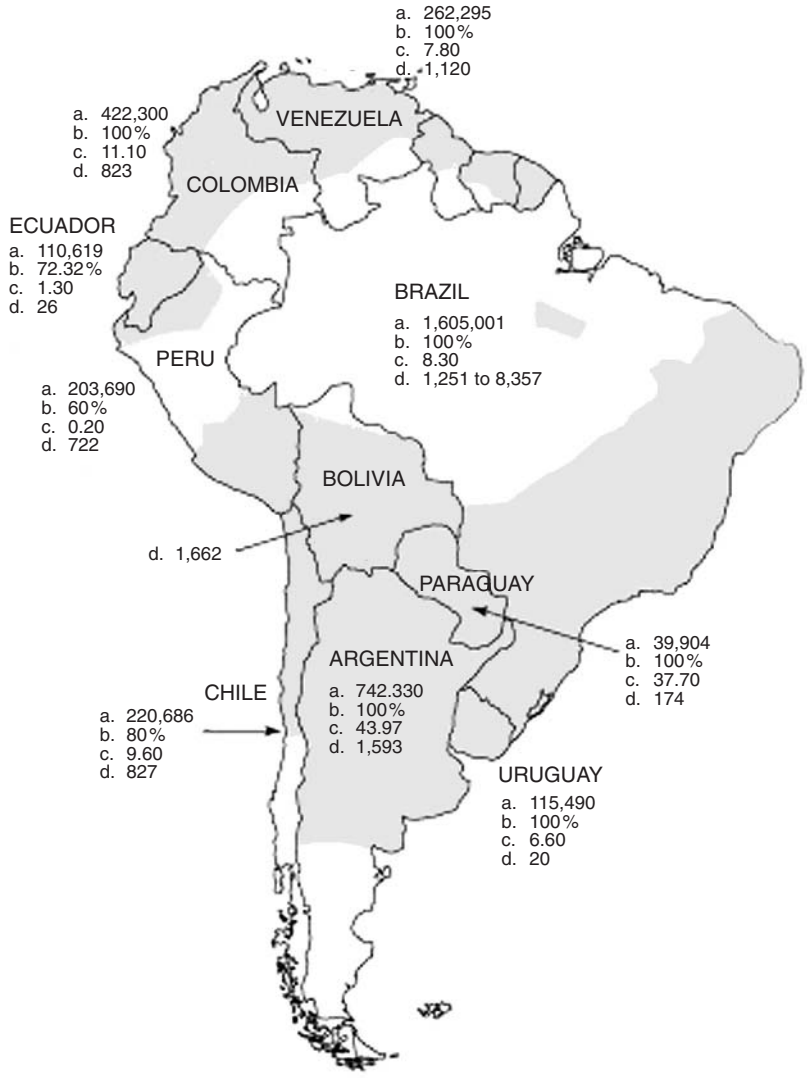

Fig. 19.3. Endemic area for human *Trypanosoma cruzi* infection and Chagas disease in South America (shaded area): (a) number of blood donors; (b) percentage of donors screened for *T. cruzi*; (c) prevalence rate per 1000; (d) estimated number of congenital cases per year in the 80s except for Brazil which was for 1995.

few of them. Figures 19.3 and 19.4 show the estimates of annual incidence in South and Central American endemic countries, considering a conservative incidence of 3% among newborns from *T. cruzi*-infected mothers, from a low 20 in Uruguay or 26 in Ecuador to a high 1593 in Argentina and 1251 in Brazil. In relation to the population, the highest annual incidence is in Bolivia, at 1662 (Schmunis, 1999a).

Clinical Findings

Recent infection

In most cases of recent infection, signs and symptoms are so mild that they are not associated with *T. cruzi* infection. In those cases where symptoms appear and are recognized as *T. cruzi* infection, the site of entry of the parasite shows a primary chagoma, which is

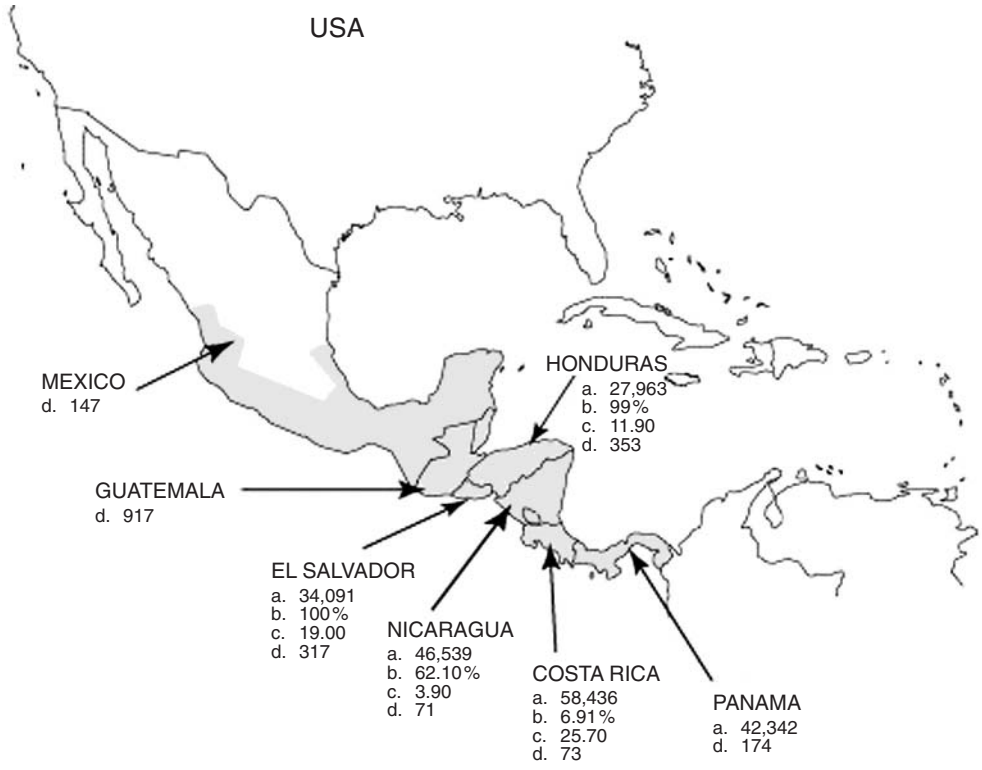

Fig. 19.4. Endemic area for human *Trypanosoma cruzi* infection and Chagas disease in Central America (shaded area): (a) number of blood donors; (b) percentage of donors screened for *T. cruzi*; (c) prevalence rate per 1000; (d) estimated number of congenital cases per year in the 80s.

often accompanied by the swelling of satellite lymph nodes. The fever that appears next may be followed by hepatomegaly and splenomegaly. Occasionally the predominant symptoms and signs are those of diarrhoea or an acute respiratory infection. Often they are not recognized as originating from *T. cruzi* infection. Young children may show symptoms of an acute myocarditis or meningoencephalitis. Mortality is usually low, except for children with cardiac or central nervous system involvement. Even without treatment, most individuals recover from the acute stage of infection.

In the majority of cases that have been diagnosed as transfusionally acquired *T. cruzi* infection, the most common symptoms that appear 4 weeks after transfusion are moderately high fever, general lymph node enlargement and splenomegaly. Few physicians associate these symptoms with the possibility of a protozoan infection originated in a transfusion that occurred 4 weeks earlier. The patient usually receives antibiotics and the fever and other symptoms disappear. However, symptoms would disappear even without treatment. The infection is then only recognized months or years later by a routine serological test, or many years later by a serological test done to confirm a clinical suspicion. Symptoms are much more severe when the transfusion is received by an immunocompromised host and such symptoms include fatigue, headaches and myalgia, followed by mild or severe cardiac failure (Grant *et al.*, 1989; Nickerson *et al.*, 1989; Cimo *et al.*, 1993).

Fetal infection may occur when mothers are in the acute phase of the infection, in the indeterminate phase or in the chronic

stage. The outcome of pregnancy may be spontaneous abortion, stillbirth, premature birth, low birthweight for gestational age, or a full-term delivery. In most cases, however, pregnancy and delivery are within normal limits. Congenital infection produces a broad spectrum, from severe illness to an asymptomatic newborn. The latter is usually delivered full term. When symptoms are present, they include fever and hepatosplenomegaly, jaundice, oedema, neurological alterations, abnormal cerebro-spinal fluid, anomalies in the fundus oculi, pulmonary involvement and cardiac alterations. The appearance of cardiac symptoms or symptoms of meningoencephalitis indicate a poor prognosis (Munoz *et al.*, 1992; Freilij *et al.*, 1994).

Chronic disease: morbidity

Considering the number of individuals infected with *T. cruzi* and the known prevalence of disease in those infected, it has been estimated that the number of individuals with chronic chagasic symptomatology in the Americas may reach up to 4.8 to 5.4 million (WHO, 1991). Other estimates suggest that 1.2 to 2.8 million people are affected (Schmunis, 1999a). In most cases the infection was acquired through vectorial transmission. Conservative estimates from the Southern Cone countries of South America suggest that chagasic cardiopathy in Argentina occurs in 300,000 individuals, 50,000 of them with severe disease; in Bolivia in 260,000 individuals (another estimate mentions 420,000; Human Development Ministry, 1994), 26,000 to 46,000 of them severe cases; in Brazil in 191,000 to 400,000 individuals with 19,100 to 40,000 severe cases; in Chile in 26,556, of which 9652 are severe; and in Uruguay in 12,000 individuals with 2400 severe cases of the disease (Fig. 19.1). These estimates were based on a prevalence of 2.07 million infected individuals in Argentina, 1.40 million in Bolivia, 1.91 million in Brazil, 142,000 in Chile and 51,000 in Uruguay.

After an asymptomatic or latent period that could last 10 years or more, a variable percentage of individuals, depending on the geographical area, will develop the cardiac or digestive manifestations that characterize the chronic stage of the disease. Chronic cardiomyopathy is the most common. It appears in up to 20% of infected individuals from Argentina, Chile and Mexico, up to 24% from Bolivia, and up to 43% of those from Central Brazil (Laranja *et al.*, 1956; Coura *et al.*, 1985; Maguire *et al.*, 1987; Storino and Milei, 1994). No correlation was observed between sex or race and presence of chronic disease but a greater incidence was noted during the second and fourth decade of life. The most common symptoms are dyspnea and palpitations. Electrocardiographic alterations such as right bundle branch block and/or left anterior hemiblock, unifocal premature ventricular beats and primary T-wave changes are considered compatible with mild chagasic cardiopathy. In a severe case, atrial-ventricular block, frequent and multifocal premature beats, and atrial fibrillation are the ECG alterations that accompany the cardiac enlargement (Laranja *et al.*, 1956; Rosenbaum, 1964).

The prognosis is poor for those patients with evident or pronounced cardiac manifestations. Usually, they do not survive more than 5 years. Sudden death due to paroxysmal tachycardia and ventricular fibrillation occurs in some patients and cardiac insufficiency occurs in more than 50% of them. Heart failure coexists with multiple electrophysiological abnormalities, from sinus node dysfunction to severe intraventricular and atrioventricular conduction abnormalities.

Digestive manifestations of Chagas disease, such as megacolon and megaoesophagus, have been reported principally in patients from Central Brazil, where the latter manifestation seems to be more frequent. Dilatation of hollow viscera in Argentina is less common, whilst in Venezuela and Central America it is rarely seen. The prevalence of digestive disturbance is three times higher in Brazil than in other countries of the Southern Cone of the Americas. More than two thirds of megaoesophagus cases appeared in individuals aged between 20 and 59 years, of which 66% of them were males. The most frequent symptoms are dysphagia and odynophagia with subsequent signs of malnutrition. Alterations of the oesophagus,

as seen radiologically, may vary from delayed emptying (stage I), to moderate oesophageal enlargement with motor incoordination (stage II), hypotonic and large achalasic oesophagus (stage III) and dolichomegaoesophagus (stage IV). A complication of megaoesophagus is oesophagitis (Rezende and Luquetti, 1994). Megacolon is more often seen in males in the fifth decade of life, appearing in 25.5% of the cases. Another 21% occurred in males aged 41–50 years and a similar percentage occurred in those aged 31–40 years, with common symptoms being constipation and meteorism. As a result of colon distension and contractions, abdominal pain is frequent. Faecalomas are a common complication (Rezende and Luquetti, 1994).

In addition to the classic lesions mentioned above, there are several indications that chronic chagasic infection is associated with poor cognitive performance: the ability to recognize familiar well-learned facts and routinely practised skills (verbal intelligence quotient) is normal but the patient is slower than non-infected individuals in dealing with new situations; impaired cognitive function is also associated with alterations in subcortical white matter (Sica, 1994).

Since the early 1980s, there has been evidence of recrudescence of *T. cruzi* infection in immunosuppressed patients such as those who have received kidney or heart transplants. It does not occur in all patients but, when present, symptoms are more severe than those usually seen in acute Chagas disease. In some cases symptoms indicate that the central nervous system is involved. AIDS can also lead to immunosuppression and reactivation of a previous infection. Brain abscesses caused by *T. cruzi* have been reported in some of those patients (Gluckstein *et al.*, 1992; Lopez-Blanco *et al.*, 1992; Kirchhoff, 1993; Altclas *et al.*, 1996). With the implementation of multi-retroviral therapy for treatment of AIDS, reactivation in AIDS patients becomes unlikely.

Chronic disease: mortality

Despite under-registration of cases, Chagas disease is perceived as a debilitating and incapacitating chronic disease that appears in 20% of those infected with *T. cruzi* and kills from 23,000 (World Bank, 1993) to 45,000 people/year, depending on the source of information. However, those estimates are not reflected in the number of deaths for Chagas disease reported from the countries, which have no relationship with the estimated prevalence. For example, in Chile only 126 deaths from Chagas disease were recorded in the period 1986–1988. In a 10-year longitudinal study in Brazil, the risk of death of a chagasic individual was twice that found in a non-infected person (Coura *et al.*, 1985). In Chile, it was found to be four times higher (Arribada *et al.*, 1987).

Despite the lack of accurate nationwide information, available data from more limited areas show the importance of the disease as a cause of death. In one Argentinian province, the annual mortality rate for Chagas disease per 100,000 population was 2.56% for 1980, 2.67% for 1983 and 3.88% for 1986. Fifty per cent of the deaths occurred in individuals younger than 50 years old. In Bolivia, based on data obtained in the 1980s it was estimated that six pregnant women and seven children died because of Chagas disease each day: overall, 13,785 individuals died of Chagas disease in 1992 (Human Development Ministry, 1994). In Brazil the disease was considered to be responsible for 8.2% of the 5,044,000 national deaths that occurred during 1977–1982; 62% of the deaths were males and 38% were females. Only 7.5% of these deaths occurred in individuals younger than 30 years old, whilst 45% of them occurred before the age of 50. For the period 1980–1990, Chagas disease was responsible for more deaths in Brazil (67,529) than malaria (9560), schistosomiasis (7599) and yellow fever (132) (Silveira, 1994). During that period there was a steady decrease in the mortality rate due to Chagas disease, from 5.1/100,000 population in 1980 to 4.5/100,000 in 1985 and 3.9/100,000 in 1990. That is the equivalent of 6191 deaths in 1980, 6124 in 1985 and 5845 in 1990. However, these data seem to indicate a sig-

nificant under-registration, as the number of deaths attributed to the disease was 17,000 in 1995 (Akhavan, 2000). In São Paulo, the disease represented 0.9% of state deaths in 1987. Chagas disease was also considered a common cause of sudden death in Brazil. In one particular city, Uberaba, it was responsible for 24% of all cases of sudden death in 1980. Of those who died, 31% were asymptomatic (Lopez and Chapadeiro, 1986; Prata *et al.*, 1986). In another study in the same city 10 years later, 6.8% of sudden death cases were still attributed to Chagas disease (Lopez *et al.*, 1995). During 1977–1978, 4.3% of the deaths that occurred in Brasilia were considered due to Chagas disease. The annual mortality rate for individuals with the disease increased from 5.5 cases/100,000 population aged between 15–24 years old to 308 cases/100,000 population aged 65 years or older. In Venezuela, the mortality rate for Chagas disease varied from 0.88 to 1.02/100,000 deaths from 1979 to 1982.

Social and Economic Costs

Chagas disease represents the cause and results of social, educational, political and economic underdevelopment. At the same time it imposes a tremendous burden on the already precarious health services within the endemic area, where it constitutes a serious negative factor for the further development of individuals and social groups.

Chagasic cardiopathy ranked third as a cause of disability in a rural area of Brazil where *T. cruzi* infection is endemic. It was also the main cause of early retirement. In another endemic area 4–9% of all disability benefits received by 30–50-year-olds were attributed to Chagas disease. The yearly potential of productive life loss (YPPLL) was 5.937 for non-chagasic cardiopathy and 4.612 for chagasic cardiopathy. That represents 12.5% and 9.7% of the total number of YPPLL from all causes. The rate of disability for individuals with chagasic cardiopathy was 147.6 per 10,000 workers. In the State of São Paulo, Brazil, ischaemic heart disease was responsible for 2.4% of YPPLL, whilst Chagas disease was responsible for 1.1%. The number of YPPLL as a result of the disease among individuals between 15 and 64 years of age was estimated at 2275 per 100,000 population for males and 1369 for females. Overall in the country it was estimated that the YPPLL was 86,000 (Silveira, 1994). In Bolivia, on the other hand, it was 105,000, with a population that was 20 times lower, reflecting the higher rate of infection in this population.

The population affected by Chagas disease is of low socio-economic level and so the cost of treatment is usually provided by the state. In Argentina, follow-up of 128 patients with cardiac lesions for 30 months showed that expenses in medical attention and drugs were US$350,000. The overall indirect costs of the disease to those patients were estimated at US$6,201,600, the indirect costs of premature mortality at US$986,880, and those of morbidity at US$2,791,680 for males and US$2,423,040 for females (Evequoz, 1993). Treatment costs of each individual with acute Chagas disease were US$591.80, with asymptomatic infection US$174.49, mild cardiopathy US$493.16 and severe cardiopathy US$1597.83 (Del Rey *et al.*, 1995). If every individual with mild or severe cardiopathy of chagasic aetiology in the country had received treatment, the cost would have been US$242.6 million. Nevertheless, this is less than the US$330 million that represents the cost of the years of work lost due to premature death of chagasic patients in Argentina. In Bolivia, costs of treating patients with acute disease were US$215,000, with chronic disease US$21 million and with congenital disease US$186,000/year. It is interesting to compare these amounts with the annual treatment costs of US$662,850 for malaria and US$2.67 million for tuberculosis. Costs directly attributed to death were estimated at US$343,000, while annual indirect costs for morbidity were estimated at US$43.8 million and US$57.5 million for mortality (Human Development Ministry, 1994). In Brazil, costs of pacemakers and surgery for megavisceras were estimated at US$250

million, whilst man-hours lost from 75,000 chagasic workers due to absenteeism accounted for another US$625 million (Dias, 1987). This amount increased to US$5000 million in 1990 (Bryan and Tonn, 1990, cited in Schofield and Dias, 1991). These costs do not include consultation, care and supportive treatment for chronic chagasic patients, which amounted to a further US$1000/year per patient (Schofield and Dias, 1991), nor do they account for disability awards which in one state alone represented US$400,000 in 1987 (Dias, 1987). Early retirement brought about by chagasic disabilities was responsible for 220,000 YPPLL, representing another US$650 million, in 1989–1990, whilst the implantation of pacemakers and surgery carried out on 75,000 individuals with cardiopathy cost US$750 million (Dias, 1987). These numbers might now be revised and reduced, considering that the prevalence of the infection in Brazil has decreased and that no more than 0.2% of those infected usually need a pacemaker. At a cost of US$5000 each, treatment costs would be closer to US$19.1 million (Schmunis, 1999a). Actual hospitalization costs may also be less than previously imagined. From 1984 to 1993 the average number of inpatients was 1700, at a cost of US$745,000 in 1991 and US$600,000 in 1993 (Franca and Abreu, 1996). However, it is possible that the number of chagasic inpatients was under reported, as these figures contrast with the estimated cost of productive years lost by premature deaths, accounting for US$723.9 million (Schofield and Dias, 1991).

In Chile, the annual cost of the disease, without taking into consideration the costs of pacemakers and surgery involved, was estimated to be US$37 million. Costs would be lower if patients used public health services. The cost of implanting 120 pacemakers would be US$120,000 and to treat each of the estimated 9652 individuals with cardiopathy would cost from US$411.5 to US$549.5. The estimated cost of work lost due to premature death mounted to US$58.1 million (Schofield and Dias, 1991). Estimates in Mexico showed that, out of 152,400 individuals, 50,800 may develop cardiopathy caused by *T. cruzi* in 1992. One in ten of them may evolve to chronic chagasic cardiopathy. The cost of losing 15 years of productive life was thought to have been US$70 million in 1996. No attempt was made to calculate the cost of treatment or patient care (Vallejo and Reyes, 1996). In Uruguay the cost of treating 12,000 chagasic cardiopaths, including 2400 with severe lesions and 3700 with megacolon, was estimated at US$15 million.

The Future

As a result of spraying with residual insecticides, house infestation rates for *Triatoma infestans* in the South Cone countries of South America, and for *Rhodnius prolixus* in Central America, are decreasing sharply. In fact, vectorial transmission of *T. cruzi* is thought to have been interrupted in most of the *T. infestans* endemic areas of Brazil, Chile and Uruguay. In addition, migration from rural to urban areas is reducing the rural population exposed to infected vectors. On the other hand, migration of infected individuals from rural areas to urban areas increases the possibility of acquiring *T. cruzi* infection by blood transfusion in cities (WHO, 1991). Since the 1980s, however, the AIDS epidemic has increased the awareness of national authorities in endemic countries of the need for serological screening to prevent transfusion-transmitted diseases. This will improve the situation in relation to transfusion-acquired *T. cruzi*, as well as hepatitis B and C. Therefore, the number of infected individuals with *T. cruzi*, and of those that develop Chagas disease as a consequence of the infection, will slowly decrease in the Americas. If control measures for vectorial transfusion and congenital infection are fully implemented now, it might be possible to look forward to the prevention of most cases of Chagas disease. The disease would become a rare event in 80–90 years time, after the deaths of those who are already infected.

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20 Economics of African Trypanosomiasis

Alexandra P.M. Shaw

Emergence of Interest in the Subject

While the depredations caused by African trypanosomiasis in both people and animals were clearly identified and recognized by the beginning of the 20th century, concerted attempts to quantify and analyse its economic impact on African agriculture really only began in the 1970s. Reviewing the literature on the economics of trypanosomiasis, Jordan (1986) stated: 'Ten years ago even this incomplete account could not have been written. There is an urgent need for more hard facts…'. Nevertheless, awareness of the economic dimension had been growing for some time; as techniques for dealing with trypanosomiasis were being developed and refined in the 1950s and 1960s, published papers increasingly included analyses of the costs of the control methods developed and implemented. Interest in the subject was fuelled primarily by the need to ensure that scarce public resources were being spent in cost-effective ways that would yield tangible economic and social benefits. During the 1960s, cost–benefit analysis moved out of its narrower financial applications in industry to look at public sector projects in fields such as transport and water supplies, then evolved into a new discipline called social benefit–cost analysis, in particular covering project planning and development activities in the Third World. Many of the basic principles of health economics were first explored during the same period. The 1970s also saw the emergence of veterinary economics as an independent field of study.

Thus interest in the economics of trypanosomiasis control grew, with the Food and Agriculture Organization (FAO) and World Health Organization (WHO) including it in their agendas in the 1960s. The first studies looking explicitly at economic aspects of the disease were undertaken in the 1970s and when FAO held a conference in 1977 on the economics of tsetse and trypanosomiasis control, the subject became firmly established on the tsetse and trypanosomiasis agenda.

Analytical Framework

Since the early 1970s, the socio-economic aspects of the disease and its control in people and their livestock have been studied in many locations, using and developing a wide range of techniques to gather data and use it in economic analyses. Much information has been amassed; this chapter provides an overview of what has been done, gives an introduction to the literature and highlights key issues to be considered.

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Direct and indirect impacts of trypanosomiasis

As a vector-borne zoonosis, with long-term implications for agricultural and livestock production systems as well as land use, trypanosomiasis presents the same degree of complexity and variability to the economist as it has to those from other disciplines who have grappled with it in the other chapters of this book. An analytical framework has to integrate the effects of the disease on people and livestock at current levels of incidence with the longer-term effects due to the way it constrains production opportunities. Many writers distinguish between the 'direct' and 'indirect' costs or effects of this and other diseases but definitions vary hugely. This is particularly so for the disease in humans. More of a consensus has emerged in the field of livestock trypanosomiasis (Putt *et al.*, 1980; Doran, 2000; Swallow, 2000) and the following definitions are generally used:

- Direct costs are the direct impacts of the disease on livestock productivity (mortality, fertility, milk yield, ability to work as traction animals) to which can be added expenditure on controlling the disease.
- Indirect effects are due to the way in which the presence of the disease limits production opportunities, influencing the choices made by livestock keepers and farmers, thus these result from decisions on herd structure, choice of livestock breed, use of animal traction, grazing patterns and migration.

The indirect effects thus reflect the 'lost potential' as described by Perry and Randolph (1999), that is, the 'total losses' due to a disease, rather than the more immediately 'avoidable losses' or direct effects. Time is also a factor; direct impacts of fertility and mortality have long-run effects on herd growth that constrain future production opportunities. When trying to find a cut-off point between direct and indirect effects, it rapidly becomes clear that, in fact, there is a continuum, with mortality and treatment costs at one end and widespread change in production systems at the other. Figure 20.1 illustrates this notion of a continuum, while setting out the framework that will be used here for assessing the economics of controlling trypanosomiasis in both people and livestock.

Economic methodology

This chapter is written to harmonize with the rest of this volume and thus primarily addresses an audience from the natural rather than the social sciences – much of the work on the economics of the disease has in the past been undertaken by entomologists, veterinarians, epidemiologists, medical doctors, etc. and probably will be in the future. The volume by Drummond *et al.* (1997) is useful for a broader understanding of health economics and the principles involved, and the article by Murray (1994) gives an introduction to measuring disease burdens using DALYs (disability-adjusted life years). In the field of veterinary economics, the volume coordinated by Perry (1999) provides a wealth of general articles and case studies.

Quantifying and valuing the costs of disease is relatively straightforward in an experimental situation but becomes extremely complex when undertaken for populations and production systems. For this reason it is important to be aware that calculations of the economic impact of trypanosomiasis inevitably rely on estimates and assumptions. Applying prices so as to evaluate control options is also complex. The costs themselves vary from situation to situation and over time. Accounting for price changes over time is theoretically straightforward but time consuming; for this reason 'updated' costs of control techniques are rare or need to be regarded with caution, all the more since techniques are evolving over time and can be very specific to particular locations.

Taking time into account leads into a second issue. A component of the methodology used in cost-effectiveness and benefit–cost analysis is assigning a lower weight to future income and expenditure as compared with present income and expenditure. This practice is called 'discounting' and has sometimes attracted controversy where long-term programmes are involved. In human health, the convention has been

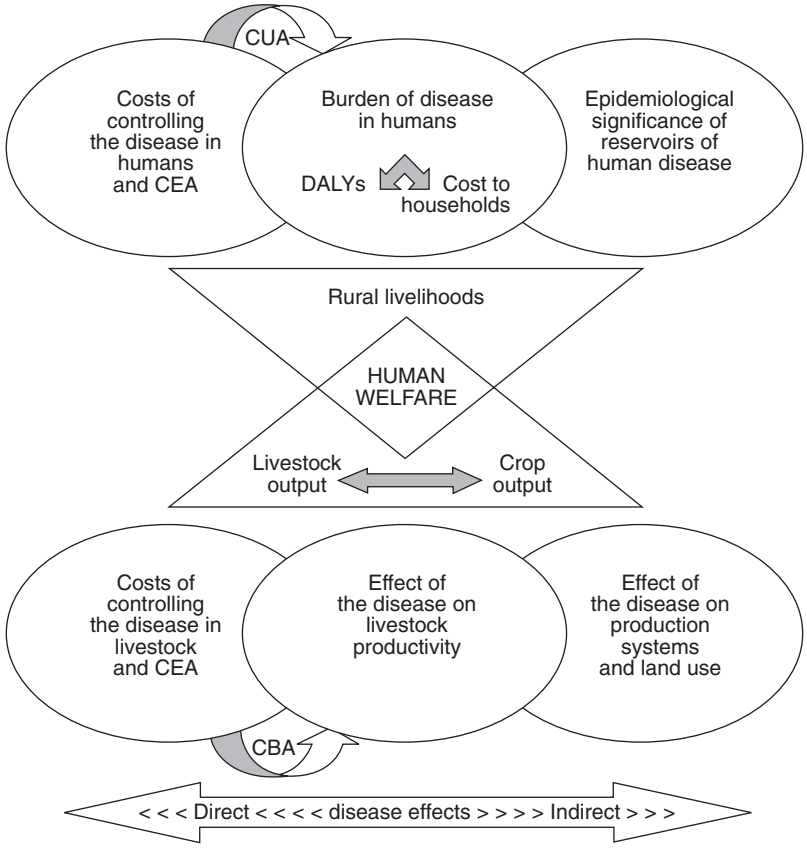

Fig. 20.1. Analytical framework: the economics of trypanosomiasis.
CUA, cost–utility analysis; CBA, cost–benefit analysis; CEA, cost-effective analysis.

to discount using a 3% to 5% compound rate (Murray, 1994; Drummond *et al.*, 1997); in livestock production, rates of 10% are more commonly used (see Shaw, 2003, for a more detailed discussion).

Lastly, a cautionary note about control costs is necessary. In an economic analysis the full costs, including all overheads and shares of fixed costs, such as salaries, should be included when comparing cost with a measure of impact, such as a human health indicator or extra production from livestock. Full costings are also required when setting up new activities, when comparing activities that are based on different health infrastructures and when comparing vector control with the use of trypanocides (see discussion in Barrett, 1997). In practice, many calculations are undertaken to compare different techniques in the field, which rely on an established infrastructure, and the costings thus omit overheads. When interpreting the costs of different ways of controlling trypanosomiasis, it is important to be aware of what is and is not included, and how appropriate each costing is in relation to the decisions to be made.

Human African Trypanosomiasis

Although the last three WHO expert committees on trypanosomiasis (held in 1976, 1985 and 1995) considered economic issues, the field of human African trypanosomiasis (HAT) has had very little formal input from health economists (Walker and Fox-Rushby, 2000). The need to deal with an epidemic resurgence of the disease in the 1990s has meant that studies to support resource allocation and determine priorities are very timely.

The burden of disease

It has been difficult to appreciate the size of the problem posed by HAT, both on a continental and on a national or local scale. Although individual countries report the numbers of patients diagnosed to WHO, it is widely recognized that these figures often only represent the tip of an iceberg of unknown proportions. It is estimated that 60 million people are continuously exposed to the risk of infection in the 20 countries where active transmission is thought to be taking place, but only 3–4 million of these people are covered by surveillance (WHO, 1998; Cattand *et al.*, 2001). There has been a well-documented re-emergence of the disease in recent years, with some 45,000 new cases being reported annually in 1998 and 1999 – about ten times higher than the number in the 1960s, when the disease was considered to be under control, and rapidly approaching the levels recorded in the 1940s. The current epidemic mainly concerns *gambiense* disease, with the most recent epidemic of *rhodesiense* disease, in southeastern Uganda, having peaked in 1988 and declined thereafter. The increase in the number of cases reported in the 1990s very closely mirrors the decline in active screening for *gambiense*. Furthermore, despite the high number of cases currently being reported, WHO estimates that these represent only 10–15% of the actual number of infected individuals, which is thought to be around 400,000 (Cattand *et al.*, 2001). This disparity between reported and actual cases reflects both the decline in health service and surveillance coverage and the inherent difficulty in diagnosing the disease. For *rhodesiense*, epidemiological work undertaken in Uganda indicates that the disparity between individuals who are diagnosed and treated and the number of individuals with the disease is of the order of 12 to 1 (M. Odiit and P. Coleman, Edinburgh, 2002, personal communication), a figure with frightening implications for human welfare and disease control.

Disability-adjusted life years

Estimates of the burden of sleeping sickness based on field data have recently been undertaken in southern Sudan for *gambiense* disease and in southeastern Uganda for *rhodesiense* disease. The disease is inevitably fatal in untreated individuals. Only a proportion of those infected are treated; between 3% and 5% of these also die. Thus the number of DALYs lost per infected person is high. In Uganda, this was estimated at 23 DALYs lost per death, and 0.21 DALYs lost per successfully treated individual (M. Odiit and P. Coleman, Edinburgh, 2002, personal communication). The data for Sudan are not yet published but estimates were of 33 DALYs due to premature mortality (D. McFarland, Atlanta, 2003, personal communication). Extrapolating these figures to a continental level is difficult, given the great uncertainty about the number of unreported cases. The total number of deaths due to sleeping sickness is very approximately estimated at 50,000 to 100,000 annually (Cattand *et al.*, 2001), which would imply an annual burden of disease of some 2 million DALYs. Two factors are vital in understanding why, with few cases when compared with malaria or AIDS, the present epidemic of sleeping sickness is considered a major public health risk. The first is due to the epidemiology of the disease, which, in the absence of effective surveillance, can rapidly reach epidemic proportions, as evidenced by the recent resurgence. The second is due to the case fatality rate. In southeastern Uganda, there were nearly 200 times as many cases of malaria as sleeping sickness, but malaria only accounted for 3 times as many DALYs lost (M. Odiit and P. Coleman, Edinburgh, 2002, personal communication).

Costs to households and the rural economy

Moving from DALYs to the impact of the disease on communities and individuals, many descriptive accounts exist but few quantitative estimates have been published. Information on age at death very clearly demonstrates that the disease above all affects economically active adults. Unpublished data from Uganda show nearly 25% of cases occurring in the 20–29 age group, and just over 60% in those aged from 10 to 39 years (M. Odiit and P. Coleman,

Edinburgh, 2002, personal communication). The nature of human–fly contact and the propensity of the disease to affect individuals in particular occupations, especially in the early stages of epidemics or at low prevalences, are well known. This exacerbates the disease's impact on households, since it tends to hit the main providers, whose income is lost to the family if they die undiagnosed. Furthermore, sick individuals require a lot of care. The initial attempt to obtain a diagnosis typically involves several trips to a rural clinic, recourse to traditional medicine and healers and travel to a more distant centre for specialist advice. For those patients who are successfully diagnosed, someone is usually needed to accompany them during treatment. If a correct diagnosis is not achieved, the patients need to be cared for until they die. Gouteux *et al.* (1987) estimated the financial costs borne by families on the basis of interviews with diagnosed patients. Payments for pre-treatment drugs, such as vitamins, plus other costs such as transport, food provided during hospitalization and treatment, came to a figure equivalent to 12% of the annual income from agriculture. Adding the 'indirect' costs represented by the time lost to the diagnosed patients and those accompanying them, resulted in a total cost to families equivalent to about a quarter of a year's income. Studies are underway to try to provide current estimates of these costs in a number of locations. These, together with the ongoing work on DALY calculations and the information from increased case detection and surveillance work on *gambiense* disease, will ensure that a much clearer idea of the true burden of sleeping sickness emerges in the next few years.

Analysing the costs of controlling trypanosomiasis in humans

Case detection in gambiense *disease*

In *gambiense* disease, surveillance and active case detection are considered to be the most effective way not just of ensuring that individuals are diagnosed and treated, but also of controlling the disease by reducing the size of the human reservoir. Numerous approaches are used, ranging from the purely passive, in which patients with marked clinical signs visiting local health clinics may eventually be referred, correctly diagnosed and treated, to active and exhaustive case-finding using mobile teams and screening large sections of the population at risk. The appropriate intensity of case-finding should be a function of the local prevalence of the disease (WHO, 1998). In order to support and direct active case-finding to control the disease, a systematic approach for surveillance has been developed (Cattand *et al.*, 2001), based on mapping foci of the disease. Villages and hamlets are categorized according to their infection status, endemic villages are grouped into foci, and decisions are taken as to where to direct control activities and where to initiate specific surveillance activities based on serologically testing a proportion of the population.

The costs of case-finding and treatment have been analysed in a number of studies. The results of the first attempt at comparing different modes of delivery were presented in WHO (1986). This adopted a full cost approach, including training, administration, depreciation of all equipment and shares of salaries of staff and costs of facilities working part time or full time on trypanosomiasis control. This study was later updated (WHO, 1998). The distinction was made between systematic testing at rural health centres, where all people visiting the centre are routinely screened for trypanosomiasis, and true passive detection, where patients presenting at health centres are eventually correctly diagnosed as having trypanosomiasis. Also costed were attempts to integrate sleeping sickness control and surveillance with the primary health-care system, by systematically collecting blood samples for screening on filter papers at rural health centres or by primary health-care workers, based on work in Uganda and Côte d'Ivoire (Laveissière *et al.*, 1998; WHO, 1998). Costs for active case detection from these calculations and from other recent studies are given in Table 20.1 and approximate to around $1/person screened.

Table 20.1. Recent costings[a] for active case-finding in *gambiense* disease.

Country (Source)	Costing method	Technique	Cost per person screened (US$)	Impact
Uganda (WHO, 1998)	Full costing, including all field costs, overheads, share of salaries and administrative costs, based on experience in the field, at 1995 prices	Filter paper sampling at rural health centres	0.8	Access only those people visiting health centre
		Community health workers using filter paper	0.6	Depends on time available to sample people in homes
		Mobile teams		
Democratic Republic of Congo (DRC) (P. Lutumba, Kinshasa, 2001, pers. comm.)	Full costing, based on experience in the field	Mobile teams	1.4	Can access virtually whole population if enough time spent in the area
			0.6–1	Can access virtually whole population if enough time spent in the area
Central African Republic (Ruiz Postigo *et al*., 2001)	Full cost of provisional programme was $ 4.1, per person including surveys, all treatment and overseas costs	Mobile teams	2.5[b]	Planned to access whole population.
Côte d'Ivoire (Laveissière *et al*., 1998)	Actual field operating costs included, depreciation on vehicles and equipment excluded	Community health workers using filter paper	0.10	Accessed 73% of population in 2 months
		Mobile teams	0.55	Accessed 42% of population in 10-day period

[a]These costs refer to situations with prevalences of around 1%. Initial screening in all scenarios involved use of the CATT (Card Agglutination Test for Trypanosomiasis), which in the DRC study was used in parallel with lymph node palpation.
[b]Fully costed as in calculations for Uganda and DRC, with some additional costs for treatment centres.

These cost-effectiveness analyses initially focused on comparing modes of delivery. However, from the point of view of controlling the disease and of patient welfare, finding and treating as high a proportion of patients as possible is the main criterion. Thus, although mobile teams are the most expensive, they are generally considered to be the most effective way of sampling a population and finding the greatest number of patients. Not only are they able to access a higher proportion of patients, but also there tends to be less 'leakage' of suspected cases from screening who do not come for further testing, or of diagnosed patients who do not present themselves for treatment. This can be a problem when using community health services. Nevertheless, for surveillance or when resources are limited, using community health services can be very cost-effective. Looking to the future, the cost-effectiveness of different diagnostic protocols will need to be examined, to see what the trade-offs are from using fewer, more sensitive and/or more expensive tests.

Treatment

Treatment regimes are evolving and vary greatly from country to country (WHO, 1998) both in terms of the timing of drug administration and duration of hospitalization as well as in terms of the health infrastructure, which itself determines hospitalization costs. Some of the costs of treatment are borne by family members, who may provide food, pay some costs or accompany the patient (e.g. Gouteux *et al.*, 1987). An estimate of treatment costs can be found in WHO (1998), which gives first-stage treatment cost at US$107 for treatment using pentamidine at a subsidized price, as against US$227 at full market cost and US$114 for suramin. For second-stage treatment the estimates are US$257 for treatment using melarsoprol and US$675 using eflornithine. Since then, WHO's public/private partnerships have assured a cost-free supply of drugs until 2006. In economic calculations, as opposed to budget estimates, the cost of drugs should still be included as they are real resources used to deal with the disease. An analysis of the cost-effectiveness of using different second-stage drug regimes can be found in Politi *et al.* (1995). Second-stage treatment regimes using melarsoprol are usually based on 20–30 days' hospitalization but a new 10-day schedule for treating second-stage *gambiense* patients (Burri *et al.*, 2000) is being tested. This would improve the cost-effectiveness of treatment and, to a certain extent, relieve the financial burden on families.

Vector control

Vector control activities primarily aimed at controlling HAT have been undertaken at various times and in different localities. For most of these schemes, careful recording of field costs has been undertaken and reported on in publications. Tsetse control activities using traps and screens and involving local communities have been undertaken for *gambiense* disease in Côte d'Ivoire and Congo and for *rhodesiense* in Uganda. Laveissière *et al.* (1994) summarized the work of many years in Côte d'Ivoire, citing costs for traps, screens and vehicle running, which came to US$39/km^2 protected and US$2.3/person protected. For Congo, summary figures for the cost of trapping materials are given in Gouteux and Sinda (1990): for a control campaign involving 25,000 inhabitants this came to US$0.23/person/year. In both Congo and Côte d'Ivoire, active case-finding accompanied the work. In Equatorial Guinea, the cost of traps accompanying active case-finding worked out at US$5.1/inhabitant (Simarro *et al.*, 1991). In Uganda, Lancien and Obayi (1993) estimated the cost per person protected at US$0.5, based on a cost for traps, staff and logistics. Thus, although the costs cited only include direct field costs and vary over time, it is still clear that there is a big variation from scheme to scheme, reflecting the different techniques used and the characteristics of each geographical focus of the disease.

Comparisons of cost and impact of different control approaches

Although the costs of controlling trypanosomiasis have been studied for several decades, it is only since the recent studies estimating

the number of DALYs lost per treated and untreated patient that it has become possible to relate costs to impact. In Shaw and Cattand (2001), the full cost of case-finding and treatment using mobile teams was calculated at different prevalences and the likely cost per DALY was estimated. Even if the number of DALYs for each case found and treated was only 15 (less than either estimate for *gambiense* or *rhodesiense*, and not including any figure for the effect that treating infective individuals has on transmission of the disease), the cost per DALY averted at a prevalence of 1% would be US$22. This would decline linearly, so that if 20 DALYs were averted the cost would be US$16 and if 30 were averted the cost would be US$11. The analysis by Politi *et al.* (1995) showed that the cost per DALY averted for melarsoprol treatment of patients in the second stage was US$8.

The most comprehensive calculations can be found in Trowbridge *et al.* (2001). They studied the situation in Tambura County in southern Sudan and concluded that 'if trypanosomiasis prevalence doubled every 1.5–2.5 years, as it did in Tambura County, and the initial prevalence was 0.5%, periodic screening would yield a cost-utility value of US$10.28 per disability adjusted life year (DALY) averted (range of US$3.84 to US$13.41) while emergency intervention after a 9-year interval would yield a cost-utility of US$17.41/DALY averted (range of US$11.97 to US$21.56)'. All these cost/DALY figures are well below the threshold of US$25, currently considered 'highly attractive', and compare very favourably with other disease control interventions in Africa. Being able to demonstrate this has made the case for investment in HAT control much more convincing.

For *rhodesiense* disease, an interesting economic scenario is unfolding with evidence that treating cattle, the main livestock reservoir, can prevent epidemics of HAT in some localities (see Chapter 11). A preliminary economic analysis undertaken by the author based on information from Uganda (M. Odiit and P. Coleman, Edinburgh, 2000, personal communication) indicates that, for this type of intervention, the cost per DALY averted will actually be negative. That is to say, the financial benefits more than cover the costs, before taking into account the effect on human health in terms of DALYs. This is because treating cattle will increase income from livestock, since the trypanocides used are effective against the trypanosomes that are pathogenic to cattle as well as against *T. b. rhodesiense*. In addition, lowering the incidence in people will save future costs of treating human patients.

The important unknown in quantifying the cost per DALY averted either for control of the human or animal reservoir or for vector control is the impact on transmission rates and hence on the disease incidence in the medium and longer term. To date, only the analysis by Trowbridge *et al.* (2001) has explicitly considered this effect, though it is implicit in the preliminary work on *rhodesiense* discussed above. Some studies have looked at the effect on prevalence of repeated screening surveys or vector control (e.g. Simarro *et al.*, 1991) and data on the changes in prevalence and population screened exists for many localities. An early study (Shaw, 1989) showed the relative economics of vector control as compared with case-finding and treatment to be hugely sensitive to epidemiological assumptions. At that time, case-finding and treatment appeared to be more cost-effective at lower incidences and when dealing exclusively with a human reservoir. The conclusion, then as now, is that epidemiological models and economic models need to be integrated to be of use in decision making.

Tsetse-transmitted Trypanosomiasis in Livestock

It is variously estimated that some 45–50 million cattle live under trypanosomiasis risk, in a tsetse-infested area of some 8–10 million km^2 (Budd, 1999; Kristjanson *et al.*, 1999; Gilbert *et al.*, 2001). Attempts to quantify the impact of the disease have been undertaken firstly by trying to measure its direct impact on key productivity parameters – mortality, fertility and (where relevant) milk production, animal traction output and

weight gain – and then to translate these into monetary terms, often using demographic herd or livestock population models. Secondly, a number of studies have tried to investigate the extent to which the indirect effects of the disease constrain livestock and crop outputs. An excellent and comprehensive summary and discussion of the work undertaken can be found in Swallow (2000). Some of the key findings are discussed here.

Direct impacts of the disease in livestock

The last three decades have seen the accumulation of a respectable body of information on how the disease affects livestock productivity, most of it relating to cattle. Table 20.2 gives the results of the major studies looking at the disease's impact on cattle productivity and Table 20.3 the results of a few studies on sheep and goats. A number of different approaches have been tried; these can be distinguished both in terms of how the data were collected and in terms of the basis for comparison used to assess the impact of trypanosomiasis. Data collection was usually linked to studies on disease prevalence and has ranged from continuous monitoring of a selection of parameters to one-off questionnaire surveys. Assessment of the impact of trypanosomiasis is fairly straightforward at an experimental level – for example, on-station or in ranch cattle, where it is relatively easy to match animals, vary treatments or exposure and quantify impacts. However, the disease's importance is to herders and farmers with mostly traditionally managed herds or smallholdings, and here valid comparisons are far more difficult to make. In these situations, comparisons have been made between: (i) infected and non-infected individual animals, in the African Trypanotolerant Livestock Network (ATLN), which developed a detailed protocol and made comparisons on the basis of parasitaemia in individual animals; (ii) infected and non-infected herds in the same area; (iii) sites with different levels of challenge; and (iv) the same site before and after disease or vector control. The results of these studies are given in Tables 20.2 and 20.3. Most striking is the wide variability, which makes it difficult to generalize. It is clear that the presence of trypanosomiasis affects most key production parameters to some extent, but that extent varies greatly from situation to situation and according to the structure of the study.

Mortality

Differentials in annual calf mortality rates mostly fall in the 0–20 percentage points range suggested by Swallow (2000), with just over half of the comparisons cited showing calf mortality to be 6–10 percentage points higher due to the presence of trypanosomiasis. In the special case of the Yalé pastoral zone in Burkina Faso, where an outbreak hit recently settled herders very hard, over 60% of calves died of trypanosomiasis according to recall data (Kamuanga *et al.*, 2001a). In southern Africa, however, the cross-site comparisons show a different picture, with mortality between 5 and 14 percentage points *lower* under high challenge than under low. Doran (2000) discussed this at some length, and concluded that it reflects the fact that calves are less at risk, both because of the way that they are managed – they often do not go out to graze with the rest of the herd – and because they are bitten less by tsetse. Results for annual mortality rates in older animals are also very variable, with most differences in the range of 2–8 percentage points. However, in the Doran (2000) study for southern Africa, high levels of treatment of oxen and cows mitigate this effect.

Calving rates

Differences in calving rates occur consistently throughout the sample, the cross-site comparisons tending to show greater differences, with calving rates in higher-challenge areas being between 6 and 19 percentage points lower than in comparable lower-challenge areas. For per animal comparisons on the basis of parasitaemia, the differences in calving rates were mostly around 7 percentage points lower in infected animals. The studies in The Gambia highlighted the fact

Table 20.2. Selection of cattle production parameters in relation to trypanosomiasis status.
(a) West Africa

Country, production system and source[a]	Data collection method and sample	Impacts measured[b]			
		Calf mortality	Adult mortality	Calving rates[c]	Other

The Gambia, N'Dama village herds (Agyemang *et al.*, 1990, 1997)

ATLN protocol[d], comparison by parasitaemia[e]

During 4 months post-partum

	1+	0
CR%	56.1	63.2
n	43	251

Calf survival to 6 months

	Alive	Died
CR%	51.9	69.0
n	275	19

Milk yield 1st 6 months

	1+	0
litres	137	172
n	41	48

The Gambia, N'Dama village herds (Agyemang *et al.*, 1997)

Comparison of 6 sites with 200 to 1200 cattle, each site monitored for up to 6 years, ATLN protocol[d]

Site	Challenge level	% Calf mortality	% Adult mortality	% Calving rate	Lactation yield: Daily milkings	kg
Gunjur	Low/Zero	12.8	4.5	55.5	2×	405
Nioro Jattaba	Low	10.8	3.7	53.1	1×	302
Pirang	Low/Medium1	8.7	2.4	58.4	2×	495
Keneba	Low/Medium2	16.2	2.4	57.7	2×	421
Bansang	Medium	15.7	11.4	45.1	1×	278
Missira	Very High	26.3	20.0	54.3	1×	203

The Gambia, N'Dama village herds (CIRDES, ILRI and ITC, 2000)

Continuous monitoring, monthly data collection, 11 herds north bank, 11 south bank, 1194 animals, comparison of:
- uninfected
- infected + untreated
- infected + treated.

Mortality 0–6 months

	Inf'd	Uninf'd
%	15	8
n	149	220

% Calving rate

Uninfected	54.2
Inf'd 1st 6 months & untreated	55.0
Inf'd 2nd 6 months & untreated	53.7
Inf'd 1st 6 months & treated	49.2
Inf'd 2nd 6 months & treated	42.8
Calf lost first month	89.5
Calf lost first 8 months	78.8

300-day Lactation yield

	Inf'd	Uninf'd
kg	236	260

Côte d'Ivoire Sedentary village herds, N'dama, Baoulé[f] and crosses (Thorpe *et al.*, 1988)	ATLN protocol[d], comparison by parasitaemia[e]								During 8 months post-partum	
									1+	0
								CR%	71.3	78.5
								n	38	44
Côte d'Ivoire Village herds – various breeds: Baoulé[f] (Bl), N'Dama (Nd), Zebu × Baoulé crosses (Z × Bl) and N'Dama × Baoulé crosses (N × Bl) (Camus, 1981a)	Comparison of parameters in infected (positive) and uninfected (negative) herds, 191 herds monitored, over 3000 cattle sampled.	% Calf mortality			% Adult mortality			% Calving rate		
		Breed	+ve	–ve	Breed	+ve	–ve	Breed	+ve	–ve
		Bl	18.5	12.1	Bl	1.6	1.7	Bl	44.5	42.7
		Nd	12.5	9.2	Nd	3.5	1.9	Nd	40.5	39.6
		N×Bl	10.0	4.7	N×Bl	2.4	0.6	N×Bl	43.8	41.2
		Z×Bl	20.5	11.6	Z×Bl	3.2	1.6	Z×Bl	40.7	46.6
Burkina Faso Pastoral zone, newly settled zebu cattle, severe outbreak of trypanosomiasis, after which tsetse controlled (Kamuanga *et al.*, 2001a)	Before and after for tsetse control comparison using, recall data of deaths due to trypanosomiasis and related diseases from 101 households 'before', 261 'after', 3+ = Aged 3 and over, M = male, F = female	Calf mortality			Adult mortality					
		% tryps. & related			% tryps. & related					
			Before	After		Before	After			
		M	64.9	8.7	M3+	69.9	7.2			
		F	63.3	7.3	F3+	70.8	10.0			
					Ox3+	61.0	5.4			
					All	63.1	7.1			
Togo Ranch N'Dama and Race Locale (Thorpe *et al.*, 1988)	ATLN protocol[d], comparison by parasitaemia[e]								During 6 months post-partum	
									1+	0
								CR%	80.7	91.9
								n	29	116

All figures refer to a period of 1 year unless stated otherwise.

[a]References as cited, adapted in part from Swallow (2000).

[b]*n* = number of animals or calving intervals in each category; Inf'd = infected; Uninf'd = uninfected; +ve = positive; −ve = negative.

[c]Calving rate (CR) refers to % of cows calving in a year, where necessary converted from calving intervals (CI) so that CR = 365/CI.

[d]ATLN protocol involved monthly weighing, bleeding, recording of mortality, births, calculation of calving intervals usually other entries and exits from herds and, at some sites, milk sampling and recording. Herders were offered some treatments for sick animals, which did improve productivity over time.

[e]Comparison by parasitaemia was by number of parasitaemic months, one or more (1+) or zero (0).

[f]Savanna West African Shorthorn, a trypanotolerant breed.

Continued

Table 20.2. *Continued*

(b) Central, Eastern and Horn of Africa

Country, production system, (source)[a]	Data collection method and sample	Impacts measured[b]			
		Calf mortality	Adult mortality	Calving rates[c]	Other
Republic of Central Africa Pastoral herds, zebu cattle (le Gall *et al*., 1995)	With and without comparison for tsetse control using traps, herd monitoring *n* = 673 cattle	All mortality No trapping 10.1% Trapping 9.3%		% Calving rate No trap 45.4 Traps 46.5	270 day lactation yield litres No trap 233 Traps 237
Democratic Republic of Congo N'Dama ranch cattle (Thorpe *et al*., 1988)	ATLN protocol[d], comparison by parasitaemia[e]			During 8 months post-partum 1+ 0 CR% 67.3 74.6 *n* 75 113	
Democratic Republic of Congo N'Dama ranch cattle (Feron *et al*., 1988)	ATLN protocol[d], cross-site comparison, high and zero challenge	% Calf mortality to 8 months High Zero % 9.5 3.3 *n* 299 432		% Calving rate High Zero CR% 74.0 85.7 *n* 193 401	
Ethiopia Susceptible zebus (Ahmedin Jemal and Hugh-Jones, 1995)	Cross-site comparison, longitudinal monitoring 90 cattle in high-challenge village and 167 in zero-challenge village	% Calf mortality Age High Zero 0–1 8.3 1.3	% Herd mortality Age High Zero All 25.9 2.6		
Ethiopia Susceptible zebus (Woudyalew Mulatu *et al*., 1999)	ATLN protocol[d], before and after comparison, for tsetse control using pour-ons; size of monitored herd: *n* = 90 to 147.	Calf mortality (CM) Stillbirths (SB) Before After CM% 8.9 5.3 SB% 13.5 4.1	Mortality age 1–5 Before After % 10.6 2.3 For age 5–10, no apparent reduction	% Calving rate Before After CR% 62 71	Tick burden also reduced

Tanzania Grade Boran ranch cattle (Fox *et al.*, 1993)	Before and after comparison, for tsetse control using deltamethrin dip. Parameters calculated from whole ranch population of 8000 head.	Pre-weaning mortality (PWM) and abortions and stillbirths (ASB)			Mortality from all diseases (AD), trypanosomiasis (TR), anaplasmosis (AN), hyena predation (HP)			% Calving rate			
			3 yrs Before	1 yr After		3 yrs Before	1 yr After		10 yrs Before	1 yr After	Tick burden also reduced
		PWM%	14.4	4.6	AD%	9.6	3.2	CR%	58	77	
		ASB%	0.75	0.33	TR%	3.6	0.6				
					AN%	0.4	0.1				
					HP%	0.6	0.1				
Kenya Boran ranch steers (Wilson *et al.*, 1986)	Comparison of steers of different Boran types with and without isometamidium chloride prophylaxis: 140 steers, 10 of each breed in untreated group				Untreated groups % mortality						Weight loss measured: % reduction in survivors' final liveweight
					Orma	50					Orma 0%
					Galana	50					Galana 14%
					Rumuruti	90					

Notes: As Table 20.2a.

Continued

Table 20.2. *Continued*

(c) Southern Africa

Country, production system, (source)[a]	Data collection		Impacts measured[b]							
	Method	Sample size	Calf mortality			Adult mortality			Calving rates[c]	
Malawi (Northwestern) Village herds (Doran, 2000)	Cross-site comparison, based on one-off survey, recall data	2843 households, 5 sites	Medium 1.5	Low/Zero 12.4			Medium	Low/Zero	Medium 36.0	Low/Zero 55.9
					Young		3.4	8.8		
					Cows		5.1	3.8		
					Oxen		5.5	3.2		
Mozambique (Western) Village herds (Doran, 2000)	Cross-site comparison, based on one-off survey, recall data	6001 households, 12 sites	Low 1 4.7				Low 1		Low 1 45.8	
					Young		13.2			
					Cows		5.0			
					Oxen		10.7			
			Low 2 4.0				Low 2		Low 2 45.5	
					Young		5.1			
					Cows		6.1			
					Oxen		3.0			
Zambia (Eastern) Village herds, 2 sets of cross-site comparisons (Doran, 2000)	Cross-site comparison, based on one-off survey, recall data	2583 households, 7 sites	High 1 8.8	Low 1 13.2			High 1	Low 1	High 1 44.1	Low 1 50.2
					Young		10.6	17.1		
					Cows		13.6	13.1		
					Oxen		11.5	14.3		
			High 2 7.1	Low 2 21.4			High 2	Low 2	High 2 37.1	Low 2 52.5
					Young		9.8	1.6		
					Cows		8.5	6.6		
					Oxen		6.5	14.6		
Zimbabwe (Northeastern) Village herds (Doran, 2000)	Cross-site comparison, based on one-off survey, recall data	328 households, 1 site	Zero 2.2				Zero		Zero 61.0	
					Young		6.5			
					Cows		6.8			
					Oxen		8.6			

Notes: As Table 20.2a.

Table 20.3. Selection of small-ruminant production parameters in relation to trypanosomiasis status.

Country, production system and source[a]	Data collection method and sample	Kidding rate (KR) and lambing rate (LR)[b]		
The Gambia Trypanotolerant sheep and goats (Mattioli *et al.*, 1997)	Comparison by parasitaemia[d] 100 sheep and 200 goats monitored		1+	0
		LR%	103	141
		KR%	92	129
Côte d'Ivoire Village sheep, Djallonké and Djallonké × Sahelian crosses (Thorpe *et al.*, 1988)		Infected during 4 months post-partum:		
			1+	0
		LR%	140.9	160.1
		n	18	134
Togo Djallonké village sheep, (Thorpe *et al.*, 1988)	ATLN protocol[c], comparison by parasitaemia[d]	Infected during 4 months post-partum:		
			1+	0
		LR%	143.1	147.2
		n	69	112
Tanzania East-African short-eared goats under traditional management (Hendy, 1988)	ATLN protocol[c] Cross-site and with and without isometamidium chloride prophylaxis comparison	Cross-site comparison		
			Kidding rate	
			Medium	Low
		KR%	137.2	160.8
		n	95	91
			Litter size	
			Medium	Low
		Size	1.51	1.48
		n	216	195
		Prophylaxis		
			Kidding rate	
			Without	With
		KR%	141.5	156.0
			Litter size	
			Without	With
		Size	1.56	1.43

All figures refer to a period of 1 year unless stated otherwise.
[a]References as cited, adapted in part from Swallow (2000).
[b]Kidding and lambing rates (KR and LR) refer to % of adult females giving birth in a year, where necessary converted from parturition intervals (PI) so that KR or LR = 365/PI; accordingly *n* refers to number of intervals recorded.
[c]ATLN protocol involved monthly weighing, bleeding, recording of mortality, births, usually other entries and exits from herds, and at some sites milk sampling and recording. Herders were offered some treatments for sick animals, which did improve productivity over time.
[d]Comparison by parasitaemia was by number of parasitaemic months, one or more (1+) or zero (0).

that a far more important factor in raising calving rates would be the early death of a calf. Results for this are shown in Table 20.2a for comparison. Camus (1981a) also discusses this effect. Thus, where trypanosomiasis of either the dam or the calf is implicated in calf deaths, the two tendencies would work in opposite directions, masking the effect of trypanosomiasis.

Other parameters

Monitoring milk output is more difficult and time consuming and so only a few results are available. Results from two studies in The Gambia showed milk yield differences of 10% and 26% between infected and uninfected cows. Monitoring in the Republic of Central Africa showed only a 2% difference

between the tsetse control area and the non-intervention area.

Weight has been monitored in many studies and is important in understanding the physiological processes involved. In terms of the economics of the disease, quantifying weight loss should be done at the point where it impacts on a 'life event', i.e. when low weight leads to death, late calving, lowered fertility or low weight at time of sale or slaughter. In ranching systems, weight is crucial, since the primary purpose is fattening stock for sale at target weights. A number of studies have emphasized this (e.g. Wilson *et al.*, 1986).

A key parameter, which merits far more investigation, is the direct impact of the disease on draught animals' mortality rates and ability to work. Farmers are very much aware of the need to protect these cattle from the disease and consequently target their expenditure on trypanocides, as discussed in Doran (2000). Thus, the cross-site comparisons in Zambia showed lower mortality in work oxen in the higher-challenge locations. The effect of trypanosomiasis on oxen's ability to work is discussed in Swallow (2000). Based on data from Ethiopia, he concludes that oxen in a high-risk area were 38% less efficient than oxen in a low-risk area.

Far fewer studies have been conducted on the effects of trypanosomiasis on sheep and goats. Table 20.3 summarizes results from some of these, showing that trypanosomiasis in village sheep and goats can lower annual lambing and kidding rates by around 20–30 percentage points and can also reduce twinning.

Discussion

Above all else, these results demonstrate how variable the impacts of the disease are on livestock productivity. In many cases the underlying use of trypanocides was difficult to document and, as Doran (2000) demonstrates, this has a major impact. The different bases for comparison also tend to yield slightly different results. It is difficult to generalize but it seems that the cross-site and before-and-after comparisons often come up with greater differences in productivity than comparisons by parasitaemia (see, for example, results for Democratic Republic of Congo and The Gambia, both using the ATLN protocol). That production parameters also vary greatly between sites with similar levels of challenge is also shown by the studies in The Gambia and in southern Africa (Table 20.2a, c). In fact, the situation can vary enormously even among herds from the same village, as shown by the studies undertaken by Wacher *et al.* (1994), which demonstrated that individual herds within a study site were subject to very different levels of exposure to tsetse, depending on their daily grazing routine.

In the case of the before-and-after comparisons, the high differential can reflect the choice of sites where trypanosomiasis has caused a particularly serious problem, as is notably the case in the study by Kamuanga *et al.* (2001a). Removing or reducing the impact of trypanosomiasis may have unexpected effects. For example, Fox *et al.* (1993) noted a reduction in deaths due to hyenas to one-sixth of their previous level, probably reflecting the fact that sick animals are those most easily predated.

These factors have important implications, both for extrapolating from data already collected and for planning future studies. Kamuanga (2001a) and Doran (2000) argue that interviewing livestock keepers is a cost-effective way of obtaining data about the economic impact of the disease, as compared with longitudinal monitoring. Snow and Rawlings (1999) outlined a tsetse/trypanosomiasis-specific rapid appraisal approach. Achieving an understanding of why different methods and comparisons yield different results is essential. One way of doing this is the approach used in studying bias in different data-collecting techniques (Misturelli and Heffernan, 2003).

Herd models

In order to analyse and integrate these production parameters so as to assess their implications for herd growth and output, demographic herd models have been widely used, often in connection with benefit–cost studies. These models use age- and sex-spe-

cific death rates, birth rates, milk yield and offtake rates (animals extracted for sale, transfer to another herd or slaughter). These parameters are then varied in order to estimate how livestock populations evolve under various assumptions – for example, using a lower calving rate for a livestock population with trypanosomiasis and gradually increasing it as the disease is controlled. Herd models are an ideal tool for analysing the effects of a disease like trypanosomiasis, which both affects the levels of livestock outputs (weight of slaughter stock, milk yield and ability to work) and has long-term implications for herd growth, via fertility and mortality effects. Table 20.4 summarizes the results of several of these studies, which are also discussed in Swallow (2000). Income and output differences are presented in very different ways in published studies but the potential effect of controlling trypanosomiasis on livestock population growth is estimated in all of them. The differences in the annual population growth rate vary from 0.5 to 6 percentage points (Table 20.4). Herd models can be used not just to simulate direct effects on livestock productivity; they can also be adapted to simulate the responses of livestock keepers to changes in productivity, through changes in the numbers sold, slaughtered or transferred into the area and the retaining of a changing proportion of draught males for traction. They can thus capture many of the indirect effects associated with the disease (Shaw, 1990), though not changes in breed.

Indirect effects of the disease in livestock

During the 1990s increased efforts to investigate the ways in which the presence of trypanosomiasis constrains rural development were made. A number of studies have looked at migration, growth of livestock populations, choice of breed and use of draught power, at the level of individual farmers, localities and on a continental scale. There is a comprehensive and structured discussion in Swallow (2000) and a very careful analysis of these effects undertaken for southern Africa by Doran (2000).

Use of animal traction

Despite the presence of trypanosomiasis, animal traction has become widely used in sub-Saharan Africa, notably in West Africa, Ethiopia and southern Africa. Extension and veterinary services, when functional, have given much support to the use of work oxen, in particular encouraging the timely use of trypanocides. Thus, in some areas, it is not so much that oxen are not used but that the cost of keeping them is very high. For example, among the villages compared in Ethiopia by Ahmedin Jemal and Hugh-Jones (1995), the proportion of work oxen among cattle in the tsetse control village was far lower (just under 30% as against nearly 60%), but in the unprotected village a third of adult males were replaced annually, reflecting the far higher mortality (see Table 20.2b). Also in Ethiopia, Swallow (2000) pointed to work oxen's lower efficiency in the area where tsetse is not being controlled, which has the effect of making traction inaccessible to those who do not own oxen, whereas in the controlled area non-owners are able to hire or borrow oxen.

For southern Africa, while some of the higher-challenge sites show lower use of draught animal power, in others there is little difference, or the relationship is reversed – a reflection of the targeted use of trypanocides (Doran, 2000). In The Gambia (CIRDES, ILRI and ITC, 2000), cross-site comparisons show little significant difference in the ownership of draught animals. With the exception of horses, there is a slight trend towards higher ownership levels in higher-challenge areas, possibly reflecting the way the animals are managed. Where the disease problem is severe, its removal can have a big impact on the use of oxen. Kamuanga *et al.* (2001a) reported from Burkina Faso, where the number of adult male cattle doubled once tsetse were controlled, while the number of draught oxen increased tenfold. In another study in Burkina Faso, the proportion of households with draught cattle increased from 64% to 93% after tsetse control (Kamuanga *et al.*, 2001c).

Table 20.4. Herd models used to assess the impact of trypanosomiasis on cattle.

Country and source[a]	Nature of model	Data source	Comparison	Effect on annual herd growth rates	Effect on output
Côte d'Ivoire (Camus, 1981b)	Dynamic steady state, meat output only	Camus, 1981a	Output from cattle in infected and uninfected herds	% Growth rate by breed Herd status +ve –ve Baoulé 2.6 3.1 N'Dama 0.4 2.1 N'Dama × Baoulé 2.9 4.6 Zebu × Baoulé –0.5 4.6	Output per head per annum for uninfected herds as compared with infected Increase in –ve % Baoulé 1.3 N'Dama 8.3 N'Dama × Baoulé 8.1 Zebu × Baoulé 14.3
Burkina Faso (Brandl, 1985)	Dynamic model, meat and milk output	Camus,1981a; FAO, ILCA and UNEP 1980	Output from cattle with and without trypanosomiasis as a result of tsetse elimination, trypanocides and other production costs included in income calculation, no change in milk yield assumed	Without project With 0.4% 4.2% medium level assumptions if offtake rates unchanged	Cumulative effect over 10 years: increase of 43% in total income (12% low, 73% for high assumptions)
Southern Africa (Shaw, 1990)		Grey literature from Zambia and Zimbabwe	Output from cattle with and without trypanosomiasis as a result of tsetse control including production costs	Without project With 0.7% 4.1% if offtake rates unchanged	23% increase per head per annum, of which a quarter due to draught
Côte d'Ivoire background calculations to (Shaw *et al.*, 1994)	Dynamic model, meat, milk and traction output	Ivorian data, other West African studies	Output from cattle with reduced incidence of trypanosomiasis as a result of tsetse control using traps, including production costs, trypanocides saved	Without project With Transhumant 2.4% 4.9% Sedentary 2.4% 3.9% if offtake rates unchanged	% Increase in income per animal per year Transhumant 14.0 Sedentary 18.2
Republic of Central Africa (Blanc *et al.*, 1995)	Dynamic model, meat and milk output	le Gall *et al.*, 1995 Model: LIVMOD EMVT-FAO	Output from cattle with reduced incidence of trypanosomiasis as a result of tsetse control using traps	Without traps With 0.05% 1.8% implied for larger herders	
Ethiopia, sub-Saharan Africa (Kristjanson *et al.*, 1999[b])	Dynamic model, meat and milk output	Woudyalew Mulatu *et al.*, 1999	Output from cattle with and without trypanosomiasis as a result of widespread use of a vaccine against trypanosomiasis	Without project With 1.1% 6.8% if offtake rates unchanged	

[a]References as cited, adapted in part from Swallow (2000).
[b]Study used the ILCA herd Model (von Kaufmann *et al.*, 1990).

Choice of breed

There are three broad categories of cattle in Africa: indigenous trypanotolerant taurine breeds; indigenous trypanosusceptible zebu breeds; and exotic trypanosusceptible breeds. Trypanotolerant cattle are widely used in West Africa and have been introduced into Central Africa with varying success (see Chapter 25; see also Shaw and Hoste, 1987). The old view that they are less productive than zebu cattle was successfully challenged by the study undertaken by the FAO with The International Livestock Centre for Africa and the United Nations Environment Programme (FAO, ILCA and UNEP, 1980). This showed that in low-challenge situations trypanotolerant stock were at least as productive as zebu. As challenge increases, trypanotolerant cattle are increasingly affected by the disease, whereas zebu cattle are sometimes unable to survive. A comparative study on the economics of keeping trypanotolerant cattle in villages in six countries (Itty, 1992) showed that they performed at least as well as susceptible breeds with prophylaxis. Thus, in terms of the indirect impacts of the disease, the keeping of trypanotolerant rather than susceptible zebu does not appear in itself to be a cost. Instead it should be regarded as control strategy. Chapter 25 discusses the various reasons why farmers nevertheless show a preference for larger animals in some situations. A review of the factors influencing farmers' preferences can be found in Kamuanga *et al.* (2001d). As regards exotic breeds, trypanosomiasis is a major reason why keeping them is difficult throughout lowland Africa, along with the presence of other (notably tick-borne) diseases.

Herd size and structure

Whilst the cumulative effect of the disease's impacts on mortality and fertility is to slow livestock population growth, a number of decisions about livestock holdings, herd composition and animals entering and leaving the herd are the prerogative of the livestock keeper. Comparisons of individual cattle herd sizes inside and outside tsetse-infested areas reflect both these direct and indirect components, as well as other variations in local circumstances and management systems. Consequently, studies have come up with mixed results. Comparing changes inside and outside the tsetse control area of Côte d'Ivoire, average herd sizes in the control zone were larger both before and after the control programme started. Within the zone they have increased very much in Korhogo but not at all in Boundiali, and have increased in both of the areas outside the study zone (Pokou *et al.*, 1998). In the sites compared by Doran (2000) in southern Africa, the average number of cattle per household tended to be higher in low-challenge areas but not necessarily the number per cattle owner. However, in The Gambia (Mugulla *et al.*, 1999; CIRDES, ILRI and ITC, 2000) herd sizes in one low-challenge site were nearly tenfold greater than in a high-challenge site. Similarly, variable results have been obtained when looking at herd structure and offtake rates.

Migration

The motivations for migration can be analysed in terms of 'push' and 'pull' elements. Pastoralists have long used movement as a strategy for avoiding or reducing the risk of disease, through regular seasonal transhumance in the case of tsetse, and to evade epidemics such as, in the past, rinderpest. The biggest recent shift of pastoral populations occurred in response to the 'push' given by the Sahelian drought of the early 1970s and, to a lesser extent, that of the early 1980s (e.g. FAO, ILCA and UNEP 1980; Shaw and Hoste, 1987; Pokou *et al.*, 1998). For example, there are now substantial populations of susceptible zebu cattle established in the middle belt of Nigeria (Bourn *et al.*, 2001). Northern Côte d'Ivoire saw its population of zebu increase from 40,000 to 250,000 between the mid 1960s and 1985. In the Republic of Central Africa numbers rose from 0.4 million in 1960 to 2.1 million in 1983, due to the drought and other push factors. All of these movements were into formerly almost wholly tsetse-infested areas and probably initially involved

heavy losses of cattle, but savannah tsetse have retreated and the settlers have remained in these zones, whilst making much use of trypanocides (le Gall *et al.*, 1995; Pokou *et al.*, 1998).

A number of studies have examined the extent to which tsetse control can act as a 'pull' factor. Studying the Nigerian eradication programme and looking back over 17 years, Putt *et al.* (1980) estimated the economic impact of in-migration by pastoralists and relocation towards riverine areas. However, it was difficult to attribute the extent to which the removal of tsetse motivated people. Similar movements towards riverine areas occurred in other parts of the country in the absence of any tsetse control (discussed in Bourn *et al.*, 2001). More recently, motivations for migration have been studied in Burkina Faso, Ethiopia and Zimbabwe (see discussion in Swallow, 2000). In Ethiopia, Swallow *et al.* (1998) studied in-migration at two sites in the Ghibe Valley. In one, migration was somewhat higher just after tsetse control started; in the other it peaked earlier, at the time of the establishment of a large commercial farm, but continued during tsetse control. In Satiri and Bekuy in Burkina Faso, Kamuanga *et al.* (2001c) found that most in-migration pre-dated tsetse control, and recent migrants did not give tsetse control as a reason for coming to the area. Although some might have been influenced by the 'pull' of the control of onchocerciasis undertaken earlier on in the area, their main reason for immigrating had been the 'push' of the Sahel droughts. Tsetse control itself was undertaken in response to local demand. In Yalé, Kamuanga *et al.* (2001a) found that, after tsetse control, old settlers brought back their cattle and newcomers immigrated, so that the number of households shortly after tsetse control was undertaken was already higher than it was before the disease became a serious problem. In their study in Zimbabwe, Govereh and Swallow (1998) related immigration to access to other amenities and crop prices as well as to tsetse control. They concluded that immigration accelerated with tsetse control but had started before it. Among reasons for emigration, 82% of emigrants listed factors related to land availability and quality in their area of origin. An important finding was that migrants were a particularly dynamic and productive subgroup, making more use of animal traction and being more likely to own cattle and to plant larger areas to cash crops. In another study in Zimbabwe, 60% of immigrants gave access to more land as the main reason for their move, 16% mentioned access to crop, grazing land and removal of tsetse, and 82% came from very heavily populated areas, so that lack of land in their home areas was a major 'push' factor (Doran, 2000). Elsewhere, in the common fly belt of Zambia, Zimbabwe, Mali and Mozambique, immigrants have settled in tsetse-infested areas and managed cattle using trypanocides. Doran (2000) concluded:

> While it is likely that the presence of tsetse fly will slow the process of migration, it cannot be argued conclusively that it will always prevent the movement of people into an area. Clearance operations may facilitate or even accelerate this process … but it cannot be argued, without qualification, that of themselves they will cause movement.

Looking at the larger picture, it seems that push factors (principally drought and land scarcity) have been very important in inducing people to migrate. The presence of tsetse does not act as an absolute impediment to immigration, nor does tsetse control on its own necessarily attract immigration to new areas. However, tsetse control is seen as an important increase in an area's amenities by potential immigrants and can accelerate the rate of immigration. Most importantly, control of tsetse prevents immigrants losing large numbers of stock or having to invest heavily in trypanocides.

The continental picture

Lastly, the relationship between tsetse and the continent's cattle distribution, and the potential relationship between control of the disease and future livestock distributions, have received much attention. Summarizing knowledge on the economic impact of the disease at that time, Finelle (1974) estimated

that with 20 million cattle living in the tsetse-infested areas, the cattle population could perhaps be increased by as much as 120 million head. Recently, the use of geographical information systems (GIS) has enabled more accurate estimates to be made. Kristjanson *et al.* (1999) estimated cattle numbers in the tsetse-infested area at 47.5 million and noted that, in eastern and southern Africa, the number of cattle per square kilometre inside the tsetse-infested areas was 8.9 as against 14.4 outside, implying a potential for a 62% increase. Gilbert *et al.* (2001) described the Programme Against African Trypanosomiasis Information System (PAATIS) geo-referenced database: using a series of predictive equations for cattle and crops, they estimated the impact of the removal of the tsetse fly on cattle and crops. These are illustrated in maps. They estimate that the potential increase in cattle numbers is about 50 million, or equivalent to doubling the existing cattle population in the tsetse-infested areas. The bulk of these increases would be in the subhumid and humid zones of West and Central Africa (from Guinea to southern Sudan), Ethiopia and the countries around the Lake Victoria basin.

Looking at it from a herd modelling perspective, to achieve a doubling of these populations in 25 years would require an annual compound growth rate of 2.8%; to achieve it in 50 years the growth rate would have to be 1.4%. Although when severe droughts or disease outbreaks occur, cattle numbers decline locally, overall it appears that cattle populations in many tsetse-infested areas are stable or growing (see Table 20.4), occasionally even at rates above 1.4%. Thus, even at today's production levels, doubling may not be an unachievable goal. However, this could be brought forward substantially if the 'with control' production parameters were applicable, which imply growth rates of around 4% (and a doubling period of 18 years).

The impact of such a potential increase in cattle populations would be not just on livestock outputs but also on crop production, via the use of animal traction and manure. This is discussed in detail in Swallow (2000) which reproduces maps produced in 1999 by Gilbert *et al.* from the PAATIS database in an estimate illustrating areas where cultivation would increase in the absence of tsetse. The main area highlighted is West Africa's subhumid zone. Swallow went on to discuss ways of quantifying the disease's impact on agriculture at a continental level and suggested that this could be done by building on macroeconomic studies which attempt to quantify the impact on total agricultural output of the presence of livestock. Based on these, Swallow suggested using an elasticity of 20% (that is, a 1% increase in livestock numbers would increase agricultural output – including that from livestock – by 0.2%) and applied this to 40% and 80% increases in cattle numbers. This idea was originally presented by Swallow in 1997 and was taken up by Budd (1999) and used to estimate that, in the long run, a doubling of the cattle population in tsetse-infested areas would yield an extra annual agricultural income of US$4500 million. This contrasts with the estimate by Kristjanson *et al.* (1999), who went on from the herd modelling approach (Table 20.4) to use an economic surplus model to estimate the value of benefits (such models factor in likely price reductions following substantial increases in output, so that producers benefit from the profits on extra production and consumers by being able to buy more at lower prices). They estimated the potential long-run benefits of controlling the disease at US$700 million per annum for meat and milk, or US$1300 million including the producer and consumer surpluses. Furthermore, it seems likely that animal traction could add as much as half again to the value of benefits from extra meat and milk production in some mixed crop livestock production systems (Shaw, 1990; Shaw *et al.*, 1994).

Costs of dealing with trypanosomiasis in livestock

Cost estimates of control programmes have been regularly published since the 1940s. However, as discussed above, published costings vary enormously in what is

included and excluded, and therefore in their suitability for comparison with benefits or for planning future work. Inflation and changes in relative prices make comparisons over time difficult, so that any figures quoted are soon out of date. In order to understand the bases for cost comparisons and their implications, some recent figures are given and discussed below.

Tsetse control

Current estimates of the cost of controlling tsetse using a range of techniques are given in Table 20.5. This kind of standard league table can be very misleading. Firstly, comparisons are difficult, because each calculation includes a different selection of cost components. Many published costings were designed to compare the field costs of different control techniques implemented by an established tsetse control department, which had already trained its staff, surveyed the tsetse distribution, and so on. These costings thus tended for the most part to exclude the overheads involved in preparing for, setting up, administering and monitoring control or elimination programmes, and concentrated on comparing the cost-effectiveness of different control techniques (as discussed and undertaken in the careful and comprehensive costings produced in Barrett, 1997). Including these overheads is likely – at least – to double the costs cited (see comparisons of operational budgets and salaries and wages in Doran, 2000, or analyses of costs in Brandl, 1988a,b and Putt *et al.*, 1980). In addition, these estimates also exclude the cost of preliminary research or pilot phases. For example, in the study by Shaw *et al.* (1994), including costs for the initial research phase would double the amount. Complete analyses of all the costs involved in larger projects are usually commissioned by donors and tend to remain confidential or in the grey literature. Furthermore, some costings are based on actual figures for work undertaken whereas others are estimates or extrapolations.

Secondly, costs vary widely between locations and according to the scale of operation. The ratio between the area treated and the area cleared of flies can vary greatly according to location, tsetse species and control method. The issue of scale has been much discussed. Budd (1999) pointed out the clear economies of scale involved in elimination work, where the relative burden of barrier costs would be reduced for larger projects. Scale is particularly important for techniques that aim at eradication. For the sterile insect technique (SIT) this was demonstrated by Brandl's calculations (Brandl, 1988b). However, it appears that, in practice, large scale projects can also involve diseconomies. These can be due to lengthy set-up and pilot phases and other initial overheads. Once again, this can only be examined by consulting project evaluation and review reports. Chapter 30 argues that where farmers themselves are involved, far more cost-effective approaches to tsetse control are possible.

Thirdly, different techniques are designed to achieve different things, so that comparisons per square kilometre can be relatively meaningless. Some aim at local eradication or elimination of the fly; others are designed for ongoing control. Comparison of control and elimination efforts would involve a situation-specific cost-effectiveness analysis, including discounting future costs for ongoing control and reflecting the reduction in disease incidence achieved by each approach.

Use of trypanocides

For the overwhelming majority of livestock keepers in tsetse-infested areas, trypanocides are the only option available for dealing with the disease in their stock. Trypanocides account for a large proportion of their cash expenditure. For example, in the Republic of Central Africa, 80% of cash expenses were on trypanocides (Blanc *et al.*, 1995). Estimates of the total number of doses used per year vary from 25 to 50 million (see, for example, McDermott and Coleman, 2001; also see Chapter 23). Costs per dose to farmers currently range from US$1 to US$2 and are usually nearer the US$2 figure. Drug resistance is becoming a problem in some areas, especially with intensive use (Chapter 23).

Table 20.5. Estimates of tsetse control costs.

Tsetse control technique	Estimated costs/km^2 (US$)	Control or eradication	Included/excluded in costs	Source, country
Ground spraying Targets Aerial spraying (SAT) Cattle treatment (pour-on, 15 cattle/km^2)	265–390 220–385 435–535 120	Eradication	*Included*: All field costs (insecticide, labour, flying charges), access development and camping, routine disease surveys only for insecticide-treated cattle, figures vary according to terrain and tsetse species present *Excluded*: headquarters-level administration, staff training, tsetse and trypanosomiasis surveys and research	Estimates for flat terrain, mixed fly populations, Zimbabwe and Zambia by Barrett (1997) updated by Budd (1999)
Linear km of barrier using targets	 2000 1600	Barrier • establishment • annual cost		
Cattle treatment (pour-on, case study with 44 cattle/km^2)	60	Control cost per annum	(Costs calculated from farmers' and project's viewpoint.) *Included*: pour-on, tsetse monitoring, farmers' time, time taken to apply to cattle, transport *Excluded*: trypanosomiasis monitoring and other research components. Based on actual figures	Woudyalew Mulatu *et al.* (1999), Ghibe, Ethiopia
Aerial spraying – sequential aerosol technique (SAT)	265–275	Eradication	*Included*: only operational costs for insecticide and aerial spraying contract, based on actual figures for 2002	R. Allsopp, Maun, Botswana, 2002, personal communication
Sterile insect technique (SIT) Eastern and southern Africa, following suppression using targets, traps or pour-ons West African riverine systems, large scale following suppression	 SIT: 800 SIT: 250–400	Eradication	*Included*: all field costs, flying, rearing costs, full investment in buildings and equipment, office overheads, local staff *Excluded*: Overseas and headquarters administration, surveys, monitoring	U. Feldmann, Vienna, 2003, personal communication
Control using mono-pyramidal traps (17 cattle/km^2)	26	Control cost per annum	*Included*: all field-level costs, capital items, local administration and salaries, donor costs, full costing based on actual 1992 figures *Excluded*: adaptive research	Shaw *et al.* (1994), northern Côte d'Ivoire

Studies on the use of trypanocides indicate that farmers tend to treat animals that are sick on suspicion, do not always distinguish between curative and prophylactic trypanocides, and focus their attention and expenditure on their most productive animals – cows and draught males (Doran, 2000). This strategy of treating these animals is optimal in economic terms, since milk and draught are the main outputs and cow fertility and survival are key to the development of the herd. Model calculations (background to Shaw *et al.*, 1994; Shaw, 1990) indicate that just preventing the disease in cows and oxen would reduce losses from the disease by two-thirds to three-quarters. Recent entomological work (Stephen Torr, Chatham, 2002, personal communication) also indicates that larger animals within a herd are more likely to be fed on by tsetse.

A number of studies have compared the use of trypanocides before and after tsetse control operations. Kamuanga *et al.* (2001a) found that expenditure per head of the population increased by nearly 30% after tsetse control and Pokou *et al.* (1998) found that a higher proportion of livestock owners used trypanocides inside the tsetse control zone as compared with outside. Both studies attribute this, in part, to pastoralists treating herds that are due to leave the tsetse control area on transhumance as well as to risk-reduction. Doran (2000) similarly found cattle owners in Zambia, in an area protected by targets, using trypanocides because water shortage forced them to graze cattle outside the area. On the other hand, le Gall *et al.* (1995), comparing herds inside and outside the trapped area, found that those outside administered 1.6 treatments per head per year, as against only 0.9 in the trapped area. In Zambia, average use was 1.5 doses per head of the herd per year, concentrated, as described above, on cows and oxen (Doran, 2000).

Trypanotolerant livestock

Trypanotolerant livestock breeds are used throughout West Africa's tsetse-infested zones. As discussed above, their use entails no extra costs. Livestock keepers tend to vary their holdings of trypanotolerant stock according to their perception of the risk from trypanosomiasis. For example, after serious outbreaks in Yalé, Burkina Faso, and despite tsetse control in the area, there was a substantial shift towards trypanotolerant stock over 3 years (Kamuanga *et al.*, 2001a). In Côte d'Ivoire a very mixed picture emerges, with the proportion of trypanotolerant cattle or trypanotolerant crosses varying from area to area. Changes since implementation of tsetse control have also been varied, with fewer changes in breed composition outside the tsetse control area but large increases in the size of cattle herds (Pokou *et al.*, 1998). Within the tsetse control area, the proportion of susceptible (zebu) cattle remained the same in Korhogo and fell dramatically in Boundiali, where there was a correspondingly large shift towards zebu–trypanotolerant crosses. The southward movements of susceptible zebu cattle due to the Sahelian droughts, as discussed above, also led to substantial increases in the numbers of cross-bred animals in parts of West Africa (FAO, ILCA and UNEP, 1980; Shaw and Hoste, 1987).

Studying farmers' inputs

How disease control is delivered and who pays for it are key issues in dealing with trypanosomiasis. As with any other disease control activity, they determine its effectiveness, how well it is targeted to local needs and whether it is sustainable and financially viable. The involvement of farmers in tsetse control is discussed in Chapter 30. Only some aspects of the economics of farmers' contributions have been studied. As outlined above, many livestock keepers already manage their herds to minimize challenge: making substantial investments in trypanocides, moving herds seasonally to avoid tsetse and varying the breed composition of their herds by keeping more trypanotolerant stock where the disease risk is perceived to be high.

A number of studies have used the technique of contingent valuation to examine how much money or labour farmers would be willing to contribute to tsetse control using traps, targets and pour-ons. The most recent study (Kamuanga *et al.*, 2001b) was under-

taken in the Yalé pastoral zone of Burkina Faso, and also reviewed previous studies using the same approach in Kenya and Ethiopia. In Yalé, livestock keepers were badly affected by a severe outbreak of trypanosomiasis. Tsetse control measures, using traps, targets and deltamethrin pour-ons, were implemented to deal with the problem. Two years later households were interviewed as to their willingness to contribute labour or money and all interviewees indicated that they were prepared to contribute. When the actual contributions of labour were assessed, only 56% of those who said they would contribute labour did so, while 3% of those who had not pledged labour did contribute it. Various factors influencing these contributions were analysed and the authors concluded that trapping could not be sustained from local contributions alone.

Swallow *et al.* (1995) studied willingness to pay for pour-on treatments in Ethiopia. They found that farmers were willing to pay for these, 67% of those interviewed having done so in the previous month, thus treating nearly half of the cattle held by the group. Farmers with a high proportion of oxen and cows were more likely to use pour-ons; nearness to the treatment centre and seasonal factors were also important.

Overall, farmers' willingness to contribute to tsetse control depends not only on the importance they attach to their cattle and the disease, but also very much on the extent to which they perceive tsetse control to be a private good, benefiting their own stock, or a public good, benefiting everyone's. Trypanocides are clearly a private good; pour-ons are often perceived to be a private good by farmers (Swallow *et al.*, 1995), whereas traps and targets are seen as more of a public good. However, the Republic of Central Africa's project described in Blanc *et al.* (1995) was very successful in persuading farmers to buy and use traps.

Economics of trypanosomiasis control strategies

To complete this discussion of the direct and indirect costs imposed by trypanosomiasis, this section looks at how the costs of the various disease control options compare with the expected benefits.

Most formal benefit–cost studies are undertaken either when a project is being contemplated (the appraisal or *ex-ante* analysis) or after the project has been running for some time or has finished (the evaluation or *ex-post* analysis). These studies are nearly always funded by a donor in order to decide on future investments or to examine how successful ongoing or past investments have been. Consequently, along with the cost calculations, these often remain as confidential documents or in the grey literature. In 1987, reviewing the studies undertaken up to that time, Tacher *et al.* (1988) pointed out: 'Economic data are not only rare, but also lack homogeneity to allow comparisons between the costs and benefits involved'. The main published studies are listed in Table 20.6. As can be seen, comparisons are still difficult because benefits and costs have been calculated in different ways. The studies from the Republic of Central Africa and Ethiopia show very high returns to farmers for their investments (Blanc *et al.*, 1995; Woudyalew Mulatu *et al.*, 1999). The larger-scale project in Côte d'Ivoire (Shaw *et al.*, 1994) gave consistently good returns and was very robust to changes in the assumptions about benefits. The Burkina Faso study (Brandl, 1988a) showed more modest returns, especially for tsetse control using the sterile insect technique and the use of prophylaxis. The study of the Nigerian tsetse eradication programme (Putt *et al.*, 1980) was the only one to try to quantify indirect benefits, and the difficulty of attributing an appropriate share of changes to tsetse control is shown by the wide divergence between the results for high and low benefit assumptions for the Sokwa District study. Two other early studies, by Jahnke (1974) and Habtemariam *et al.* (1983), looked at the feasibility of introducing cattle into areas where tsetse had been cleared by ground spraying or game elimination, but all the options considered were only marginally profitable. Turning to the use of trypanocides, only a few published studies examine the economics of these and have been based on prophylaxis and extrapolated

Table 20.6. Results from a selection of published benefit–cost calculations of interventions to control tsetse and trypanosomiasis in cattle.

Country (source)	Intervention	Benefits	Costs	Benefit–cost ratios[a]
Ethiopia (Woudyalew Mulatu *et al.*, 1999)	Pour-on trial, ex-post evaluation	Increased output of milk, meat, herd growth and savings in trypanocides using herd model	Pour-on (paid for by farmers), farmers' time, project costs for transport and application of pour-ons	Benefit–cost ratios, over 5 years, discounted at 10% Farmers 8.0 Project 4.3
Republic of Central Africa (Blanc *et al.*, 1995)	Trapping, extrapolation of effects from localized ex-post evaluation	Increased output of milk, lowered mortality and improved fertility, weight of slaughter stock, savings in trypanocides	Traps (paid for by farmers), project costs for extension and adaptive research	Benefit–cost ratio for farmers buying traps Large herds 5.9 Smaller herds 3.1
Côte d'Ivoire (Shaw *et al.*, 1994)	Low-density trapping, work evaluated 15 years retrospectively and projected for a further 10 years	Increased output of traction, milk, meat, herd growth and savings in trypanocides using herd model	All costs of traps, trap deployment, initial pilot programmes and adaptive research, also increased producer costs for keeping more animals	Project benefit–cost ratio, over 25 years, discounted at 10% Baseline 3.2 High Benefits 4.0 Low Benefits 2.3
Burkina Faso (Brandl, 1988a)	Sterile insect technique (SIT) actual programme for local elimination, compared with other hypothetical approaches in the area: helicopter spraying, targets at 300 m and prophylaxis of cattle	Potential benefits estimated using herd model, including milk for first time, also meat output, trypanocides saved and herd growth	Herders' production costs, cost of control technique, including overheads	Benefit–cost ratios, for medium-level benefits, discounted at 10%: (No. of years) (10) (20) SIT 0.49 1.39 Helicopter 1.23 2.56 Targets 1.56 2.43 Trypanocides 1.14 1.65
Nigeria (Putt *et al.*, 1980)	Ground spraying and helicopter spraying, localized elimination of tsetse, evaluated 17 years afterwards (Sokwa district) and 8 years afterwards (Burra District)	Lowered mortality and increased meat output from cattle, milk omitted, indirect benefits from increased use of traction and increased cultivation	Full cost of ground and helicopter spraying operations, including overheads	Benefit–cost ratio, over 20 years, discounted at 12%: (a) Sokwa District High benefits 8.0 Low benefits 2.7 (b) Burra District High benefits 6.5 Low benefits 4.7
Kenya (Wilson *et al.*, 1986)	Isometamidium chloride prophylaxis	Mortality and weight losses avoided	Trypanocide, equipment, labour and mustering cost	3-monthly regime 6.0 Strategic regime 52.3

[a]Benefit–cost ratios refer to the total value of discounted benefits divided by the total value of discounted costs over the time period analysed. They are cited here as values (i.e. 3.3) rather than ratios (i.e. 3.3:1).

from trials on station or ranch (e.g. Wilson *et al.*, 1986; Table 20.2b).

Generalizing, it is possible to state that well-targeted tsetse control interventions, which are sustained, usually realize high benefit–cost ratios of 2.5 and over (discounted at 10%) within 10–20 years. For tsetse elimination projects, whether or not they are sustained is obviously crucial. For example, Brandl's 10- and 20-year projections for the SIT project in Burkina Faso were not borne out by events, but such projections were valid for work in Zimbabwe and northern Nigeria.

It is worth commenting at this stage that, although there is no published analysis covering a broad range of techniques, the costs of many forms of tsetse control have fallen considerably and some are likely to be reduced further. The higher-return projects studied in recent years have moved away from trying to clear large areas of tsetse to focusing on places where there is a tsetse and trypanosomiasis problem, which has been identified as a priority by local farmers and livestock keepers. The resulting projects have involved local communities and looked at ways in which they could contribute (see also Chapter 30).

The wide range of results from sensitivity analyses of individual projects in Table 20.6 should serve as a reminder of the extent to which this type of economic analysis has to depend on assumptions about the time period and level and nature of benefits. For global studies (e.g. Budd, 1999; Kristjanson *et al.*, 1999), levels of uncertainty are very much greater. Examining the returns to the successful adoption of a possible vaccine against trypanosomiasis, Kristjanson *et al.* (1999) obtained an internal rate of return of 33%. (The internal rate of return can be simply defined as the average annual percentage return on the sums invested which is obtained by the benefits, over the life of the project, expressed as a compound interest rate.) Budd (1999) estimated a relationship of 2.6 to 1 between the very long-run sustained maximum benefit level and hypothetical investments in tsetse elimination, without specifying or accounting for time periods.

Despite these uncertainties, it is possible to make some generalizations about the economics of dealing with the disease in livestock. Firstly, where livestock population densities are low, using trypanocides can be more cost-effective than tsetse control, since trypanocides mount up on a per animal basis, whereas tsetse control costs are per riverine or square kilometre. This applies where there is no drug resistance (see Chapter 23), such as in pastoral and smallholder production systems where trypanocides are strategically administered to economically important animals (as described in Doran, 2000). At higher livestock and human population densities, tsetse control tends to be slightly cheaper, because vegetation cover is less dense, there is better access and there is a source of local labour and farmers to contribute to the activities. Such comparisons need to be viewed in relation to epidemiological models of the disease. In their description of transmission models, McDermott and Coleman (2001) implied that, all other things being equal, tsetse control has relatively more impact on transmission per unit increase in control effort, and argued for linking biological and economic models, incorporating real estimates of benefits and costs based on practical experiences in the field.

Secondly, in comparing the overall costs of tsetse control as opposed to elimination from the economic point of view, the key variables are whether elimination can be sustained, the relationship between the likely true cost of elimination (including overheads) and the same estimate for control discounted over say, 25 years, and the impact that each approach has on the disease. For example, discounted at a normal rate for livestock projects of 10% over 25 years, a one-off expenditure on elimination of US$1000/km^2 is equivalent to an annual expenditure on control of US$100. If the unusually low discount rate of 5% were used, it would be equivalent to an annual expenditure of US$70. If control were half as effective, it would need to cost half as much to be competitive, i.e. US$35–50. Analyses of this type will be necessary whatever the relative and absolute numbers involved (Shaw, 2003).

Thirdly, when switching from one way of dealing with a disease to another, the correct methodology is to compare the extra benefits gained, plus any cost previously incurred, but now saved, due to the switch, to the costs of the new control method. As discussed above, farmers are themselves dealing with the disease year in and year out, mostly by using curative drugs for their cows and oxen and, in West Africa, by protecting stock during transhumance. This means that they are already avoiding a significant proportion of the losses due to the disease, and in a sense are 'capturing' a proportion of the potential benefits of formal disease control measures. In the future, if drug resistance becomes more widespread this would again change the parameters in the analysis.

There are a number of other considerations that should also be factored into strategic assessments. Autonomous control of tsetse and trypanosomiasis is occurring in many locations across Africa, due to human population pressure and agricultural expansion (Ford, 1971; Bourn *et al.*, 2001). Under such circumstances, the problems of the disease will gradually decline, so that while short-term interventions are very cost-effective, long-term ones will be less so. On the other hand, throughout the tsetse-infested areas, the benefits from tsetse control will be higher where there are not only higher livestock population densities, but also higher human populations, so that agriculture benefits from the availability of draught animals and manure.

The Present Situation

Finelle (1974) stated that 'accurate data are so limited that it is almost impossible at present to draw up even an approximate report'. So, after more than three decades of effort, what has been achieved?

In the field of human trypanosomiasis, there are now reliable estimates of the costs of surveillance and case-finding. The burden of disease in specified locations has been estimated in terms of DALYs and more is being discovered about DALYs and the costs to families in terms of expenditure and lost income. There is also more information about the likely relationships between reported and unreported cases. Controlling the reservoir is crucial for both forms of the disease, but knowledge of how effective this is in different situations is variable and the situations themselves are very diverse. Ideally, economic analyses need to be linked to epidemiological models. In the mean time, the best use should be made of the available historical data showing what might be optimal levels of surveillance for *gambiense* disease. For *rhodesiense*, in situations where cattle are the main reservoir, it appears that controlling the disease in cattle may pay for itself financially in terms of livestock productivity and human treatment costs saved, before DALYs are even considered. For *gambiense* disease, its focal nature means that interventions are highly cost-effective, with costs per DALY averted well below the current US$25 threshold of 'good value for money'. Thus, there has been success in proving that, among health interventions, controlling human trypanosomiasis ranks very highly in terms of economic profitability.

In the field of livestock trypanosomiasis, the amount of information on the impact of the disease has increased enormously. There are the results of numerous studies trying to quantify the impact of the disease on cattle production parameters in village and pastoral settings and, particularly in recent years, a number of carefully structured studies have examined the indirect effects it has on agricultural development. What emerges above all else is an impression of great variability. This applies whatever the parameter being studied, both for direct and for indirect effects, and often within the same production system and country. This variability is a reflection of different epidemiological situations, different farming systems, different management practices, different livestock breeds, different ways in which the disease affects individual animals and the different ways in which people deal with it and react to its absence or presence.

Where present, the disease acts as a con-

stant drain on livestock productivity and livestock keepers' financial resources throughout its range. It consistently impacts on mortality and fertility in livestock, thus constraining the rate of livestock population growth. It also lowers milk yields and the ability of draught animals to work. However, these effects are to varying extents mitigated by the actions of livestock keepers, particularly their use of trypanocides and animal husbandry risk-reduction practices. Losses are particularly high when susceptible cattle colonize new areas. In parts of West Africa, the use of trypanotolerant stock helps to reduce the impact of the disease, without any lowering of livestock productivity, and farmers do vary the breed composition of their herds in line with their perception of risk from the disease.

The extent to which the presence of trypanosomiasis constrains and shapes the pattern of rural development is difficult to ascertain. Even more difficult is finding out to what extent changes in the trypanosomiasis status of areas will cause changes in the pattern of settlement, cultivation or use of animals. Supposedly susceptible breeds are kept and used for draught in many tsetse-infested parts of the continent; in other areas they are markedly absent. Rural development and migrations in Africa have a momentum of their own, which is not necessarily dictated by the absence or presence of tsetse. As Doran (2000) states, 'Human reactions are complex, influenced by a range of endogenous and exogenous factors, and not always easy to predict.' Some studies show accelerated migration once tsetse have been controlled; in other situations, migration pre-dates control, or the demand for control is generated by immigrants suffering extremely heavy losses in their stock. In some areas the use of draught animals is restricted by the presence of tsetse; in others, farmers find ways of keeping them anyway. Thus, although it is possible to extrapolate losses to a continental scale, there is no economic evidence for the existence of a continental solution or universally applicable approach to control. Nevertheless, the way in which the disease constrains livestock population growth consistently points to an untapped potential for expanding livestock production and harnessing the benefits it confers on crop production.

There have been many interesting developments in the economic methodology applied to the study of trypanosomiasis, using herd models, contingent valuation and economic surplus models, as well as GIS, various statistical models and the more traditional techniques of benefit–cost analysis. Paradoxically, since work on the subject started over 50 years ago with the careful reporting of the field costs of dealing with tsetse, this has become the area where far more rigorous work is needed now that there are few established tsetse control infrastructures. There is a need to delve further into the grey literature on past projects to get a better idea of what is really involved, and to appreciate the extent to which different delivery systems affect costs and sustainability.

Delivery systems and scale are big issues that need to be underpinned by appropriate and accurate economic analyses, bearing in mind that economic analyses can only be as good as the technical information on which they are based. Past projects also hold much information on the successes and failures of different approaches in different situations and need to be studied. From the benefit–cost studies that have been undertaken, it is very clear that well-targeted tsetse control schemes, underpinned by consumer demand and involving farmers, are consistently highly profitable investments. Where livestock and human populations are low, benefits are lower; and where ambitious schemes are not sustained, benefits can be substantial but transitory.

Economics is essentially concerned with assisting decision making and guiding resource allocation. The nature of the decisions to be made in the field of tsetse and trypanosomiasis control, as in any other field, change over time. Thus, the key issues faced at the start of a new millennium may seem dated to a reader in a decade or two, as they are overtaken by events, or diverted by changing circumstances, most particularly by the expansion and redistribution of human and livestock populations. This chapter has tried both to present the knowledge

acquired, so that it can be weighed in the light of the issues currently being faced, and to demonstrate the need for consistent collection, analysis and presentation of economic data in the future.

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21 Current Chemotherapy of Human African Trypanosomiasis

Christian Burri, August Stich and Reto Brun

History of Sleeping Sickness Treatment

The historical background of the treatment of sleeping sickness is of importance for a better understanding of the use and properties of the current drugs. Detailed reviews of historical aspects can be found in the predecessor of this book (Mulligan, 1970), in Sicé (1937) and in Williamson (1962). This chapter will deal with the administration and use of drugs currently applied to the treatment of Human African Trypanosomiasis.

Registered Drugs for Treatment of the First Stage

Pentamidine

Pentamidine is a synthetic aromatic diamidine chemically related to the antidiabetic drug phenformin (Fig. 21.1). The drug, introduced in 1940, has an established place in the treatment of Gambian trypanosomiasis, antimony-resistant leishmaniasis and *Pneumocystis carinii* pneumonia (PCP) (Sands *et al.*, 1985). Until recently, two salts of the molecule were available: pentamidine isethionate (Pentacarinat®, Aventis) and methanesulphonate (Lomidine®, formerly Rhône-Poulenc Rorer), but the latter is no longer produced. Pentacarinat is supplied in 200 mg ampoules.

Clinical application

Pentamidine is the drug of first choice for first-stage *gambiense* sleeping sickness, where cure rates are as high as 98% (WHO, 1986). Failures in treating *rhodesiense* sleeping sickness are quite considerable (Apted, 1980), possibly because pentamidine's uptake is lower in *Trypanosoma brucei rhodesiense*, and it is seldom used for East African trypanosomiasis. Some cures of second-stage infections have been reported, but cerebrospinal fluid (CSF) drug levels are usually insufficient to guarantee a trypanocidal effect in the central nervous system. One or two pentamidine injections have been used as a pre-treatment before melarsoprol in second-stage *gambiense* disease. Pentamidine's former use in chemoprophylaxis of *gambiense* sleeping sickness is no longer recommended (WHO, 1986).

Because of the frequent occurrence of hypotension after intravenous (i.v.) application, the drug is usually given as deep intramuscular (i.m.) injection. Intravenous infusion, given in normal saline over 2 h, may be used if hospital conditions are suitable. The main advantage of pentamidine over suramin is the brevity of the treatment course and ease of application.

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Fig. 21.1. Pentamidine.

THERAPY SCHEDULES The most commonly used dosage regimen is 4 mg/kg body weight daily or on alternate days for seven to ten injections of pentamidine isethionate.

ADVERSE DRUG REACTIONS Generally pentamidine is well tolerated, especially as intramuscular injection. However, minor adverse reactions (usually reversible) are common (Sands *et al.*, 1985).

Reported immediate adverse drug reactions include hypotension (9.6%) with dizziness, sometimes collapse and shock. Intravenous injection can increase the frequency of a hypotensive reaction to 75%. Simultaneous application of plasma expanders and a prolonged infusion time may alleviate this reaction, but close monitoring of pulse rate and blood pressure are still mandatory. Nausea and/or vomiting have been reported in 2.4%.

Local reactions at the site of injection include pain (18.3%) and sterile abscesses or necrosis (6.7%). Systemic reactions are acotaemia due to a nephrotoxic effect (23%), leucopenia (14.5%), abnormal liver function tests (11%), hypoglycaemia (8.4%) and hyperglycaemia (5%), rarely with persistent manifestation of diabetes. There is no clinical or laboratory evidence for mutagenicity or fetotoxicity.

Pharmacology

PHYSICO-CHEMICAL PROPERTIES Pentamidine is a synthetic aromatic diamidine with a molecular weight of 340 g/mol (base), 533 g/mol (methanesulphonate) or 593 g/mol (isethionate). The pKa of 11.4 indicates that the large majority of the drug is positively charged at physiological pH.

Pentamidine is soluble in water: 1 g of the isethionate dissolves in 10 ml of water. It is not absorbed orally, hence administration is by injection. Care should be taken with dose calculation, depending on whether the dose is indicated for the base or the salt.

Tissue binding is high and total plasma protein binding is estimated at 70% (Bronner *et al.*, 1995a). Significant deposition occurs in the liver and spleen and nephrotoxicity is sometimes observed as an adverse effect of the drug.

PHARMACOKINETICS Several high-performance liquid chromatography (HPLC) methods have been developed for the determination of pentamidine (e.g. Yeh *et al.*, 1993). Early studies on the pharmacokinetics were incomplete and only in the 1990s did reliable data from patients treated against PCP and *gambiense* trypanosomiasis emerge.

In AIDS patients with PCP the elimination half-life after the first i.m. dose of 4 mg/kg was 9.4 h and 6.4 h after i.v. administration; with this latter route, the peak plasma concentration was three times higher than after i.m. injection (Conte *et al.*, 1986). A pharmacokinetic study on single dose administration of pentamidine isethionate, given by 2 h i.v. infusions of 3.0–4.8 mg/kg, to patients with *Trypanosoma brucei gambiense* infection, showed a rapid distribution phase of 10 min followed by a slower distribution phase and an elimination phase over weeks to months. The average terminal elimination half-life was 265 h. There was a threefold variation of the clearance and volume of distribution, which may reflect individual differences in metabolism. About 5% of the administered dose of pentamidine was found unchanged in the urine (Bronner *et al.*, 1995a).

Patients receiving multiple doses of 3 mg/kg of pentamidine for PCP showed progressive drug accumulation and increasing

trough concentrations, without achieving steady state, throughout treatment. The elimination after the first dose followed a three-compartment model and the terminal half-life ($t_{1/2}\gamma$) was estimated at 29 h. After the last of an average of 13 daily injections, the mean elimination half-life was prolonged to about 12 days with pentamidine still found 6 weeks after the last application (Conte, 1991). Extensive binding of pentamidine to tissues has been suggested, with extremely large apparent volume of distributions (11,850 l after a single dose application and 35,000 l after multiple dosing).

Similar results were found in patients treated for *T. b. gambiense* with ten i.m. injections of pentamidine methanesulphonate (3.5–4.5 mg/kg on alternate days). Maximum plasma levels were generally reached within 1 h of injection and varied extensively (420–13,420 nmol/l). The median plasma concentration after the last dose was about five times higher than after the first. Data obtained after ten i.v. short infusions (3.8–4.2 mg/kg) on alternate days were fitted to a three-compartment model with median half-lives associated with the first, second and third phase of 4 min, 6.5 h and 512 h, respectively (Bronner *et al.*, 1991).

Since renal clearance accounts for only 2–12% of plasma clearance, dose adjustment is not recommended for renal impairment.

Small amounts of pentamidine (0.5–0.8% of the plasma concentrations) have been found in the CSF of patients after the last dose of a 10-day treatment course and pentamidine persisted in CSF for at least 30 days (Bronner *et al.*, 1991). Concentrations as low as 10 ng/ml have a trypanocidal effect during prolonged exposure *in vitro*, hence pentamidine may be active in so called 'early-late stage' patients with a white blood cell count (WBC) of up to 20 cells/mm^3 instead of 5 cells/mm^3 (Doua *et al.*, 1996). In a preliminary study on such patients, the rate of relapse after pentamidine treatment was only slightly higher than after melarsoprol (6% vs. 3.7%) and the new 'early-late stage' definition is now used as a standard in Angola; the publication of the result is pending.

Some pharmacokinetic evidence indicates that three injections may be as effective, but less toxic, than seven to ten injections (Bronner *et al.*, 1991). TDR (UN Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases) is currently carrying out a randomized trial to compare effectiveness and toxicity of the 3- and 7-day regimens (4 mg isethionate/kg/day i.m.).

Pentamidine metabolites Pentamidine is converted to at least seven primary metabolites by cytochrome P-450-dependent oxygenases in rat liver homogenates and rat liver microsomes. The two main metabolites, the 2- and 3-pentanol analogues of pentamidine, were found to be conjugated with sulphate or glucuronic acid. Mass spectroscopy revealed that about 1% of the drug was unchanged in the urine while the majority was metabolized prior to elimination, mainly in the urine (Bronner *et al.*, 1995b).

Mode of action Mechanisms that may conceivably play a role in drug action have been reviewed (Wang, 1995). Various bindings to nucleic acids, disruption of kinetoplast DNA, inhibition of RNA-editing in trypanosomes and inhibition of mRNA trans-splicing may be involved, but the actual mode of action remains unknown. The drug is actively transported by several different transporters into bloodstream forms of *T. b. brucei*, leading to an accumulation to very high levels that may affect multiple cellular processes.

EFFICACY Pentamidine has a very high cure rate for first-stage sleeping sickness, i.e. CSF WBC counts $\leq$ 5 WBC/mm^3. Reasonable cure rates can also be obtained with 'early-late stage' patients ($<$ 20 WBC/mm^3). The cure rate of first-stage patients proved to be lower for *T. b. rhodesiense* than for *T. b. gambiense* patients (Apted, 1980). *In vitro* studies revealed that pentamidine acts slowly, reaching optimal activity only after a 3-day drug exposure with a minimum inhibitory concentration (MIC) of 2–5 ng/ml for *T. brucei* spp. Reported relapse rates after a course of five injections of between 7% and 16% have been reported, though these could be explained by misdiagnosed second-

stage infections, rather than by pentamidine-resistant trypanosomes, which have not been described so far in the field. Chemoprophylaxis using a 6-monthly application of the drug was previously used but this approach was stopped because it favours progression of undiagnosed first-stage infection to second stage and, in principle, selection for drug resistance.

Availability and cost

Pentamidine isethionate is produced by Aventis under the trade name Pentacarinat®. One 300 mg vial costs about US$30 for the treatment of *P. carinii* in AIDS patients. Pentamidine is one of three drugs (along with melarsoprol and eflornithine) that will be provided by Aventis free of charge for use against sleeping sickness until 2006. WHO coordinates the allocation of drugs to the national sleeping sickness programmes and non-governmental organizations (NGOs) through the logistics department of Médecins sans Frontières (MSF).

Suramin

Suramin (Fig. 21.2) was introduced in 1920 in Germany for the treatment of trypanosomiasis. It is effective against the first stage of both forms of the disease but pentamidine is preferred for *T. b. gambiense* today. Suramin is produced in ampoules of 1 g (Germanin®, Bayer) and is also an effective macrofilaricide against *Onchocerca* spp. and *Brugia pahangi*. Suramin is an inhibitor of reverse transcriptase but it was superseded by more efficient protease inhibitors, nucleoside analogues and other non-nucleoside reverse transcriptase inhibitors for the therapy of HIV/AIDS. The investigations on treatment of hormone refractory prostate cancer are summarized in Kuyu *et al.* (1999).

Clinical application

Suramin's use in sleeping sickness is confined to treatment of first-stage disease, especially the Rhodesian form, as the drug does not reach therapeutic levels in the CSF.

Suramin is usually given as monotherapy. Combinations with pentamidine may reduce the adverse drug reactions of both components and suramin has also been administered prior to melarsoprol chemotherapy in second-stage *rhodesiense* sleeping sickness. Its application as a chemoprophylactic agent is historical.

Intramuscular injection is very irritant and so suramin is injected intravenously after dilution in distilled water.

THERAPY SCHEDULES The most commonly used dosage regimen consists of a test dose of 4–5 mg/kg body weight on day 1, followed by five injections of 20 mg/kg body weight every 3–7 days (e.g. day 3, 5, 12, 19, 26) (WHO, 1986). The maximum dose per injection is 1 g.

ADVERSE DRUG REACTIONS Adverse drug reactions of suramin are dependent on nutritional status, concomitant illnesses (especially onchocerciasis) and the general clinical condition of the patient. Life-threatening events and lethal outcomes have been described but the drug remains one of the safest in trypanosomiasis treatment. Adverse reactions, other than pyrexia and a usually mild, reversible nephrotoxicity, are rare. The drug accumulates more in the kidneys than in other organs. First symptoms of renal impairment are albuminuria, later cylinduria and haematuria. Regular urine checks during the course of treatment are therefore mandatory.

Other adverse effects are reported at frequencies $< 5\%$ (though there might be considerable under-reporting in the African situation). Reactions include those typical of early hypersensitivity, such as nausea, circulatory collapse and urticaria, and those typical of late hypersensitivity, including exfoliative dermatitis and haemolytic anaemia, peripheral neuropathy, and bone marrow toxicity with agranulocytosis, thrombocytopenia and reactive encephalopathy. Adrenocortical degeneration and insufficiency have been reported after high but not after normal dose treatment. In patients experimentally treated for HIV/AIDS, suramin-induced adverse effects have been more severe but usually reversible.

Fig. 21.2. Suramin.

Pharmacology

PHYSICO-CHEMICAL PROPERTIES Suramin is a polysulphonated naphthyl urea that is strongly negatively charged at physiological pH. Its molecular weight is 1297 g/mol (acid) and 1429 g/mol (sodium salt).

The compound is freely soluble in water. It deteriorates in air and should be injected immediately after preparation (Gustafsson *et al.*, 1987).

Suramin is poorly absorbed from the intestine and causes intense local irritation when given intramuscularly. Therefore it is given by a slow intravenous injection in trypanosomiasis treatment and by continuous infusion in experimental cancer treatment.

Suramin is among the most extensively bound of all drugs, with 99.7% of the drug bound to plasma proteins (e.g. albumin, globulins, fibrinogen) in the body.

PHARMACOKINETICS Several HPLC and micellar electrokinetic chromatography methods have been developed for determination of suramin in serum.

High interpatient variability in pharmacokinetic profile was recognized very early. After a single 1 g dose, suramin was detected in the plasma for 5–8 days; after a course of five to six doses, it could be detected for 150–250 days. The interval to detectable parasitaemia varied from 73 to 327 days (suramin dose of 1 g per patient) and 103 to 180 days (suramin dose of 2 g per patient), respectively, after direct inoculation of trypanosomes or through bites of infected tsetse flies.

Suramin's half-life is among the longest of all drugs applied to humans. After a test dose of 200 mg, 1 g of suramin was given i.v. once a week for 5 weeks to HIV/AIDS patients. The drug accumulated during the time of administration and then diminished with a half-life of 44–54 days. Total plasma levels remained above 100 μg/ml for several weeks. The volume of distribution was 38–46 l and total clearance was $<$ than 0.5 ml/min. Renal clearance is the main route of elimination. In experimental onchocerciasis treatment, the elimination was also slow with a median half-life of 91.8 days and a plasma clearance of 0.1 ml/min.

No dose modification for renal or hepatic dysfunction is recommended at this time and there is no indication for the existence of drug metabolites.

MODE OF ACTION Suramin is slowly trypanocidal, eliminating parasites from blood and lymph nodes 12–36 h after injection. As a large polyanion it inhibits numerous enzymes, such as L-α-glycerophosphate oxidase, glycerol-3-phosphate dehydrogenase, RNA polymerase and kinases, thymidine kinase, dihydrofolate reductase, hyaluronidase, urease, hexokinase, fumarase, trypsin, reverse transcriptase and the receptor-mediated uptake of low-density lipoprotein (LDL) by trypanosomes (summarized in Wang, 1995), although whether any of the reported effects influences trypanocidal activity is unknown. Suramin is taken up by trypanosomes by pinocytosis, as a plasma protein-bound complex (Fairlamb and Bowman, 1980). The accumulation of suramin in the trypanosomes was hypothesized to be one of the reasons for the differential toxicity between the host and the parasite.

EFFICACY Suramin is the drug of choice for first-stage *T. b. rhodesiense* infection. Cure

rates of over 95% have been reported (Apted, 1980) but failure rates in the range of 25–35% are common. Since pentamidine is easier to administer, it is used in preference to suramin for treatment of *T. b. gambiense*. The value of suramin/pentamidine combinations is uncertain.

In vitro, a drug exposure of 1 μg/ml for 24 h is sufficient to inactivate bloodstream forms. Suramin-resistant *T. b. gambiense* or *T. b. rhodesiense* strains are not known in the field today. Observed relapses could be attributed to second-stage infections that were not diagnosed as such. Experimental CNS infections in mice could be cured with high doses (> 40 mg/kg/day for 4 days), indicating that some drug crosses the blood–brain barrier. Suramin in combination with other drugs (including eflornithine, metronidazole, nifurtimox and 5-nitroimidazoles) was effective against second-stage *T. b. rhodesiense* infection in rodents (Jennings, 1993) but combinations of suramin and other drugs have not been evaluated in clinical studies.

Availability and cost

Suramin is produced by Bayer under the trade name Germanin®. It comes in vials of 1 g. One vial costs about US$5, i.e. a course consisting of five injections amounts to about US$25. Availability in the near future should be assured following negotiations between WHO, MSF and Bayer.

Registered Drugs for Treatment of the Second Stage

Melarsoprol

The organo-arsenical drug melarsoprol, Mel B (Arsobal®, Aventis) (Fig. 21.3), was developed in 1949 by the addition of the heavy metal chelator, dimercaptopropanol (British Anti-Lewisite, BAL), to the trivalent arsenic of melarsen oxide. The adduct was reported to be about 100 times less toxic than melarsen oxide (Friedheim, 1949) (Fig. 21.4) but only 2.5 times less trypanocidal.

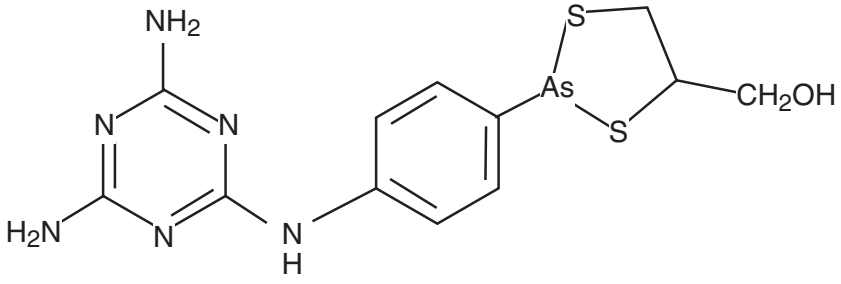

Fig. 21.3. Melarsoprol.

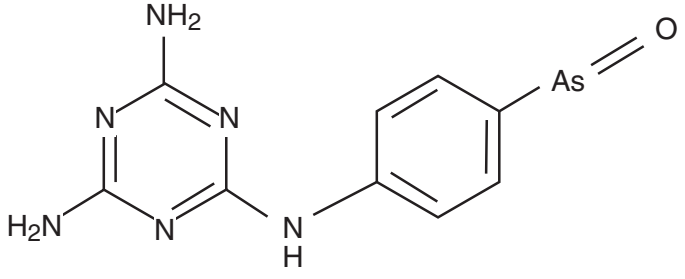

Fig. 21.4. Melarsen oxide.

Despite the high frequency of severe adverse drug reactions and an increasing number of patients refractory to treatment, a lack of valid alternatives means that the drug is still the first choice for treatment of second-stage sleeping sickness.

Clinical application

Until the latter half of the 20th century, second-stage Rhodesian trypanosomiasis was untreatable. The introduction of melarsoprol in 1949 was a landmark in patient management. It has saved many lives but the high rate of severe adverse drug reactions limits its application to the second stage of the disease.

Melarsoprol clears trypanosomes from blood and lymph within 24 h in most patients. Due to the irritating properties of the solvent, propylene glycol, a paravasal deposition of the drug has to be avoided and melarsoprol must be given strictly as intravenous injection.

THERAPY SCHEDULES A wide range of different treatment regimens has been used, most of them not supported by prospective clinical trials. The schedules all apply the drug in repeated series of three to four injections, spaced by 7–10 days. Recently acquired knowledge about the pharmacokinetics of the drug led to the suggestion of a new, concise mode of application, which is currently being evaluated (Burri *et al.*, 2000). Examples for currently used standard dosage regimens for melarsoprol are given in Fig. 21.5 (WHO, 1986, 1998).

ADVERSE DRUG REACTIONS Adverse drug reactions of melarsoprol may be severe and life threatening. The most important one is an encephalopathic syndrome, which occurs in 5–10% of all treated cases and which is fatal for about 10–70% of those patients (Pepin and Milord, 1994; WHO, 1998). Clinically, the symptoms have been defined either as convulsions, the rapid deterioration of neurological symptoms and progressive coma, or as psychotic reactions or abnormal behaviour.

The syndrome has been termed in different ways, indicating that the exact cause is not known. An immune reaction is generally thought to underlie the syndrome but the detailed mechanisms remain unclear. The presence of trypanosomes in the CSF or a WBC count of 100/mm^3 or more in the CSF and malnutrition is associated with an increased risk for encephalopathic syndrome. The association of fever with the occurrence of an encephalopathic syndrome was found to have a poor prognosis (Blum *et al.*, 2001). Simultaneous administration of corticosteroids (prednisolone, 1 mg/kg body weight, maximum of 40 mg daily) has been shown to reduce the incidence of encephalopathic syndromes by two thirds and the mortality by half (Pepin *et al.*, 1989).

Pyrexia, headache and general malaise occur in nearly all patients during melarsoprol treatment. Skin reactions including pruritus and thrombophlebitis are common, but severe complications such as exfoliative dermatitis occur in $< 1\%$ (WHO, 1998). Cardiac failure is common during treatment and can be a cause of death but it is still unclear whether this is due to an adverse drug reaction or the well-known cardiac involvement of HAT itself. Other adverse reactions have been reported occasionally: peripheral motoric (palsy) or sensorial (paraesthesia) neuropathy, cardiac failure, renal dysfunction (proteinuria and hypertension) as well as hepatotoxicity (elevated liver enzymes, bilirubinaemia) (Van Nieuwenhove, 1999).

The potential for teratogenicity is unknown but the risk of leaving second-stage trypanosomiasis untreated outweighs any potential dangers of melarsoprol treatment.

Pharmacology

PHYSICO-CHEMICAL PROPERTIES The molecular weight of melarsoprol is 398 g/mol. The pK is 9.2, and the coefficient of partition between buffer and *n*-octanol at pH 7.4 is approximately 160 (Keiser and Burri, 2000).

The compound is insoluble in all common solvents, and is marketed as a 3.6% solution in propylene glycol in 5 ml ampoules, so must be strictly applied by the intravenous route. Experimental oral appli-

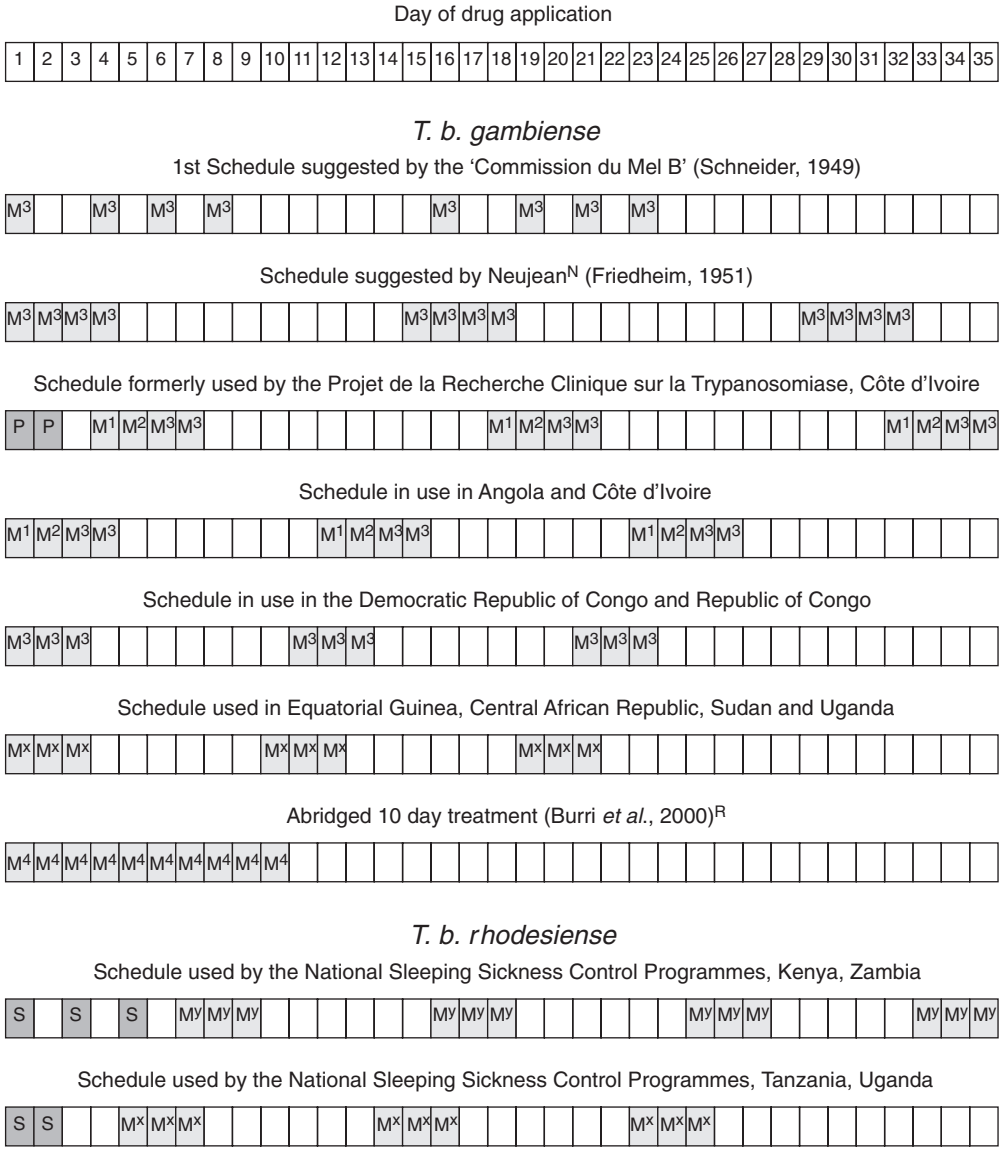

Fig. 21.5. Comparison of selected treatment schedules used for late-stage sleeping sickness treatment with melarsoprol 1949–2000. P, pentamidine pretreatment 4 mg/kg body weight (bw); S, suramin pretreatment; M^1, melarsoprol 1.2 mg/kg bw; M^2, 2.4 mg/kg bw; M^3, 3.6 mg/kg bw; M^x, one series of 1.8, 2.16, 2.52 mg/kg bw, one series of 2.52, 2.88, 3.25 mg/kg bw, one series of 3.6, 3.6, 3.6 mg/kg bw; M^y, one series of 0.36, 0.72, 1.1 mg/kg bw, one series of 1.4, 1.8, 2.2 mg/kg bw, one series of 2.2, 2.9, 3.6 mg/kg bw, one series of 3.6, 3.6, 3.6 mg/kg bw; M^4, melarsoprol 2.16 mg/kg bw; N, one series for patients with white blood cell (WBC) count of $< 20/mm^3$, two series for patients with WBC count of > 20 but $< 100/mm^3$, three series for patients with WBC count of $> 100/mm^3$, four series for relapse patients.
R, recommended by ISTRC 2003 for use in *T.b. gambiense* upon request by WHO.

cation has been reported but never appropriately documented.

A total serum protein binding of 79% (albumin binding 79%; α-acidic glycoprotein 70%) was measured by ultrafiltration (Keiser and Burri, 2000). The *in vivo* situation might

be more complicated, because sleeping sickness patients often have elevated IgG or IgM levels, which may result in an increased total protein binding. Additionally, concomitant diseases such as malaria or hepatitis and malnutrition may cause a modification of the plasma concentrations of albumin and α-acidic glycoprotein. Serum and CSF concentration differ by up to tenfold when determined by a biological assay at equal time points (Burri *et al.*, 1993).

Biological assays indicated a 35 h half-life of the drug while HPLC methods indicated a half-life of melarsoprol itself of < 1 h, indicating that the drug was metabolized to other trypanocidal derivatives (Bronner *et al.*, 1998). Melarsen oxide was the only product that could be detected by HPLC analysis after microsome incubation. A short half-life of 3.9 h for this metabolite is due to both rapid elimination and an irreversible binding to plasma proteins. The pharmacokinetic parameters calculated after determination of the total activity by bioassay were confirmed by atomic absorption spectroscopy (AAS), which showed that all active metabolites contain arsenic. Metabolites other than melarsen oxide could not be identified by HPLC, although whether melarsen oxide, melarsoprol, or further (so far unidentified) metabolites are bound to proteins was not certain (Keiser *et al.*, 2000).

PHARMACOKINETICS For determination of melarsoprol concentrations different methods have been developed, including a bioassay that measures total activity, including all active metabolites, and HPLC methods that specifically determine melarsoprol. AAS to identify the arsenic moiety has also been described.

Prior to the 1990s virtually nothing was known on the pharmacokinetics of melarsoprol. An array of different treatment schedules was used, all based on three to four series of three to four daily injections, spaced by 7–10 days. The origin of repetitive serial application lies in the treatment schedules of the older arsenicals, which required prolonged and repetitive drug application because of insufficient penetration of the CSF and drug resistance. Early drug-level investigations with melarsoprol indicated that < 20% of the injected arsenic was found in the urine. Arsenic could not be detected after 3 days and this was interpreted to mean that the drug was retained within the body, so recommendations not to exceed three injections in one series of application were made to prevent drug accumulation. Melarsoprol is actually eliminated predominantly in the faeces; 6 days after the last injection of a course, a total of 85% of melarsoprol has been eliminated. This information was neglected for many years and changes to the classical administration regimens were slow in coming.

More recent studies (Burri *et al.*, 1993) revealed that maximum serum concentrations of melarsoprol showed interpatient variation in the range of 2.5–6 μg/ml. The mean concentration 120 h after the last injection was 220 ± 80 ng/ml. Elimination was biphasic with a pronounced β-phase. The mean terminal elimination half-life of active metabolites, determined by bioassay, was in the range of 35 h, the volume of distribution was about 2 l/kg and total clearance 1 ml/min/kg.

HPLC determination of melarsoprol revealed concentrations much lower than those determined by bioassay, except in samples collected immediately after injection. The average maximum plasma concentration of melarsoprol after 15 min determined by HPLC was found to be 0.7 μg/ml compared with 4.9 μg/ml determined by bioassay. The half-life of melarsoprol was calculated to be < 1 h with HPLC and no melarsoprol was found by HPLC in samples taken 3 h after application. However, < 1% of the drug was found in the urine and melarsoprol could not be detected in plasma or CSF 24 h after the fourth injection. This discrepancy between results obtained with HPLC and bioassay indicated that the drug is transformed into active metabolites. These pharmacokinetic investigations were used to simulate possible alternative therapy schemes, which were the basis for clinical investigations of a concise, uninterrupted 10-day schedule (Burri *et al.*, 2000).

Levels of melarsoprol in the CSF,

obtained by spinal tap, are generally very low, in the range of 2% of those found in serum. The mean CSF levels in 19 patients, 24 h after the last drug application, varied from below the limit of determination (9 ng/ml) to 100 ng/ml (Burri *et al.*, 1993). Similar results were found in early studies in primates with CSF levels 0.5–5% of those found in plasma.

MODE OF ACTION Melarsoprol appears to be a non-discriminatory inhibitor of a large number of mammalian and trypanosome enzymes that contain dithiols (Wang, 1995). However, in spite of a great deal of speculation, the mode of action remains unknown. The drug appears to enter cells via an unusual nucleoside transporter, termed P2 (Carter and Fairlamb, 1993; Mäser *et al.*, 1999). Loss of this transporter leads to low levels of resistance to the drug but recent data indicate that other events are associated with high-level resistance and the drug possibly also enters cells via another route.

EFFICACY Melarsoprol remains the drug of choice for second-stage infection caused by *T. b. rhodesiense* or *T. b. gambiense*. In spite of the fact that CSF levels reach only 1–3% of those found in plasma, its efficacy against CNS infection is attributed to the drug's ability to pass the blood–brain barrier. Melarsoprol is a fast-acting drug that kills trypanosomes within 12–24 h *in vitro* with an MIC of 10–25 ng/ml, and IC_{50} values determined over 72 h are between 1 and 5 ng/ml. Thus it is sufficiently potent to kill parasites in the CSF in spite of its being weakly penetrative.

The minimum effective dose of melarsoprol depends on the stage of infection as well as parasite and patient characteristics. Patients with first-stage *gambiense* infection could be cured with a single application with a relapse rate of $< 5\%$. Cure of second-stage infection, however, requires several applications with at least three series of three injections.

The relapse rate after melarsoprol treatment was usually in the range of 3–10% for both forms of HAT. Recently, this proportion has increased in some epidemic *T. b. gambiense* areas: 30% in northwest Uganda, 21% in southern Sudan, 25% in northern Angola (Brun *et al.*, 2001). The reasons for these relapses are not yet clear but may involve a complex interaction between parasites with reduced susceptibility to drug and interpatient variability in drug accumulated into different extravascular compartments. The P2 nucleoside transporter involved in melarsoprol uptake into trypanosomes has been reported to be altered in several drug-resistant isolates from the field (Matovu *et al.*, 2001), though the extent to which these mutations underlie resistance remains to be elucidated.

Availability and cost

Melarsoprol is produced by Aventis (formerly by Rhône Poulenc Rorer) under the trade name Arsobal®. It comes in vials of 5 ml as a 3.6% solution in propylene glycol. One vial costs about US$5, i.e. a full course consisting of 9–12 injections amounts to about US$45–60 (injection materials and hospitalization not included). The agreement between Aventis and WHO secures the availability for free for the next 5 years of melarsoprol.

Eflornithine

Eflornithine (α-difluoromethylornithine, DFMO) (Fig. 21.6) is a selective irreversible inhibitor of ornithine decarboxylase (ODC) which blocks polyamine biosynthesis. It shows anti-tumour effects and is active against *P. carinii* and several protozoa. Trypanocidal activity was shown in animal models and humans. In 1990 the US Food and Drug Administration approved eflornithine for the treatment of *gambiense* sleeping sickness.

Eflornithine is the only alternative for patients after treatment failure with melarsoprol. Eflornithine's use is hampered by several major problems: (i) the high cost of treatment; (ii) the innate lack of susceptibility of *T. b. rhodesiense* to the drug, due to a more rapid ODC turnover in this parasite (Iten *et al.*, 1997); and (iii) the suboptimal pharmacokinetic and pharmacodynamic profile, which requires frequent applications of large doses; and (iv) the logistic complica-

Fig. 21.6. Eflornithine (DFMO).

tions due to the need for large amounts of infusion fluids.

Clinical application

Eflornithine is principally considered as a second-line treatment for melarsoprol refractory cases of *gambiense* sleeping sickness. The drug can be applied orally, but intravenous administration leads to a better bioavailability and is currently the preferred mode of application. Eflornithine should be administered by short infusions over a period of at least 30 min. A continuous 24 h application is also possible if permitted by the treatment facilities.

THERAPY SCHEDULES The most commonly used dosage regimen consists of 100 mg/kg body weight at 6 h intervals for 14 days (150 mg/kg body weight in children).

ADVERSE DRUG REACTIONS The adverse drug reactions of eflornithine are frequent and the characteristics are similar to other cytotoxic drugs for the treatment of cancer. Their occurrence and intensity increase with the duration of treatment and the severity of the general condition of the patient. The most frequent adverse effects are bone marrow toxicity (anaemia, leucopenia, thrombocytopenia) in 25–50% of the treated patients. Gastrointestinal symptoms (including nausea, vomiting and diarrhoea) can be observed in about 10%, alopecia (usually towards the end of the treatment) in about 5–10% and neurological symptoms (including convulsions) in 7% of treated patients. Generally, adverse drug reactions of eflornithine are reversible after the end of the treatment course.

In vitro studies did not reveal mutagenic changes and teratogenicity has not been reported in animals. However, the drug arrests embryonic development in mice, rats and rabbits. Documentation from humans is lacking. The excretion into breast milk is unknown.

Pharmacology

PHYSICO-CHEMICAL PROPERTIES Eflornithine is a white to off-white odourless crystalline powder. The formula weight of the base is 182 g/mol and of the hydrochloride 237 g/mol. The pK is not known. The compound is freely soluble in water and sparingly soluble in ethanol. Based on its physico-chemical properties, the drug may be given orally or intravenously. Eflornithine does not bind significantly to plasma proteins.

PHARMACOKINETICS Specific HPLC methods have been described for the determination of eflornithine (Smithers, 1988; Cohen *et al.*, 1989). Its pharmacokinetics has been studied in humans. In one study, after low oral doses (5–10 mg/kg), peak plasma concentrations were reached 1.5–6 h after ingestion. The mean half-life was 3.3 h and the volume of distribution in the range of 0.35 l/kg. Renal clearance was about 2 ml/min/kg (i.v.) and accounted for more than 80% of drug elimination. Bioavailability of an orally administered 10 mg/kg dose was estimated at 54%. The interpatient concentration variation is about 2. With this dosage range, the amount of drug absorbed was directly proportional to the dose given. However, no further increase of the plasma levels after application of more that 3.75 g/m^2 (corresponding to about 95–110 mg/kg) was observed in dose escalation studies, indicating a non-dose linear kinetics at high doses.

Eflornithine produces CSF/plasma ratios between 0.13 and 0.51 or even higher ratios at the end of a 14-day i.v. regimen: 0.91 in adults and 0.58 in children less than 12 years of age (Milord *et al.*, 1993). The mean steady-state serum concentration in children was only half as high as in adults, while the mean CSF concentration was only one-third, possibly due to enhanced renal drug clearance in children. CSF/serum ratios were higher in patients with melarsoprol-refractory infections, possibly because severe impairment of the blood–brain barrier due to chronic meningoencephalitis increased its permeability. Successful treatment depends on the CSF drug level reached during treatment with levels above 50 µmol/l required to attain consistent clearance of parasites. This concentration may not be consistently attained in patients treated at 100 mg/kg/6 h orally.

MODE OF ACTION ODC catalyses the conversion of ornithine to putrescine, the first and rate-limiting step in the synthesis of the polyamines spermidine and spermine (Bacchi *et al.*, 1980). Polyamines are essential for the growth and multiplication of all eukaryotic cells. Trypanosomes are more susceptible to the drug than human cells, possibly due to the slow turnover of ODC in *T. b. gambiense* meaning that a pulse of eflornithine effectively inhibits ODC activity and depletes polyamines in trypanosomes for a relatively prolonged period. This induces a non-dividing state that is vulnerable to host immune attack.

EFFICACY Eflornithine is used as a back-up drug for melarsoprol-refractory *T. b. gambiense* cases. It is a slow-acting drug that takes 4 days to eliminate trypanosomes from the blood. The frequent application by infusion is very demanding and requires sophisticated logistics. Therefore eflornithine in the intravenous form cannot generally replace melarsoprol as the first-line drug, despite the current availability at very low cost.

The drug was first used in the mid-1980s for melarsoprol-refractory second-stage *T. b. gambiense*. High relapse rates of 17% were accounted for mostly by children who require higher doses, due to a higher clearance of the drug, and other studies have revealed much lower relapse and mortality rates of 5.3% and 6.9%, respectively.

In a recent study a 7-day and a 14-day i.v. schedule were compared in first-treated and melarsoprol-relapse patients. It yielded an unacceptably high relapse rate for treatment of new cases. The reported superior outcome for treatment of relapsing cases will yet have to be confirmed, since the number of patients enrolled in this study arm was very small (Pepin *et al.*, 2000). There are indications that eflornithine cannot cure HIV-positive HAT patients, since the drug is trypanostatic and an intact immune system is required to eliminate the parasites.

Eflornithine seems not to be effective against *T. b. rhodesiense*, probably due to differences in polyamine metabolism and the ODC half-life in these cells compared with *T. b. gambiense*.

Availability and cost

Eflornithine was produced until 1999 under the trade name Ornidyl® by Marion Merrell Dow. The drug is very expensive; last estimates were US$350 per patient (injection materials and fluids not included). Bristol-Myers-Squibb and Aventis will produce the drugs and make it available for free for 5 years, until 2006, as part of the agreement with WHO and MSF.

Alternative (Non-registered) Drugs

Nifurtimox

Nifurtimox (Lampit®, Bayer) (Fig. 21.7) is a drug that was introduced in the late 1960s for use against Chagas disease. It is not registered for sleeping sickness and its use is restricted to the compassionate treatment, in combination with eflornithine or melarsoprol, of patients not responding to the melarsoprol standard therapy.

Clinical application

Small-scale clinical trials of nifurtimox alone yielded unsatisfactory results, with

Fig. 21.7. Nifurtimox.

high rates of relapse (Van Nieuwenhove, 1988). However, despite a high frequency of severe adverse drug reactions, the drug has a place as second-line treatment of melarsoprol-refractory cases in combination chemotherapy, in situations when eflornithine is not available.

THERAPY SCHEDULES Different empirically derived treatment schedules are in use. The standard treatment protocol for Chagas disease (*T. cruzi*) is 10 mg/kg for adults and 15 mg/kg for children for 60–90 days. Based on pharmacokinetic considerations and preliminary results of ongoing trials, a schedule of 1.8 mg melarsoprol/kg for 10 days and 15 mg nifurtimox/kg for 10 days was selected for further investigation against melarsoprol-refractory cases of sleeping sickness.

ADVERSE DRUG REACTIONS Nifurtimox is generally not well tolerated, and only about one-third of patients remain free of adverse drug reactions. Adverse effects are usually not severe; they are dose related and very rarely lethal. In one study gastrointestinal disturbances with nausea, abdominal pains and vomiting occurred in six out of 25 patients, when treated with 15 mg/kg body weight over 60 days. Two out of 25 patients developed a reversible cerebellar syndrome. In another treatment study with a dose of 30 mg/kg body weight over 30 days, toxicity was significantly higher, leading to a higher rate of neurological adverse reactions (8/30) with general convulsions, ataxia and tremor. The development of a peripheral polyneuropathy and a generalized skin reaction were seen as occasional events.

No teratogenicity was found in rats and mice. A high frequency of benign and malign tumours was found in rats after the application of very high nifurtimox doses, but since this represented no significant increase over the rate observed in the control group, the results are equivocal.

Pharmacology

PHYSICO-CHEMICAL PROPERTIES Nifurtimox is a 5-nitrofuran derivative with a molecular weight of 287 g/mol. Its pK is not known. The substance is slightly soluble in water. The drug can be administered by the oral route.

PHARMACOKINETICS A specific HPLC method has been described for the determination of nifurtimox (Paulos *et al.*, 1988) and this has superseded the thin layer chromatography (TLC) methods that were formerly used.

Volunteers given single oral doses of 15 mg/kg of the drug showed average peak plasma levels of 751 ng/ml (range 356–1093 ng/ml) within 2–3 h. The drug has an apparent volume of distribution of about 755 l and a high apparent clearance of 3200 ml/min. It is quickly eliminated with an average plasma elimination half-life of 3 h (range 2–6 h). The drug is probably almost completely absorbed, and $< 1\%$ of the orally administered dose was excreted in the urine as the parent drug in several mammals.

Nifurtimox is extensively metabolized in animals and humans. Radiolabelled drug revealed relatively high concentrations throughout rats, including brain and spinal

cord. More recent results suggested that nifurtimox is biotransformed partially by cytochrome P450, but mostly by NADPH P450 reductase. The formation of reactive metabolites may be responsible for the long-term deleterious effects of the drug.

MODE OF ACTION The mechanism of action of the drug has not been completely elucidated but its trypanocidal action may be related to its ability to undergo partial reduction to form chemically reactive radicals causing the production of superoxide anions, hydrogen peroxide and hydroxyl radicals. Those free radicals may then react with cellular macromolecules and cause membrane injury, enzyme inactivation, damage to DNA and mutagenesis (Docampo and Moreno, 1986).

EFFICACY Nifurtimox is only used as a compassionate treatment in combination with other trypanocides as a back-up drug for melarsoprol-refractory *T. b. gambiense*. It has not been used for the treatment of *T. b. rhodesiense*. Only a limited number of patients have been treated, under well-controlled conditions, and more research needs to be done.

Availability and cost

Nifurtimox is produced by Bayer under the trade mark Lampit® as tablets of 120 mg. Availability has been a problem until recently, since the drug is registered only for Chagas disease and the final product was produced by Bayer Argentina. The cost for a 3-week treatment course is about US$20. Negotiations between WHO, MSF, and Bayer led to renewed production of Lampit® in 2001.

Diminazene aceturate

Diminazene aceturate (Fig. 21.8) was developed against animal trypanosomiasis and is not registered for sleeping sickness therapy. However, it has been applied by the intramuscular route against first-stage human *T. b. gambiense* and *T. b. rhodesiense* infections in about 400 patients when other drugs were not available. Its use by the oral route (Bailey, 1968) and as a pre-treatment before melarsoprol use has also been reported.

Clinical application

Diminazene is very cheap, regularly produced and easily obtainable. However, registration for human use has not been attempted and diminazene cannot be recommended for routine use prior to further toxicological studies.

THERAPY SCHEDULES During its limited use in humans, the drug was given as an intramuscular injection at doses of 2–5 mg/kg for 7–10 days.

ADVERSE DRUG REACTIONS Moderate adverse drug reactions were frequent and included nausea, vomiting and albuminuria, but severe events including reversible paralysis and coma were rare. The main adverse drug reactions seen in animals were severe cerebral haemorrhages, which may be species specific, but these reactions caution against use in humans before extensive trials have been conducted.

Pharmacology

PHYSICO-CHEMICAL PROPERTIES Diminazene aceturate is a diamidine derivative analogue

Fig. 21.8. Diminazene azeturate.

to pentamidine, with a molecular weight of 587 g/mol. It is freely soluble in water.

PHARMACOKINETICS The pharmacokinetics of diminazene has not been studied in humans. In animals, the half-life is between 11 and 14 h for sheep, dogs and goats and 63 h for cattle (Peregrine and Mamman, 1993). In sheep the volume of distribution was 0.56 l/kg, the clearance 1.1 ml/kg/min, with plasma-protein binding estimated at 65–85%. Diminazene is to some extent absorbed when given orally but the evidence gathered is insufficient.

MODE OF ACTION The mode of action is not certain but it is at least partially through irreversible inhibition of trypanosomal *S*-adenosyl-methionine-decarboxylase, an enzyme involved in the synthesis of polyamines, and it also selectively blocks kinetoplast DNA synthesis. It appears to enter *T. brucei* group parasites via the P2 nucleoside transporter that is also capable of transporting other diamidines and melamine-based arsenicals.

EFFICACY *In vitro* studies revealed that the drug is slow acting but effective, with MIC values of 10–30 ng/ml for a 3-day exposure. Resistance to the drug seems to arise when the P2 transporter, responsible for its uptake, is mutated in such a way that uptake is diminished.

In one study, 16 of 17 patients with first-stage *T. b. gambiense* infection were cured with 2 mg/kg/day i.m. for 7 days, while 85% of a set of several hundred patients treated with diminazene were cured with 7 mg/kg/day three-times every second day. For first-stage *rhodesiense* infections, three doses of 5 mg/kg on alternate days were used in two trials, with $< 5\%$ relapses. Diminazene appears to be highly effective against first-stage *rhodesiense* and *gambiense* infections and the low relapse rate is probably accounted for by early second-stage infections misdiagnosed as first-stage. The drug has several advantages over pentamidine (e.g. shorter treatment period, lower cost) but, since it is not registered for use in humans, it cannot be recommended for routine use.

Availability and cost

Diminazene aceturate is produced by Hoechst under the trade name Berenil® restricted to veterinary usage. It comes in sachets containing 1.05 g diminazene aceturate, which dissolves readily in water. A three-injection course costs approximately US$2 (injection materials not included). This veterinary drug is currently widely available.

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22 Current Chemotherapy of American Trypanosomiasis

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Introduction

Chemotherapy of American trypanosomiasis in humans was largely empirical until 1960. Several drugs were tried, based on their action on morphologically similar parasites (such as antimonials for leishmaniasis) or because they were introduced for other infectious diseases. The first known attempt at chemotherapy was by Mazza *et al.* (1937) who treated a child in the acute phase with a quinoline derivative (Bayer 7602 Ac). Several other compounds with recognized trypanocidal activity were tested later, as reviewed by Prata (1963). Conclusions on their effect were dubious, mainly due to poor methodology. Drugs were given for a few days and evaluation criteria were based on negative direct parasitological methods and brevity of acute-phase symptoms.

The first improvement was experimental demonstration by Brener (1961) of the benefits of long-term use of a trypanocidal drug (nitrofurazone) for destruction of *Trypanosoma cruzi*. Further progress was establishment of methodology and criteria for inclusion and evaluation of patients in treatment trials (review in Reunião de Debates sobre Doença de Chagas, 1963). Another contribution was recognition of the effect of some drugs, such as nitrofurazone, on the intracellular amastigote form (Andrade and Brener, 1969).

Following the accepted criteria, several drugs were later used on patients, e.g. nitrofurazone (Eaton), levofuraltadone (Eaton NF-602 and NF-902), furazolidone (Eaton), nifurtimox (Bayer 2502), a piperazine compound CL 71-366 (Lederle), benznidazole (Roche Ro 7-1051), BW 349.C.59 (Wellcome), ticlopidine (Sanofi), allopurinol (Wellcome), ketoconazole (Johnson and Johnson) and itraconazole (Johnson and Johnson). Other compounds (megazol, gossipol, MK-436), reviewed by Brener (2000), were experimentally effective but could not be used in humans, due to either their toxicity or mutagenicity. Of those drugs used in humans, only two were recognized as useful, according to both their tolerance and their efficacy, namely, nifurtimox (Nf) and benznidazole (Bz). Nevertheless, neither is an ideal drug, since they are not always effective and sometimes may cause serious side effects. New drugs currently under evaluation include D0870 (Zeneca) and SCH56592 (Schering-Plough) (Urbina, 1999).

Basis of Treatment

Aetiological treatment of American trypanosomiasis is aimed at eliminating all *T. cruzi* from infected individuals, whether in

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circulation or in the tissues. Even if there is autoimmune involvement in the pathogenesis, it is clear that the continued presence of the parasite may contribute to the progression of the disease (Higuchi, 1997). Thus, theoretically, treatment may be beneficial for any infected individual. Nevertheless, as treatment is not always effective, and may have significant side effects, each physician needs to balance the benefits against the consequences for each individual patient. It should be noted that, depending on the geographical region, nearly half of infected individuals may not develop overt disease.

Patients may be in either the acute phase or the chronic phase, which, for treatment purposes, may be subdivided into early chronic phase (mainly children) and late chronic phase. Recommendations from a meeting of experts sponsored by the World Health Organization and the Pan-American Health Organization (PAHO/WHO, 1999) indicate treatment for all individuals in the acute phase, whatever the origin, whether vectorial, transfusional, congenital or reactivation through immunosuppression. Treatment during the early chronic phase (i.e. children under 12 years old) has been recommended after early observations of cure (Rassi, 1981; Ferreira, 1990), confirmed by two randomized trials in Brazil and Argentina (Andrade *et al.*, 1996; Sosa Estani *et al.*, 1998). For those in the late chronic phase, aetiological treatment must be a judicious decision between physician and patient, and the former should know that correct follow-up exceeds a 10-year period.

Selection of Patients to be Treated

In the acute phase, all patients with demonstrable parasites by direct methods should be treated with one of the two available drugs (Luquetti, 1997).

In the chronic phase, diagnosis should be by epidemiological (history) and clinical assessment, and sound laboratory diagnosis, based on presence of antibodies against *T. cruzi*, detected by at least two different serological techniques and with an antibody concentration that is secure for diagnosis. Borderline results should be assessed by other techniques, since cross-reactions do occur, mainly in regions with leishmaniasis (Luquetti and Rassi, 2000).

Parasitological tests, if available, are desirable, such as xenodiagnosis or haemoculture; it is possible to have a positive result in 25–40% of infected individuals with a single examination (Luquetti and Rassi, 2000). PCR has higher sensitivity but is only available in a few research centres and is currently under evaluation (PAHO/WHO, 1999).

Either of the available drugs should be given during 60 (Bz) to 90 (Nf) days, with proper controls for adverse reactions.

Follow-up and Assessment of Treatment

As the infection is defined by the presence of *T. cruzi* and antibodies against *T. cruzi*, the aim of successful treatment should be the permanent absence of both. In relation to assessment, two 'dogmas' have been established (Cançado, 2000):

1. Chagas disease is caused by the parasite. The infected individual develops antibodies against it.
2. Cure of the infection means the eradication of the parasite, and, as a consequence, the specific antibodies against it should disappear as well.

Amendments to the dogmas include: (i) that the presence of *T. cruzi* should be recognized by indirect classical parasitological methods, i.e. xenodiagnosis and/or haemoculture (PCR is still under evaluation and not yet considered to be a classical method); and (ii) that antibodies are measured conventionally by serological techniques, with crude antigens. Five tests have usually been recognized: complement fixation (CF), indirect haemagglutination (IHA), indirect immunofluorescence (IIF), enzyme-linked immunosorbent assay (ELISA) and direct agglutination with 2-mercapto-ethanol (DA2ME). The former and the latter are no longer used for operational reasons. Recombinant antigens, synthetic peptides, purified antigens, Western blot, complement-mediated lysis, flow cytometry, chemi-

luminescence and other combinations of antigens or methods are not universally accepted; not all patients will respond and such methods are as yet considered inappropriate for certification of cure (Moncayo and Luquetti, 1990; PAHO/WHO, 1999).

Once antibodies (formerly detected by any of the three conventional methods with crude antigens) disappear permanently, the patient will be considered to be cured, even if recombinant antigens or other tests show positive results.

Dogmas may be modified with time, but reliable comparisons with other methods must be clearly established by several groups.

To achieve assessment of treatment, it is necessary to assess each treated patient by serial parasitological and serological examinations. As parasitaemia is usually low, yet antibodies are usually consistent and abundant, the meaning of parasitological and serological results is different.

Parasitological tests have absolute value only when the results are positive, unequivocally indicating therapeutic failure. Repeated negative parasitological results have only relative value for determination of cure; a positive examination may be found later. Once therapeutic failure has occurred, the clinician should consider treatment with another drug or different schedule (doses or time).

Serological tests are useful when antibodies disappear, which may take from months to decades. Once antibodies, initially present, are undetectable on a sustained basis in repeated blood collections, the interpretation is that treatment was successful, there are no more parasites and cure has been achieved (Cançado, 2000).

The time to achieving successful treatment varies according to the phase of the disease in which the patient was treated. Those treated during the acute phase take from several months up to 5 years. For those treated during the recent chronic phase, seronegativity usually takes longer – from 3 to 10 years. To obtain serological conversion in the late chronic phase, it is necessary to wait more than 15 years. For this reason, earlier investigators described events after aetiological treatment during the chronic phase, in terms of sustained negative parasitological tests with persistence of positive serology, when follow-up was only for a few years. Note that the first patients were treated with the effective drugs in 1965 (nifurtimox) or 1971 (benznidazole). The first chronic-phase cured cases (repeated negative parasitological tests and negative serology) after treatment with benznidazole became evident only 20 years later, i.e. in the early 1990s, and investigators realized that evaluation of successful cure was time dependent. As more late chronic-phase cases were treated, the rates of negative serology with required follow-up time have risen from 0% to around 25% (A. Rassi, unpublished observations). Furthermore, antibody concentration does not decrease at once; several reports describe a progressive decrease in titres after 10 years or more. Thus decrease in titres only indicates a trend to be confirmed later. Clear outcomes are either positive parasitological and serological tests, indicating treatment failure, or negative parasitological and serological tests (cured). For treated acute-phase patients, 5 years of follow-up is adequate to determine whether cure has been achieved (Rassi, 1981; Ferreira, 1990).

Around 40% of those treated during the chronic phase are therapeutic failures, because parasites were demonstrated shortly after treatment; about 35% are still under evaluation, of which some maintain undiminished antibody titres and some have significantly reduced antibody titres; 25% have negative parasitology and serology after long-term follow-up. Only parasitology and serology were available for follow-up of treatment. Xenodiagnosis was the reference parasitological test, especially after standardization (Cerisola *et al.*, 1974); early results with haemoculture were disappointing. Both procedures have been optimized in the last 25 years. The first results with PCR and infected humans were published by 1993; only recently has it been applied to follow-up of treated patients and so more experience is required.

Pitfalls

Conventional tests, whether parasitological or serological, may occasionally give false results. A false-positive parasitological test by haemoculture could result from growth of *Trypanosoma rangeli*. In regions with *T. rangeli*, a smear of the positive haemoculture should be fixed and stained with Giemsa, and checked for long epimastigotes of *T. rangeli*, with a small kinetoplast. False-positive xenodiagnosis may be due to contamination of the triatomine colony by *Blastocrithidia triatomae*.

For serology, samples should be stored in glycerol at −20°C to allow repetition of all tests in parallel. This will minimize reagent differences and allow detection of subtle differences in titres (Ministério da Saúde, 1997; Luquetti and Rassi, 1998).

Drugs Available

Two drugs are considered to be useful based on their efficacy and tolerance.

Nifurtimox

This drug, synthesized by Bayer, was designated Bayer 2502. It is a 3-methyl-4-(5′-nitrofurfurylidene-amino) tetra hydro 4H-1, 4-thiazine-1, 1-dioxide marketed as Lampit® and used orally at the dose of 8–10 mg/kg body weight/day for adults, and 10–15 mg/kg body weight/day for children in divided doses every 8 h, for 60–90 days (Rassi and Luquetti, 1992; PAHO/WHO, 1999).

Benznidazole

Benznidazole is *N*-benzyl-2-nitro-1-imidazol acetamide. It was synthesized by Roche and designated Ro 7-1051, marketed as Rochagan® (Brazil) and Radanil® (Argentina), and used at the dose of 5 mg/kg body weight/day for adults and 5–10 mg/kg body weight/day for children, as twice-daily doses for 60 days. Clinical trials with this drug started in 1971.

Adverse Effects

The most important adverse effects of nifurtimox include anorexia with weight loss, nausea, vomiting, peripheral sensitive polyneuropathy (mainly of distal parts of the lower limbs), allergic dermopathy and several central nervous system alterations such as excitation, insomnia and seizures (rarely). All symptoms are reversible after drug withdrawal.

Adverse effects of benznidazole include localized or, sometimes, generalized allergic dermopathy (non-bullous polymorphic erythema) which is not dose dependent, is of slight to moderate intensity, and usually starts around the 9th day of treatment or occasionally later. Treatment should be discontinued only when this effect is severe or associated with fever and lymph node enlargement. The dermopathy is reversible and the use of antihistaminic drugs does not make it shorter. Another adverse effect, which may appear towards the end of the treatment and is dose dependent, is a peripheral sensitive neuropathy, mainly of distal parts of the lower limbs: in this case interruption of treatment is indicated. The neuropathy may take several months to subside slowly and it is not relieved by the administration of B-complex vitamins.

Leucopenia with marked decrease in granulocytes has been described rarely, sometimes followed by acute tonsillitis (agranulocytopoenic sore throat) at 15–20 days of treatment: leucopenia occurs by day 20 of treatment and is a formal indication for treatment withdrawal. To monitor this rare and severe side effect, it is necessary to follow up all patients clinically and by leucocyte counts. Leucopenia usually subsides a few days after withdrawal of treatment and tonsillitis should be treated. Other side effects, rarely observed and that disappear spontaneously, are loss of taste and onycholysis.

A high incidence of malignant lymphomas (8/21) was observed experimentally by Teixeira *et al.* (1990) in rabbits infected with the Ernestina strain of *T. cruzi* to which benznidazole was administered intraperitoneally at 8 mg/kg/day for 60 days; no lymphomas were found in 22 control rabbits.

However, no cases of lymphoma have occurred in several thousand treated patients and several authors were unable to repeat the experimental observation (reviewed in Cançado, 1997).

Moya and Trombotto (1988) referred to a clastogenic effect of nifurtimox and benznidazole in three patients during treatment, with a high percentage of micronuclei and an increased frequency of expression of fragile sites: in six patients with follow-up of 1–15 years this effect was not observed.

The author (AR) treated 27 patients with nifurtimox (between 1963 and 1972) and 160 patients with benznidazole (since 1973), both children and adults of both sexes in the acute and chronic phases, without diagnosis of any malignant tumour during follow-up. This applies to a further group of 72 patients treated with nifurtimox and 1602 treated with benznidazole over the same period but with less rigorous follow-up.

In general, children show fewer side effects than adults, even when higher doses and the same period of treatment are employed; all those treated during the acute phase have a similar lack of side effects even if they are adults.

Experience with Nifurtimox

Nifurtimox has been used since 1965. Early studies were with the acute phase in Argentina (reviewed in Lazzari and Freilij, 1998) with a cure rate of 81% (Cerisola *et al.*, 1972). For the chronic phase, negative xenodiagnosis was achieved in 88% of those followed up. Due to adverse effects, the drug was withdrawn from 18% of patients (Lazzari and Freilij, 1998). Children and newborn (n = 45) have also been treated and the cure rate was related to age when the drug was given (Freilij and Altcheh, 1998), with better results when treatment was given before 16 months of age (93.8%) (Moya *et al.*, 1985).

In Chile, Schenone *et al.* (1981) treated 15 patients in late chronic phase, achieving negative xenodiagnosis in 13 during follow-up of 1–12 years. In a later study (Schenone, 1998) 105 children yielded negative xenodiagnosis after treatment, including all of the 81 with follow-up for more than 2 years.

In Brazil, several trials have been performed with acute- and chronic-phase patients but with less success than in Argentina and a cure rate of only 26.1–38.4% in the acute phase (reviewed in Rassi *et al.*, 2000a). Reasons for this discrepancy were suggested to be differences in strains circulating in these areas. Studies performed in a Brazilian boundary state with Argentina (Rio Grande do Sul) showed a similar cure rate as Argentina for 15 chronic-phase patients followed up for 4 years (Silva *et al.*, 1974). Negative xenodiagnosis was reported by Macêdo and Silveira (1987) in 77.8% of treated chronic-phase patients after 4–10 years of follow-up. Prata *et al.* (1975) found 17 of 30 chronic-phase patients with positive xenodiagnosis after 1 year of follow-up.

In Bolivia, nifurtimox has been used in the acute phase, with negative serology achieved in 70% of 32 treated cases after 1 year of follow-up (Gianella, 1998). Sporadic laboratory accidents in the United States have been treated with nifurtimox provided by the Center for Disease Control (CDC).

Experience with Benznidazole

Benznidazole has been employed since 1971, mainly in Brazil, Argentina, Chile, Venezuela, Bolivia and Paraguay.

Benznidazole has been used extensively in Brazil, because it was shown to be more active and better tolerated than nifurtimox. Cançado (2000) obtained 76% cure (16 of 21) for patients treated during the acute phase but only 8% (nine of 113) for the chronic phase followed up for between 6 and 18 years. Rassi *et al.* (2000a) found 51.6% cure in the acute phase and 25% in the late chronic phase with a mean follow-up of 15 years (A. Rassi, unpublished observations). Ferreira (1990) reported 70% cure in the acute phase and Shikanai-Yasuda *et al.* in 1990 found 56.2% cure.

In Argentina the first treatment started in 1974, with patients in the acute and chronic phase; negative xenodiagnosis was obtained

in 75–95% of chronic-phase patients (reviewed in Lazzari and Freilij, 1998). Viotti *et al.* (1994) gave 30 days treatment to 110 patients in the late chronic phase and compared them with an untreated group after a mean of 8 years follow-up; negative serology was obtained in 19% of those treated and in 6% of controls. They also found low antibody titres in those treated and high titres in the control group: new electrocardiographic alterations were present in 5% of those treated and in 23% of controls.

Experience with Allopurinol

After reports from Argentina of apparent success for chronic-phase patients treated with high doses of allopurinol (900 mg daily) for 60 days (Storino *et al.*, 1994), a multicentric study sponsored by the World Health Organization (WHO) was started in 1994. In one of the three centres involved, positive xenodiagnoses were obtained for all patients treated (Rassi *et al.*, 1996); and in the second centre for 16 of 17 patients (Gianella *et al.*, 1997). This demonstrates that allopurinol lacks effect in the chronic phase.

Results with Aetiological Treatment

Acute phase

Both nifurtimox and benznidazole have been used extensively during the acute phase. Cerisola *et al.* (1972) treated with nifurtimox and obtained 81% cure with 232 patients in Argentina. Rassi and Ferreira (1971) obtained 38.4% and Prata *et al.* (1975) 26.1% cure rates with the same drug in Brazil, indicating important regional differences (see Rassi *et al.*, 2000a). Benznidazole cured seven of ten patients in Brazil (Ferreira, 1990), nine of 16 patients in Sao Paulo (Shikanai-Yasuda *et al.*, 1990), 53.8% of patients treated by Cançado in 1985 and 51.6% of 37 patients treated by one of us (AR) (see Rassi *et al.*, 2000a). In the latter study, absence of disease progression was noted in the cured patients, whereas cardiac or digestive involvement arose in ten of 18 without cure (Rassi *et al.*, 2000a).

Early chronic phase

Rassi (1981) and Ferreira (1990) were able to obtain serological tests in patients treated during the early chronic phase. Based on these reports, two randomized multicentric trials with 235 subjects in Brazil and Argentina gave an efficacy of 55–62% when evaluated by serology (PAHO/WHO, 1999).

Late chronic phase

Evaluation of cure of the late chronic phase gave three different types of parasitological and serological outcome: (i) positive parasitology and serology, i.e. therapeutic failure; (ii) sustained negative parasitology with positive serology or 'dissociated cases'; and (iii) sustained negative parasitology and doubtful serology with irregular titres. In the last two outcomes, cure was not possible to ascertain. After prolonged follow-up a fourth outcome emerged, i.e. sustained negative parasitology and serology demonstrating cure, indicating that reversal of serology takes 10, 15 or more years. Importantly, halting disease progression – mainly cardiopathy – by treatment has been demonstrated by several authors (Rassi *et al.*, 2000b) (Table 22.1).

Conclusions and Perspectives

So far, two drugs (nifurtimox and benznidazole) are effective in the treatment of American trypanosomiasis. Long-term follow-up is necessary to monitor effects of treatment. The criterion for cure is serological, i.e. demonstration of absence of antibodies, in repeated examinations, when they were formerly present. Accepted serological tests at present are conventional IIF, IHA and ELISA, with crude antigens. Parasitological tests are desirable but are of absolute value only when positive, demon-

Table 22.1. Evolution of cardiopathy in benznidazole treated and untreated chronic-phase infected individuals.

	Evolution for cardiopathy (ECG)			
Authors	Material	Drug (%)	Control or placebo (%)	Follow-up (years)
Macedo and Silveira (1987)[a]	Adults (n = 171)[b]	6.7	8.8	7
Ianni *et al.* (1993)[a]	Adults (n = 33)	13.3	0.0	8
Miranda *et al.* (1994)[a]	Adults and children (n =120)	10.5	63.6	10–16
Viotti *et al.* (1994)	Adults (n = 201)	4.2	30.0	8
Fragata F. *et al.* (1995)[a]	Adults (n = 120)	7.0	14.3	7–8
Andrade *et al.* (1996)	Children (n = 129)	1.7	6.9	3

[a]References in Rassi *et al.* (2000b).
[b]Included cases treated with nifurtimox.

strating therapeutic failure. Cure or therapeutic failure is readily observed in patients treated during the acute phase: the outcome for most patients will be defined by up to 3 years follow-up. The time required to define the outcome for chronic-phase infected patients is longer: for the recent chronic phase, follow-up should extend for 5–10 years and for late chronic phase it should extend for decades, unless a positive parasitological examination is obtained. Recent evidence points to clear benefits of treatment in terms of arrest of electrocardiographic alterations and lack of progression to megaviscera. Efficacy of treatment may depend on geographical area and is nearly 100% for congenitally acquired infection, 50–80% for the acute phase and so far 25% for the late chronic phase. This latter rate should rise as more adults reach the 20 years of follow-up required to demonstrate cure, since wide use of the most efficient drug, benznidazole, occurred after 1980. Several drugs have entered clinical trials in the last 30 years and each has been shown to be less effective or less well tolerated than nifurtimox or benznidazole. New drugs are currently being tested. For the future, we envisage extensive *in vitro* testing of alternative drugs, the use of multi-drug schedules and testing of longer periods of treatment with promise of drugs devoid of side effects.

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23 Current Chemotherapy of Animal Trypanosomiasis

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Introduction

Over most of sub-Saharan Africa, bovine trypanosomiasis continues to be controlled primarily by trypanocides. Only three compounds – isometamidium chloride, homidium (bromide and chloride) and diminazene aceturate – are available and all of these drugs have been on the market for over 40 years. A full account of the history and properties of these drugs was provided by Mulligan (1970) and later reviewed by Leach and Roberts (1981).

Isometamidium is principally used as a prophylactic drug and can provide up to 6 months' protection against tsetse challenge. Whilst homidium has limited prophylactic properties, it is primarily used as a therapeutic agent. Diminazene has only therapeutic properties. It is currently estimated that about 35 million doses of these drugs are used in Africa each year and this has remained relatively constant. There are estimated to be between 40 million and 60 million cattle at risk.

Over the past 40 years these three drugs have been primarily provided by a few large European pharmaceutical companies but recently several generic forms of these compounds from a wider range of companies have become available on the African market.

Although there is a consistent demand for trypanocides by African farmers, the total value of the market (about US$30 million) is not considered sufficient to justify investment by large pharmaceutical companies in the development and licensing of new animal trypanocides, the costs of which may exceed US$250 million for a single compound. The challenge, therefore, remains to make optimal use of the three relatively old compounds until new methods of treatment emerge, possibly through serendipitous cross-reactivity with new broad-spectrum anti-protozoal compounds such as those currently being developed for the treatment of malaria and cryptosporidiosis.

Perhaps the greatest risk to the future use of the existing three trypanocides is the development and spread of drug resistance to the point where they become ineffective over large areas of Africa, and so a large part of this chapter is devoted to this issue. However, other risks exist. One is that, because of the risk of drug resistance (real or perceived), the market will shrink and manufacture will become unprofitable. Secondly, the spread of generic products, some of which are of doubtful quality, may undermine farmers' confidence in trypanocides. Thirdly, it is possible that extensive vector control may remove the need for trypanocides over large parts of the

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existing market. Fourthly, there may be growing concerns over the toxicity of these compounds and the potential threat they pose to human health. Despite these risks, the three established compounds remain available and popular and their use is very well established across Africa. In many African countries they remain the most frequently used veterinary drugs. A list of the currently available trypanocidal drugs for use in domestic livestock is presented in Table 23.1 (modified from Peregrine, 1994).

Strategies for Trypanocidal Drug Usage

Current use of trypanocides for the control of African bovine trypanosomiasis is generally practised according to one of a number of defined treatment strategies, as described below.

Routine block treatments

These are generally carried out using prophylactic drugs, notably isometamidium chloride, at predetermined intervals based on the perceived duration of prophylaxis. All animals in a herd may be treated, or treatment may be targeted at a particular group of valuable or 'at-risk' animals. When routine block treatment with isometamidium is practised it is recommended that, once a year, the animals are separately treated with diminazene in order to delay the development of drug resistance following the concept of the 'sanative pair' (Whiteside, 1962).

Strategic block treatments

These are generally carried out using prophylactic drugs, though curative drugs may also be used. All animals in a herd, or particularly valuable or 'at-risk' stock, are treated when challenge (as measured by the number of animals succumbing to infection) reaches a predetermined threshold.

Monitoring and treatment of individual infected animals

Cattle are monitored using standard parasitological methods, e.g. wet blood film, haematocrit centrifugation technique or buffy coat technique. Treatment of infected animals is generally conducted using a therapeutic drug, usually diminazene aceturate.

Monitoring and treatment of clinical cases

This is similar to monitoring and treatment of individual infected animals, but not all infected animals are treated. Cattle are treated, usually with a curative trypanocide, only if the packed cell volume (PCV) falls below a predetermined threshold, or if clinical signs of trypanosomiasis are detected. The rationale for this strategy is the belief that cattle may acquire a degree of immunity or resistance to locally circulating strains of trypanosomes, and that treatment of animals with subclinical infections is unnecessary and may interfere with this process.

In practice, a combination of these strategies may be used, such as routine or strategic block treatments together with monitoring and treatment of individual infected animals. The number of individual animal treatments given may then be used in deciding when to administer the next strategic block treatment. Routine and strategic block treatments are options frequently used in large-scale ranching situations, or under government or donor-funded control campaigns. However, there is an increasing trend towards the use of individual treatment on the basis of clinical signs alone, as livestock keepers become of necessity increasingly self-reliant in the diagnosis and treatment of bovine trypanosomiasis. Finally, where drugs are unavailable or beyond their financial means, many farmers resort to traditional ethnoveterinary medicines for the treatment of cattle diseases. However, as yet none has proven efficacy against bovine trypanosomiasis and, moreover, farmers rarely use this approach where they do have the option of the use of trypanocidal drugs.

Table 23.1. Currently available trypanocidal drugs for use in domestic livestock.

Drug	Trade names[a]	Dose (mg/kg)	Route	Use	Activity	Animal
Diminazene aceturate	Berenil® Many others	3.5–7	i.m.	T	*T. congolense* *T. vivax* (*T. brucei*) (*T. evansi*)	Cattle Small ruminants [Dogs] [Equidae]
Homidium chloride Homidium bromide	Novidium® Ethidium®	1	i.m.	T/P[b]	*T. congolense* *T. vivax*	Cattle Small ruminants Pigs [Equidae]
Isometamidium chloride	Samorin® Trypamidium® Veridium®	0.25–0.5 0.5–1	i.m. i.m.	T P	*T. congolense* *T. vivax* *T. brucei* *T. evansi*	Cattle Small ruminants Equidae Camels
Quinapyramine dimethylsulphate Quinapyramine dimethylsulphate:chloride (3:2 w/w)	Trypacide sulphate® Trypacide Pro-salt®	3–5 3–5[c]	s.c. s.c.	T P	*T. congolense* *T. vivax* *T. brucei* *T. evansi* *T. simiae*	Camels Equidae Pigs Dogs
Suramin	Naganol®	7–10 g per animal	i.v.	T (P)	*T. evansi*	Camels Equidae
Melarsomine	Cymelarsan®	0.25	s.c./i.m.	T	*T. evansi*	Camels

i.m., intramuscular; s.c., subcutaneous; i.v., intravenous; T, therapeutic; P, prophylactic.
( ), limited activity; [ ], small therapeutic index.
[a]Incomplete list.
[b]Prophylaxis observed in areas of low tsetse challenge.
[c]Dosage of sulphate.

Factors Influencing Drug Usage

Veterinary services in Africa are in a state of transition, notably contracting and consolidating in response to global economic forces and the process of structural adjustment. Privatization of veterinary services means a shift from project-driven to demand-driven campaigns and from external funding to cost recovery from the livestock owners themselves. Adequate access to animal health services is key to livestock development. Unfortunately the governments of most African countries now lack the funds and institutional capability required to provide adequate public veterinary services and the emerging private sector has been slow to fill the void. This is particularly so in terms of services to resource-poor farmers, which represent the least financially attractive end of the new market. Hence most rural livestock disease diagnosis and drug administration is now in the hands of farmers rather than professional animal health workers. Recent studies have indicated that currently more than 60% of treatments are given by livestock owners. The problem is compounded because many veterinary diagnostic laboratories are underused by fee-paying farmers and, in the absence of public support, quickly fall into disuse.

Functional and sustainable drug delivery in the post-privatization context requires that social structures involved in the relationship between village-level organizations and privatized or public veterinary services and research institutes need to be taken into consideration. Table 23.2 shows some of the principal stakeholders in the veterinary aspects of bovine trypanosomiasis control before and after privatization.

In spite of decreased farmer motivation for animal health interventions as a result of privatization, there is nevertheless a tendency among farmers to purchase and use drugs from a reliable and affordable supply rather than to dispense entirely with veterinary care. Trypanocidal drugs are clearly regarded as a priority by smallholder farmers and sales of these compounds have remained in the region of US$30 million/annum over the last decade. In several countries there have been attempts to provide trypanocides through government-supervised cost-recovery programmes, with farmers purchasing drugs through a local supply network. In others, where there has been less government supervision, informal markets in trypanocidal drugs have expanded, with many small-scale pharmacies selling products directly to farmers.

The increasing practice of treatments being given by livestock owners is not without serious drawbacks. Most farmers do not have adequate knowledge on diagnosis and appropriate drug use even in areas of high prevalence of trypanosomiasis, and trypanocides are frequently used in

Table 23.2. Stakeholders in veterinary aspects of trypanosomiasis control.

Level	Pre-privatization	Post-privatization
National	Central veterinary research institutes	Government veterinary laboratories
	Pharmaceutical companies	Private veterinary laboratories
		Pharmaceutical companies
Provincial	Regional veterinary laboratories	Private veterinarians
District	District veterinary office	Animal health assistants
		Farmers' associations
		NGOs
Village	Animal health assistants	Women's groups
	Extension workers	Schools
		Churches
		Extension workers
		Pharmaceutical retailers
Farm	–	Smallholder farmers

the absence of diagnosis or used to treat conditions for which they are ineffective. Many surveys have shown that the amount spent by livestock keepers on trypanocides is not related to the prevalence of the disease. A further factor is that the choice between the use of therapeutic drugs and prophylactic drugs may be made on the basis of cost per dose, without a clear understanding by farmers of the advantages of prophylactic drugs in appropriate circumstances. Surveys in Zambia, for example (Van den Bossche *et al.*, 2000), have shown that despite farmers administering most of the trypanocide treatments there was little evidence of underdosing, though there was a strong tendency to use curative (diminazene) rather than prophylactic drug (isometamidium). Furthermore, the majority of trypanocidal treatments were given to clinically sick animals that were not necessarily infected with trypanosomes and, irrespective of the type of drug used, oxen and cows received the majority of treatments. This indicates that farmers prefer to treat their most productive animals as a priority.

Misuse or overuse of drugs is uneconomic and environmentally unsound and may lead to drug resistance and other problems. However, despite the widespread use of trypanocides, it has been estimated that over half of the cattle raised under trypanosomiasis risk are not treated with trypanocidal drugs.

Although farmers do administer a high proportion of treatments to their own animals, most owners seek advice from others on which medications to use. Many seek advice from animal health assistants and community-based animal health workers. These individuals, although without full professional veterinary training, represent an increasingly important cadre of animal health service providers. Projects to support private-sector community-based animal health workers to deliver veterinary drugs and advice to small-scale livestock farmers are being developed in several countries. In such schemes cattle owners themselves undertake control operations, but are coached by project field personnel and private veterinarians to ensure timeliness and correct treatment of cattle. Clearly, enabling these groups to make better diagnoses and treatment decisions can have a major impact on the health status of livestock of the rural poor.

Drug Resistance

Resistance to one or more of the trypanocidal drugs used in cattle has been reported in at least 13 countries of sub-Saharan Africa (Geerts and Holmes, 1998). Most of the currently available information on drug resistance is derived from case reports and does not give any indication of the prevalence of resistance in a region or a country. Very few systematic surveys have been carried out using a representative number of randomly selected trypanosome isolates. In the few surveys that have been conducted, the occurrence of drug resistance was found to be greater in those regions where drug use was more intensive. It was shown that resistance is widespread in some regions of Africa where drug pressure is high enough to select resistant strains. Since there is a lack of baseline data, however, it is not known whether the increasing number of resistance reports is due to a higher prevalence of resistance or simply to a growing interest in drug resistance by scientists.

How do trypanosomes develop resistance to trypanocidal drugs?

Selection by drugs essentially takes place during asexual multiplication in the animal or human host, though there is some evidence that, during passage through the tsetse fly, genetic exchange (sexual recombination) may occur at least in *Trypanosoma brucei*. The genetic structure of a parasite population (clonal or panmictic) is an important parameter influenced by the transmission intensity, and this in turn might influence the rate of development of drug resistance. This has been suggested by studies on drug resistance in *Plasmodium*, which have shown that parasite populations in infected hosts are polyclonal with differ-

ing drug sensitivities between the clones making up the population. It is likely that a similar situation occurs in trypanosome infections.

Therefore drug resistance in trypanosomes is likely to occur under the same circumstances as for many other parasites, i.e. under large-scale drug use, by using inadequate dosing and by using correct dosing with drugs that are slowly eliminated from the body. In the past the development of drug resistance in trypanosomes was mainly ascribed to their exposure to subtherapeutic concentrations of trypanocidal drugs. Although this is certainly an important aspect, the intensity of drug pressure (i.e. the treatment frequency and the degree of exposure of the parasite population) is probably even more important. The immunocompetence of the host also appears to play an important role, since it has been clearly shown that drug resistance develops more rapidly in trypanosomes present in immunosuppressed mice than in normal mice. Furthermore, some trypanocidal drugs such as ethidium are well-known mutagenic compounds and might induce mutations, the most resistant of which would be selected under drug pressure. Taking into account the high basic mutation rate in trypanosomes, which is estimated at 10^{-9} per base pair per cell generation in *T. brucei*, the effects of this phenomenon should not be underestimated. Finally, the phenomenon of cross-resistance has been well established. For instance, quinapyramine usage has been shown to induce resistance to isometamidium, homidium and diminazene (Ndoutamia *et al.*, 1993).

Mechanisms and genetics of resistance to trypanocides

Isometamidium

The trypanosome kinetoplast is the primary site of isometamidium accumulation. The main mode of action of the drug is the cleavage of kDNA–topoisomerase complexes. The mechanism of resistance to isometamidium is less clear. Several workers have shown that accumulation of isometamidium is significantly lower in resistant populations than in sensitive ones. It remains to be shown whether this is due to a decreased number of protein transporters of isometamidium in the plasma membrane or to changes in the balance between influx and efflux. The role of nucleoside transporters in resistance to isometamidium by *Trypanosoma congolense* remains to be examined but changes in these transporters have been associated with resistance to arsenical drugs in *T. brucei*. Changes in mitochondrial electrical potential have also been demonstrated in isometamidium-resistant *T. congolense*.

Homidium salts

Homidium chloride and especially homidium bromide or ethidium are still widely used as trypanocidal drugs, though they are known mutagenic compounds. Their mechanism of action is not well understood but it has been shown that the drugs interfere with glycosomal functions, the function of an unusual AMP-binding protein, trypanothione metabolism and the replication of kinetoplast minicircles. The mechanism of resistance by trypanosomes to these drugs is unknown but there are indications that it is similar to that described for isometamidium.

Diminazene

Diminazene probably exerts its action at the level of the kinetoplast DNA but other targets cannot be excluded. As for other trypanocides, the molecular basis of resistance to diminazene in trypanosomes is not clear. The accumulation of diminazene has been shown to be markedly reduced in arsenical-resistant *T. b. brucei* due to alterations in the nucleoside transporter system (P2). However, there might be other resistance mechanisms.

The genetic stability of drug resistance remains uncertain but recent reports from Ethiopia based on cloned populations have shown that drug-resistance phenotypes had not altered over several years.

In conclusion, it is clear that much more work is required in order to elucidate the mechanism of resistance to the three currently available trypanocidal drugs. The same is true for the genetics of drug resistance in trypanosomes. There are strong indications that several genes are involved in resistance to isometamidium. However, the mono- or polygenic nature of drug resistance, its stability over time, and the dominance or recessiveness of the gene(s) involved need to be further examined, because of the implications for the effective control of drug resistance.

Pathogenicity of drug-resistant parasites and the impact on livestock productivity

Several authors have observed a loss of virulence and/or a loss of fitness in drug-resistant trypanosomes but this has not been a consistent finding from the limited number of studies that have been conducted. The loss of fitness in other drug-resistant parasites is a well-known phenomenon and is probably also present in trypanosomes. Well-designed experiments in trypanosome-naive definitive hosts using significant numbers of resistant and sensitive isolates should provide valuable data on this controversial but important topic.

There have been few studies to assess accurately the impact of drug-resistant trypanosomes on livestock productivity but it is generally assumed that uncontrolled infections will have a severe impact on both survival and productivity. A useful study to assess the impact of drug-resistant trypanosomes on the productivity of the local cattle was carried out in the Ghibe valley, Ethiopia, by scientists from the International Livestock Research Institute (ILRI) in the early 1990s and a high prevalence of multiple drug resistance was reported. The study showed that profitable cattle production was possible in a problem area with high prevalence of drug-resistant *T. congolense* and cattle production was able to generate attractive economic returns for herd owners (Itty *et al.*, 1995). Similar studies should be carried out in other regions with different host genotypes and under different management conditions.

Detection of drug resistance

Three types of techniques are commonly used to identify drug resistance: tests in ruminants, tests in mice and *in vitro* assays. Standardized protocols for the tests in animals have been developed, which should allow better comparisons of data on a temporal and spatial basis (Eisler *et al.*, 2001), but none of these tests is ideal. Other tests are still in the phase of development or validation. The advantages and disadvantages of each of the different techniques are briefly summarized below.

Tests in ruminants

These tests provide direct information from studies in ruminants using recommended doses of trypanocide. The tests commonly consist of infecting a group of cattle or small ruminants with the isolate under investigation and later, when they are parasitaemic, treating them with various dosages of trypanocide. It is preferable to use at least three animals in each group, because it has been shown that results obtained after inoculation and treatment of one animal are not always reliable. The animals are regularly monitored over a period of 100 days to determine the efficacy of standard drug doses in terms of their ability to provide a permanent cure. For these studies the cattle or small ruminants must be kept in fly-proof accommodation or in a non-tsetse area in order to eliminate the risk of reinfection during the study (Table 23.3). A variation on this technique is to inoculate blood from several different infected cattle into a single recipient calf. This technique is useful in situations where laboratory facilities are very limited but it only allows a qualitative assessment and does not indicate how many of the isolates inoculated into a single calf were resistant. Further constraints to this technique are that not all populations grow equally well and that sensitive isolates might overgrow resistant ones when inoculated together.

Table 23.3. Standardized protocols for testing trypanocidal drug resistance in mice and cattle (Eisler *et al.*, 2001).

	Single-dose test in mice[a]	Multi-dose test in mice[a]	Test in calves[b]
Number of groups of animals			
Treatment groups per drug	1	5	1
Control groups	1	1	not necessary
Number of animals per group	6	6	3 to 6
Inoculum			
Number of trypanosomes	10^5	10^5	10^5
Route of administration	i.p.	i.p.	i.v.
Drug dosages (mg/kg bw)			
Isometamidium chloride	1	0.01, 0.1, 0.5, 3.0, 20	0.5
Diminazene aceturate	20	1.0, 3.0, 10, 20, 60	3.5
Homidium bromide/chloride	ND	ND	1
Drug administration			
Time	24 h post inoculation	24 h post inoculation	1st peak parasitaemia
Route	i.p.	i.p.	i.m.
Parasitological examination			
Method	Tail blood wet smear	Tail blood wet smear	Buffy coat
Frequency	2 ×/week[d]	2 ×/week[d]	3 ×/week
Duration of follow-up	60 days	60 days	100 days
Interpretation of results for treated animals			
Isolate sensitive	At least 5/6 cured[e]	Probit or logit analysis of number of mice cured at each dose	3/3 cured
Isolate resistant[c]	Less than 5/6 cured		Less than 3/3 cured

[a]Mice should be weighed to an accuracy of 1 g and body weights (bw) should not vary by more than 10%; strain, sex and age of mice should be recorded.
[b]Calves must be kept in a fly-proof stable or other environment non-endemic for trypanosomosis.
[c]Resistance of individual stabilates in mice should not be extrapolated to cattle.
[d]When *T. brucei* are being tested, the frequency of parasitological monitoring should be increased to three times a week for the first 2 weeks.
[e]At least five out of six control mice must become parasitaemic; if not, the test must be repeated.
ND: not yet determined; i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous.

Tests in mice

Tests in mice can be used as a single-dose test or as a multi-dose test. In the latter case the objective is to obtain more detailed information by determining the CD_{50} or CD_{80} values (curative dose that gives complete cure in 50% or 80% of the animals) for a given trypanocidal drug. In the case of a single-dose test, a large number of trypanosome isolates is tested at a single discriminatory dosage – 1 mg/kg for isometamidium and 20 mg/kg for diminazene (Eisler *et al.*, 2001) – with the objective of characterizing the geographical area of origin of the isolates in terms of the extent of drug resistance, rather than in-depth characterization of individual isolates. The details of both tests are presented in Table 23.3. The advantage of the mouse assay is that it is cheaper than the test in cattle. There are several disadvantages, however. Firstly, most *T. vivax* isolates, and also some *T. congolense* isolates, do not grow in mice. Secondly, although there is reasonable correlation between drug sensitivity data in mice and in cattle, higher doses of drug must be used in mice in order to obtain results comparable to those from cattle because of the vast difference in metabolic size. The results in mice cannot be directly extrapolated to calculate the curative dose to be

used in cattle. Thirdly, precise assessment of the degree of resistance requires a large number of mice per isolate. This makes it a rather labour-intensive test. Finally, it takes as long as 60 days to evaluate the drug sensitivity of an isolate.

In vitro *assays*

For the *in vitro* evaluation of drug sensitivity procyclic, metacyclic or bloodstream forms of trypanosomes can be used. The advantage of *in vitro* assays is that large numbers of isolates can be examined. However, there are several disadvantages. The use of metacyclic and bloodstream forms is considered more reliable than the use of procyclic forms. Tests with metacyclic trypanosomes correlate well with field observations, but it may take up to 40 to 50 days of *in vitro* incubation to generate metacyclic trypanosomes. *In vitro* cultivation of bloodstream forms is only possible using preadapted lines and not using isolates directly from naturally infected animals. A simplified axenic culture system has been developed, but further research is still necessary to study the correlation with field data. A potential problem associated with this lengthy time of adaptation is the possible selection against trypanosomes that have the phenotype of the original population. *In vitro* assays are expensive to perform and require good laboratory facilities and well-trained staff. In contrast to *T. brucei*, it is very difficult to cultivate *T. congolense*. If techniques can be improved to adapt trypanosome isolates to grow *in vitro* more rapidly, these assays may become more popular, especially in those laboratories where culture facilities are already established. An interesting alternative is the drug incubation *Glossina* infectivity test (DIGIT), in which the trypanosomes are exposed to the drug *in vitro* for a short time and thereafter fed to tsetse flies to check whether or not they develop into metacyclic forms (Clausen *et al.*, 1999). This technique distinguishes resistant from sensitive isolates and does not require experimental animals, but it does require a ready supply of teneral tsetse flies from an artificially reared colony.

Trypanocidal drug-ELISAs

As an alternative to the tests mentioned above, the use of trypanocidal drug-ELISAs in combination with parasite detection tests has given promising results for the detection of resistant trypanosomes. A competitive ELISA allowing the detection of small amounts of isometamidium in serum of cattle (Eisler *et al.*, 1996) has been validated in cattle under experimental and field conditions. The test is both sensitive, detecting subnanogramme concentrations and specific. It allows the monitoring of drug levels over extended periods and the evaluation of factors influencing drug disappearance rates from the plasma.

The available data indicate that there is a considerable individual variation after i.m. injection of isometamidium in cattle. One interesting finding has been that the drug disappears more rapidly in animals challenged and becoming infected with drug-resistant trypanosome isolates than in those challenged but protected against infection with sensitive trypanosomes. Observations showed that the presence of trypanosomes in animals with an isometamidium concentration of > 0.4 ng/ml suggests resistance; the higher the drug level detected the greater the degree of resistance that could be inferred. Further research is necessary, however, in order to confirm these results in a larger number of animals. Similar drug-ELISAs have been developed for the detection of subnanogramme amounts of homidium bromide and diminazene.

Block treatment studies and longitudinal parasitological data

Analysis of data on the frequency of infections after block prophylactic drug treatment can give clear indications of drug resistance but comparisons with untreated cattle in the same environment are required in order to confirm the level of challenge and efficacy of the drug treatment. Alternatively, longitudinal parasitological data may be used to compare trypanosome incidence and prevalence in herds under a therapeutic drug regimen, using PCV to distinguish new from recurrent infections.

Potential new tests for the detection of resistance

All of the available tests for drug resistance have significant disadvantages and none is ideal. There is an urgent need to develop cheap reliable tests that can be used easily in developing countries. None of the recent new tests have fulfilled these essential criteria but some show potential.

For example, it has been known that the rate of isometamidium accumulation in *T. congolense* is a good indicator of the degree of drug resistance and since the mitochondrial electrical potential appears to be closely linked with the rate of drug uptake, it might be possible in the near future to develop a quantitative *in vitro* test based on these findings. If such a test could be carried out using a small number of trypanosomes directly on whole blood samples, it might provide a rapid indication of the level of resistance of a given trypanosome isolate. Some progress has also been made in identifying genetic markers for resistance. These might be developed into reagents for the identification of resistant trypanosomes using PCR in the future.

Guidelines to Delay the Development of Drug Resistance

Until now the most important guidelines to avoid or to delay the development of drug resistance were considered to be to use of the 'sanative' pair of drugs (isometamidium or ethidium and diminazene) and to avoid the exposure of trypanosomes to subtherapeutic drug concentrations (Boyt, 1986). It is clear, however, that the application of these guidelines may not be sufficient to maintain the efficacy of the existing drugs, especially since any recommendation is lacking concerning a reduction of the treatment frequency.

Based on current knowledge in the field of trypanocide resistance and on experience in the control of resistance to insecticides, anthelmintics, antibiotics and other drugs the following recommendations are proposed in order to delay the development of resistance.

Reduction of the number of treatments by integrating drug usage with other control measures

It is widely agreed that the most efficient way to delay the development of drug resistance remains the reduction of selection pressure by the drugs, i.e. decrease the number of treatments. This is of particular importance in areas of high tsetse challenge, which are commonly associated with reduced periods of chemoprophylaxis (Whiteside, 1962). It is therefore strongly recommended that control of trypanosomiasis should not rely solely on drugs and an integrated approach should be adopted using vector control, to reduce the tsetse challenge, along with reduced frequency of drug dosing. Where such measures have been adopted the results have been impressive (Fox *et al.*, 1993; Peregrine *et al.*, 1994). In situations in West and Central Africa the use of trypanotolerant livestock and drugs may be appropriate in areas of high tsetse challenge.

Use of the correct dose

Underdosing is one of the major causes of resistance development. Subtherapeutic drug concentrations exert a strong selective pressure for the emergence of resistant clones that pre-exist in the trypanosome population. Unfortunately, underdosing occurs very frequently. Given the fact that in many countries drugs are often administered by unskilled persons, errors may easily occur in calculating the correct doses for the treatment of the animals. Farmers have the tendency to underestimate the weight of their animals when they have to treat them. Furthermore there is an increasing number of generic products available on a somewhat loosely regulated market, and some of these have questionable efficacy and many contain lower doses of drug than the stated amount. This has now been shown to be the case for many drugs for veterinary and human use as well as trypanocides.

Avoiding exposure of the whole parasite population to a drug

In contrast to the approach to human sleeping sickness, mass treatments are commonly used to control animal trypanosomiasis and can be highly successful over many years for example in ranch cattle (Trail *et al.*, 1985). However, this form of treatment exerts a strong selection pressure on the trypanosome population. The higher the proportion of the trypanosome population exposed to the drug and the lower the proportion *in refugia* (e.g. the trypanosomes present in the fly population or in other hosts), the higher the selection pressure. The percentage of the total parasite population, which is exposed to the drug at the time of treatment, might thus have an impact on resistance development. Therefore in well-monitored situations there is a strong case for limiting treatment to individual clinical cases; this is also desirable on grounds of minimizing drug residues, avoiding potential toxicity and reducing costs. In such situations drug resistance problems can be minimized and acquired immunity encouraged (Scott and Pegram, 1974).

Banning the use of quinapyramine in cattle

Quinapyramine was widely used in cattle in Africa during the period 1950–1970. In 1976 it was withdrawn from sale for cattle use because of problems with toxicity and resistance development. However, it is still available for use in camels and it is likely that it is still used in cattle in some situations in Africa where both species exist in proximity to the margins of tsetse belts. The use of quinapyramine was the suggested cause of the multiple drug resistance problem in the Ghibe Valley of Ethiopia referred to earlier. Ndoutamia *et al.* (1993) showed that after artificial induction of resistance to quinapyramine in *T. congolense*, multiple resistance to isometamidium, homidium and diminazene was expressed at the level of the individual trypanosome and could be transmitted by tsetse flies. This confirms the results of Whiteside's (1962) earlier field studies. Therefore, the use of quinapyramine as a trypanocide in cattle is completely contraindicated.

Guidelines for Action when Drug Resistance is Detected

Single drug resistance

Based on the examination of a representative number of trypanosome isolates from a given area, using tests in mice or in ruminants (see earlier section), the frequency of expression of resistance to a single trypanocidal drug can be calculated. The following guidelines for action (Table 23.4) based on the frequency of expression of trypanocidal drug resistance were proposed following a meeting of international experts in Nairobi in 1999 under the auspices of the EU Concerted Action programme entitled Integrated Control of Pathogenic Trypanosomes and their Vectors.

Multiple drug resistance

Resistance to both isometamidium and diminazene, if present at the level of individual trypanosomes, may be demonstrated by testing cloned populations in mice or in ruminants. If resistance to both isometamidium and diminazene is present at the level of individual trypanosomes similar guidelines to those recommended for cases of $> 60\%$ frequency of drug resistance should be followed (Table 23.4).

Recommendations on the use of isometamidium prophylaxis

Classically, regular prophylactic treatment with isometamidium has been advocated as a means of prevention of bovine trypanosomiasis. However, recent concerns about the development of drug resistance have led to the recommendation that the number of treatments should be minimized to reduce the exposure of the parasite population to the drug. In view of these considerations, the following guidelines on the use of isometamidium prophylaxis are proposed.

Table 23.4. Guidelines for action based on frequency of drug resistance.

Frequency of drug resistance in trypanosome infections of cattle	Guidelines for action
Absent	• Use 'sanative pairs' • Minimize use of routine block treatments • Minimize drug use by vector control and/or by decreasing vector–host contacts
1–30%	• Use 'sanative pairs' • Target trypanocide treatment on clinical cases • Investigate drug usage patterns • Introduce vector control (targets, traps and/or bait techniques) • Monitor situation over a wider area and over time
31–60%	• Use 'sanative pairs' and monitor the efficacy of treatment • Strengthen other aspects of integrated disease control to minimize impact of intercurrent infections with other pathogens • Intensify vector control • Improve management/nutrition • Introduce trypanotolerant genotypes if feasible
> 60%	• Restrict use of trypanocides to clinical cases • Use zero grazing and/or fly-proof housing where appropriate, e.g. in smallholder dairy systems • Intensify vector control • Consider change from cattle to other types of livestock

- Avoid the use of continuous isometamidium prophylaxis and minimize the frequency of routine isometamidium block treatments.
- Consider the use of prophylaxis only in cattle exposed to heavy challenge for a defined period, e.g. transhumance or high seasonal challenge.
- Never administer isometamidium more frequently than every 3 months.

When routine block treatment with isometamidium is practised it is recommended that, once a year, the animals are separately treated with diminazene in order to delay the development of drug resistance following the concept of the 'santative pair' (Whiteside, 1962).

Quality Control of Trypanocidal Drugs

The increasing availability of generic trypanocides in Africa has created new problems over the quality assurance of these products. Recent pilot studies have indicated wide discrepancies in the quality of many of the products currently being sold to livestock owners.

Whilst some products are plainly fraudulent and possess no trypanocidal activity, the most common problem is variability in the content of active ingredient. This is partly caused by the lack of agreed specifications for these products but also reflects differences in batch quality and poor product control in the manufacturing or packaging plant. Whilst some products contain more than the stated quantity of active ingredient, many others contain less than that stated and it is these latter products that could lead to serious underdosing of livestock and both inefficacy of treatment and the resultant enhanced risk of the development of drug resistance. There is an urgent need to establish regional testing laboratories in Africa for trypanocides and other veterinary pharmaceutical products. These should be supported by international laboratories, which can provide back-up standardization and verification facilities.

Conclusions

Since it is unlikely that new trypanocidal drugs for treating animal trypanosomiasis will be released on to the market in the near future, it is essential to try to maintain the efficacy and supply of the currently available drugs. The most important and most efficient measure to achieve this is to adopt an integrated disease management strategy so that trypanocides are used within the broader context of trypanosomiasis control. In many respects the current privatization of veterinary services is mitigating against this policy but if more area-wide tsetse control is successfully implemented under the Pan-African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC), which was launched in 2001, there is an opportunity for a more managed level of integrated control of animal trypanosomiasis to emerge.

In the meantime farmers should be supported in their use of trypanocides and measures adopted that assist this process. These include the provision of better data on the true prevalence of trypanocidal resistance and its impact on the productivity of livestock. In order to address the issue of true prevalence and to allow a reliable comparison of the data on a temporal and spatial basis, it is of crucial importance that tests for drug resistance are carried out across Africa according to standardized protocols as described in this chapter and elsewhere (Eisler *et al.*, 2001). These methods should be promoted as routine monitoring tools in all areas where trypanocidal drugs are used.

In view of the increased trafficking in trypanocides in some regions of Africa, the proliferation of generic products and the pilot studies, which have identified substandard products, there is a need for drug quality control measures to be introduced along with an effective licensing system for trypanocides. Regional laboratories should be established which fulfil the criteria of technical competence, impartiality and acceptability to all stakeholders. Dialogue with the pharmaceutical industry should be stimulated as part of the solution to the problem of quality assurance, since the safeguarding of the long-term efficacy of products is of importance to the industry as well as to the users.

The frequent observation that curative trypanocides are administered without an accurate diagnosis of trypanosomiasis being made prior to treatment highlights the lack of practical and affordable field tests for trypanosomiasis. There is an urgent need to assist livestock owners in this regard and thereby reduce the inappropriate use of trypanocides.

Other priorities for future research include studies on the population dynamics of drug-sensitive and drug-resistant trypanosomes and determination of the risk factors that bring about drug resistance. There is also a need for more research on the genetic basis of drug resistance both to facilitate the development of markers for drug resistance and to model the development and spread of drug resistance in trypanosome populations. The relationship between the level of tsetse challenge and the rate of emergence of drug resistance in trypanosome populations requires further examination. It is also important to determine the extent to which control of tsetse can be used to limit the rate of development and spread of drug-resistant trypanosomes in the future.

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24 Future Prospects in Chemotherapy for Trypanosomiasis

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Introduction

Chemotherapy remains the principal means of controlling the African trypanosomiases, but current drugs are not satisfactory. For Chagas disease, adequate therapy has never been available. New drugs are urgently required for these diseases.

In this review, the difficulties in treating trypanosomiasis and problems with current drugs are discussed. It is hoped that recent discoveries on parasite biology, including drug target identification and evaluation, and chemical synthesis of candidate inhibitors will facilitate novel drug development. However, pharmacological criteria related to the biology of the host–parasite interface remain obstacles that must be taken into consideration. The problems they present are not insurmountable, but socio-economic barriers that stand between novel ideas and useful therapies temper optimism that new drugs will emerge from technological advances.

Current Drugs

Drugs used against the trypanosomiases

Four drugs are licensed for use against human African trypanosomiasis (Pépin and Milord, 1994; Keiser *et al.*, 2001). Two of these, pentamidine and suramin, are used prior to central nervous system involvement for the diseases caused by *Trypanosoma brucei gambiense* and *T. b. rhodesiense*, respectively. Against late-stage disease, the major drug of use is melarsoprol, though eflornithine (DFMO) is also useful against *T. b. gambiense*. Berenil (diminazene) has been used without licence and nifurtimox has been shown to have limited efficacy against late-stage melarsoprol-refractory disease.

Chemotherapy of animal trypanosomiasis (Geerts and Holmes, 1998) depends on isometamidium, homidium and diminazene, whilst cymelarsan is used against *Trypanosoma evansi* in camels. Suramin remains in use for some animal trypanosomiases but quinapyramine was discontinued due to its capacity to induce multi-drug resistance.

Chagas disease therapy is limited to two nitroheterocyclic compounds: benznidazole and nifurtimox (Urbina, 1999). Neither is effective against chronic forms of the disease, efficacy against acute forms is limited and both are associated with significant toxicity against the host.

Problems with currently used trypanocides

Toxicity

Most trypanocidal drugs were introduced prior to stringent safety controls required for registration and most would not receive

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licences today (Barrett, 2000). Most of the currently used drugs induce toxic side effects. Melarsoprol, for example, causes many side effects, including reactive encephalopathy in 5–10% of patients receiving therapy (Pépin and Milord, 1994).

More detailed studies are now being performed on some trypanocides as these reagents enter trials against other conditions. For example, the induced hypoglycaemia associated with pentamidine's administration may relate to its ability to bind to pancreatic imidazoline receptors. The pharmacological basis for many of suramin's side effects is also becoming clearer following studies on the drug's anti-cancer activity. Nifurtimox and benznidazole are genotoxic in Ames' tests and thus have carcinogenic potential. Moreover, nifurtimox has been associated with neurological sequelae. Side effects have been reported for all of the drugs used in animal trypanosomiasis (Geerts and Holmes, 1998).

Drug resistance

Resistance to the animal trypanocides, isometamidium and homidium, is widespread (Geerts and Holmes, 1998; Geerts *et al.*, 2001). Suramin resistance has also been reported in many loci. In the treatment of human African trypanosomiasis, the current epidemics in sub-Saharan Africa have been characterized by a higher than usual incidence of relapse following treatment with melarsoprol (Legros *et al.*, 1999). It is probable that treatment failure relates to drug-resistant parasites. Cross-resistance between melamine-based arsenicals and diamidines is relatively easy to select in the laboratory (Barrett and Fairlamb, 1999). Under these circumstances the transporter that carries these drugs into the cells is altered (Carter and Fairlamb, 1993; Mäser *et al.*, 1999), giving rise to diminished drug uptake. Cross-resistance of human infectious parasites resistant to melarsoprol and its metabolites may therefore, in principle, arise when resistance to berenil is selected in livestock (Barrett, 2001).

Manufacture and supply

Manufacturers of drugs do not consider treatment of the trypanosomiases to offer profitable potential (Veeken and Pecoul, 2000) and thus investment in new drugs against these diseases is low. Moreover, manufacture and supply of the currently licensed drugs is in jeopardy and the World Health Organization has been engaged in a long campaign to retain supplies of essential drugs (Barrett, 2000).

In 2001, the pharmaceutical company Aventis guaranteed production of several trypanocides (eflornithine, melarsoprol and pentamidine) for at least 5 years. However, it is imperative that the situation is kept under review and that means are found to engage the pharmaceutical industry in the search for new drugs and to guarantee the production of existing ones.

Drugs used in animal disease are generally subject to lower standards of quality control than those used in human disease. Multiple generic variants of the drugs, including isometamidium and diminazene, are available and the variability in quality of these products is striking. The release of preparations that contain low quantities of active drug engenders ideal conditions for the selection of drug resistance as well as leading directly to therapeutic failure.

Even where a supply of the drugs is guaranteed, it is frequently difficult to distribute them to areas where the diseases that they treat are endemic. This is because the infrastructure of most of the affected countries cannot currently sustain the distribution and administration of the drugs. Problems relate to the lack of roads or other transport routes, lack of public health services to mobilize patients, and lack of personnel to administer the drugs. Moreover, events related to frequent military insurgencies can actively block drug distribution.

Pharmacokinetics and difficulties in drug administration

The biology of both African and South American trypanosomes leads to particular pharmacokinetic difficulties in drug administration (Croft, 1999). Human African trypanosomiasis is seldom diagnosed prior to the late stage, when parasites are manifest in the cerebrospinal fluid and so drugs must cross the blood–brain barrier to be effective.

Neither suramin nor pentamidine reach therapeutic levels in the cerebrospinal fluid and so their use is confined to treating early-stage disease. Melarsoprol, or its active metabolites, does reach therapeutic levels in the cerebrospinal fluid but only a relatively small proportion of the total injected dose crosses the barrier (Keiser *et al.*, 2000). Crossing the blood–brain barrier is not an issue with drugs used against *Trypanosoma congolense* or *Trypanosoma vivax*, since the central nervous system is not involved in these infections.

It is a problem for drugs to reach *Trypanosoma cruzi*, which replicates within the cytosol of various mammalian cell types. A drug aimed at proliferative parasites must also enter many mammalian cell types, which diminishes the quantity of drug available to interact with the parasites as well as increasing risks of host cell toxicity.

The failure of most trypanocidal drugs to cross the intestinal epithelia is also problematic as it precludes the preferred oral route of administration for these compounds. Syringes and needles add to the cost of treatment and the reuse of needles enhances the risk of transmitting other blood-borne diseases, including malaria and AIDS.

Prior to making a pharmacological assessment on how to overcome these difficulties, it is critical to pinpoint targets within the parasite against which drugs might act, and to identify lead compounds that can be directed at these targets. A significant proportion of research into trypanosomes over the last 20 years has been related to target identification and development of lead compounds that interact with these targets. It is now crucial that action is taken to ensure that some of the lead compounds are developed to produce new, clinically relevant drugs.

Modes of Action and Drug-resistance Mechanisms

Drug targets

Targets identified through comparative physiology and comparative genomics

Trypanosomes apparently diverged early in the eukaryotic lineage and their biochemical constitution differs in many respects from that of mammalian cells. Pathways common to trypanosomatids but absent from mammalian hosts have long been considered to be interesting, since they may provide selective drug targets (Wang, 1995). Among these, the enzymes involved in biosynthesis and metabolism of the low-molecular-weight thiol, trypanothione, have attracted particular attention. Other pathways and enzymes occur in both trypanosomes and mammals and yet have been proved to be good drug targets, such as ornithine decarboxylase, the target of eflornithine. Other enzymes, such as 6-phosphogluconate dehydrogenase, are related to the enzymes of prokaryotic or plastid-type lineages, indicating that they have a prokaryotic origin. Accordingly they reveal major structural differences when compared with counterparts in mammalian cells, which suggest that they may be good drug targets.

A natural progression from comparative biochemistry has been to proceed to trypanosomatid genome-sequencing projects to identify new drug targets (Degrave *et al.*, 2001). The *T. brucei* genome project is anticipated to be complete in 2004 and that of *T. cruzi* soon after. The genomes of key animal pathogens (*T. congolense* and *T. vivax*) will also be sequenced in due course.

Since the human genome project has been completed, it will be possible to make a direct comparison of the entire predicted proteome of this host species with that of the trypanosomes that they harbour. It is likely that this comparative genomics will yield surprising discoveries and a plethora of unexpected potential novel targets for chemotherapy.

Target validation and analysis of targets

A wide range of genetic manipulation methods are now available for analysis of gene function in *T. brucei* and *T. cruzi* (Clayton, 1999). These include episomal expression vectors, gene-targeted disruption using homologous recombination with antibiotic resistance genes and inducible gene expression. These techniques can be used to determine whether a gene (and hence its encoded product) is essential, or not, and thus a credible target for inhibitory drugs (Barrett *et al.*,

1999). The discovery that *T. brucei* contains the enzymatic machinery required for gene silencing through RNA interference (RNAi) in response to the production of double-stranded RNA has further advanced the field of target validation (Ngo *et al.*, 1998). RNAi causes dominant interference with gene expression, similar to a gene knockout but frequently resulting in incomplete removal of the RNA and hence the process is termed gene knockdown. This is particularly useful for target validation of large numbers of gene products and can potentially be used for genome-wide analyses.

Drug targets can also be validated using the more classical approaches, such as specific inhibitors or analysing gene changes that result in drug resistance.

Once a target has been validated genetically, the next step, frequently, is the large-scale production of recombinant protein for drug screening studies. *Escherichia coli*, baculovirus vectors for insect cells and the yeast *Pichia* are commonly used to produce target proteins in large quantities. Chemical libraries can be rapidly screened against targets provided these assay systems exist. The advent of combinatorial chemistry techniques means that new agents can be produced and tested against validated drug targets on a scale hitherto unknown. Candidate inhibitors can also be obtained through structural analyses of the proteins using X-ray crystallography and modelling inhibitor–enzyme interactions *in silico* to select compounds from chemical databases (Hunter, 1997). Thus the discovery of inhibitors of validated targets is now a relatively rapid process. However, the process of developing these lead compounds into clinically useful compounds may still take many years and a great deal of expense and can only realistically be achieved in conjunction with a pharmaceutical company.

Potential targets, validated targets and novel trypanocidal reagents

Glucose metabolism and the glycosome

African trypanosomes in the mammalian bloodstream are absolutely dependent upon glucose to generate ATP (Opperdoes, 1987). Glucose catabolism ends at pyruvate which is released from the parasite. A functional Krebs cycle and cytochrome-containing electron transport chain are absent and only two moles of ATP are produced per mole of glucose consumed. The enzymes of the glycolytic scheme from glucose to 3-phosphoglycerate are located within a specialized microbody-like organelle termed the glycosome (Fig. 24.1). Structures of the glycolytic enzymes are correspondingly unusual and allosteric regulation of key enzymes differs between mammalian and trypanosomatid glycolysis. Inhibitors that exploit the unusual structural and functional attributes of trypanosomatid glycolytic enzymes are being pursued (Verlinde *et al.*, 2001).

By excreting pyruvate rather than reducing it to lactate or passing it into the Krebs cycle bloodstream forms of *T. brucei* need an alternative system to reoxidize the NADH produced in glycolysis. A shuttle carrying dihydroxyacetone phosphate and glycerol 3-phosphate between the mitochondrion and glycosome performs this task. Oxygen acts as a final electron acceptor in a system involving ubiquinone, cytochrome Q and the trypanosome alternative oxidase (Tielens and Van Hellemond, 1999). Salicyl hydroxamic acid (SHAM), an inhibitor of the alternative oxidase, alone or in combination with high concentrations of glycerol, has trypanocidal activity. Other inhibitors of the alternative oxidase, such as ascofuranone, are active *in vitro* and future improved inhibitors could be good drugs.

Glycolysis is also important to *T. cruzi*, although amastigotes carry out much more extensive oxidation of glucose than does bloodstream-form *T. brucei*, being rather more akin to the procylcic form of the parasite found in the tsetse fly midgut (Tielens and Van Hellemond, 1999). They also possess enzymes of the Krebs cycle and an associated cytochrome-containing electron transport chain and thus can use other substrates for respiration.

The pentose phosphate shunt is operative to some extent in both trypanosomes (Barrett, 1997). This pathway is the principal source of NADPH for reductive biosynthesis and protection against oxidant stress (Fig. 24.2). The

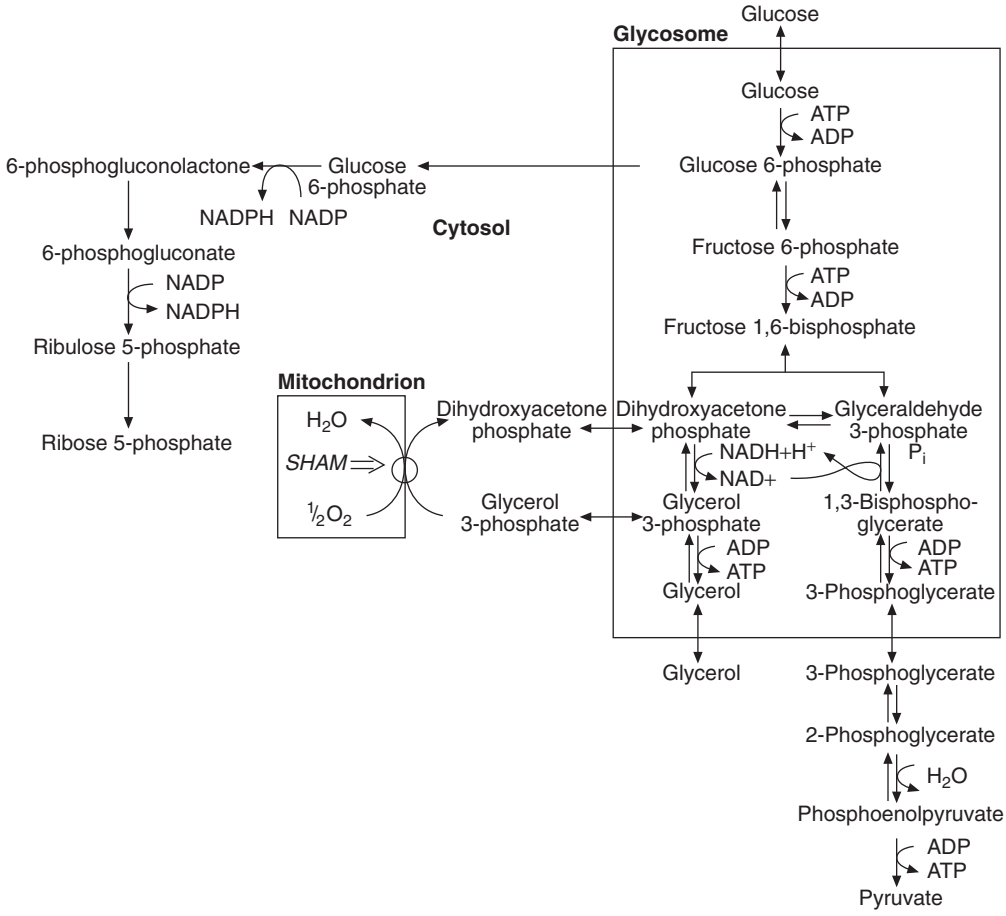

Fig. 24.1. Glucose metabolism in bloodstream-form *Trypanosoma brucei*. Bloodstream form *T. brucei* are totally dependent upon the metabolism of glucose via the glycolytic pathway for the provision of ATP. Many of the enzymes of glycolysis are localized within a microbody-like organelle termed the glycosome. A glycerol 3-phosphate:dihydroxyacetone phosphate shuttle involving the mitochondrion and a plant-like alternative oxidase is critical in regenerating NAD^+ to sustain glycolysis. A second pathway of glucose metabolism, the oxidative pentose phosphate pathway, is also operative in these cells. Although here it is shown only in the cytosol, the pentose phosphate pathway may be localized to both the cytosol and the glycosome.

pathway also provides ribose 5-phosphate and other phosphorylated carbohydrate intermediates. The enzyme 6-phosphogluconate dehydrogenase of this scheme has been proposed as a good candidate for chemotherapeutic attack. Inhibition of this enzyme leads to the accumulation of its substrate, 6-phosphogluconate, which is toxic to eukaryotic cells, possibly since it inhibits phosphoglucose isomerase and thus glycolysis. The 6-phosphogluconate dehydrogenase of *T. brucei* is structurally and pharmacologically distinct from its mammalian counterpart. This may be because the enzyme in *T. brucei* appears to be encoded by a gene with cyanobacterial ancestry, suggesting that it is a remnant of an early symbiotic event possibly involving a photosynthetic organism that was secondarily lost.

Computer modelling of flux through the pathway (Bakker *et al.*, 2000) has been useful in assessing the types of inhibitor that would be best in diminishing the glycolytic flux to levels that would be lethal to the trypanosomes. Because levels of phosphorylated

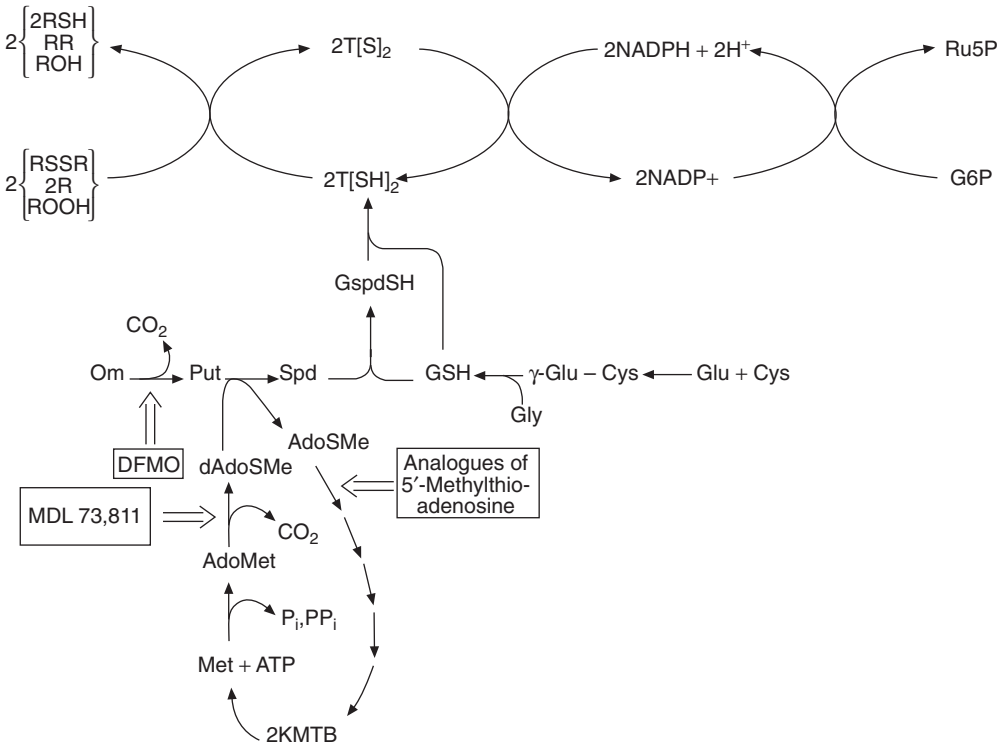

Fig. 24.2. Polyamine metabolism and trypanothione biosynthesis in trypanosomes. Trypanothione ($T[SH]_2$) is a unique low-molecular-weight thiol found in trypanosomatids. It plays many roles in maintaining cellular redox balance and protecting against oxidant stress (as indicated in the top left part of the figure). NADPH, provided by the oxidative branch of the pentose phosphate pathway (the steps between glucose 6-phosphate (G6P) and ribulose 5-phosphate (Ru5P), shown in the top right-hand part of the figure) is the primary reductant used in this system. Trypanothione comprises two molecules of glutathione (GSH) joined by the polyamine spermidine (Spd). The synthesis of glutathione involves the linking of glutamate (Glu) with cysteine (Cys) and glycine (Gly). Trypanothione synthesis then proceeds in two steps via glutathionyl spermidine (GspdSH). The polyamine biosynthetic pathway, in which ornithine (Orn) is converted to putrescine (Put) and then spermidine, is a target for chemotherapy. DFMO inhibits ornithine decarboxylase that converts ornithine to putrescine. Polyamine biosynthesis also depends upon the cycling of methionine (Met) to *S*-adenosylmethionine (AdoMet) which is decarboxylated by *S*-adenosylmethionine decarboxylase (which is inhibited by MDL 73,811). Decarboxylated AdoMet provides amino-propyl groups involved in polyamine synthesis. The 5-methylthioadenosine (AdoSMe) produced from this reaction is then recycled into methionine via a number of enzymatic steps. The final step in this cycling involves the transamination of 2-keto-4-methylbutyrate (2KMTB) using aromatic amino acid transaminases.

carbohydrate intermediates of the pathway are high, inhibitors that are competitive with these substrates must have substantially lower inhibition constants than the substrate K_m values. Transition-state analogues frequently bind enzymes with extremely high affinity, and irreversible inhibitors or those affecting coenzyme binding could also be effective in reducing the glycolytic flux by inhibiting glycolytic enzymes.

Conservation of active sites in glycolytic enzymes of mammals and trypanosomes that otherwise differ substantially in structure could make it difficult to design inhibitors that specifically inhibit the parasite enzymes. However, other parts of the enzymes can be targeted, as demonstrated in the case of glyceraldehyde-3-phosphate dehydrogenase, where potent selective inhibitors of the trypanosomal enzyme were

shown to have activity against both *T. brucei* and *T. cruzi* but not mammalian cells (Aronov *et al.*, 1999). The compounds were directed to the NAD^+ cofactor binding site of the parasite enzyme which differs in structure from that of the host version, and they inhibited glycolysis within minutes of administration.

Thiol metabolism and oxidant stress

Under certain circumstances, reactive oxygen species kill trypanosomes (Docampo and Moreno, 1984; Mehlotra, 1996). Defending against oxidant stress is thus vital. NADPH, mainly provided by the pentose phosphate pathway (Barrett, 1997), is crucial since it provides the reducing power to maintain low-molecular-weight thiols (Krauth-Siegel and Coombs, 1999) involved in this protection in a reduced form (Fig. 24.2).

Superoxide is a key intermediate in the breakdown of reactive oxygen species. In mammalian cells, production of superoxide is usually dealt with by superoxide dismutase (SOD), which converts superoxide anions to hydrogen peroxide. Glutathione peroxidase (which utilizes the low-molecular-weight thiol, glutathione, as a reductant), peroxidoxins and catalase detoxify this resultant hydrogen peroxide. Trypanosomes do have SOD but it differs in metal cofactor dependence from its mammalian counterpart, being iron-dependent.

Catalase and glutathione peroxidase are apparently absent from trypanosomatids, where some of the roles that glutathione performs in most eukaryotes are carried out by trypanothione (N^1,N^8-*bis*-glutathionylspermidine), a unique low-molecular-weight thiol that comprises two glutathione molecules linked by spermidine (Fairlamb and Cerami, 1992) (Fig. 24.2).

Trypanothione plays multiple roles, including complexing of xenobiotics and maintaining intracellular thiol redox balance. It also plays a critical role in defending against hydroperoxides through a system involving tryparedoxin, a trypanosomatid equivalent of glutaredoxin. The tryparedoxin system is also crucial to DNA synthesis through ribonucleotide reductase, which reduces ribonucleoside diphosphates to the corresponding deoxynucleotides using trypanothione to provide the reducing equivalents.

Trypanothione is synthesized from glutathione and spermidine in two steps involving glutathionyl-spermidine synthase and trypanothione synthase. These enzymatic activities are considered good drug targets, as mammals do not have isofunctional proteins.

The enzymes involved in trypanothione metabolism, particularly the essential enzyme trypanothione reductase which maintains trypanothione in its reduced form, are considered excellent candidate targets for the rational approach to drug design.

Nifurtimox and benznidazole, the two nitroheterocycles licensed for *T. cruzi*, appear to act through the generation of oxidant or reductive stress. Activity of nifurtimox seems to depend upon a one-electron reduction of the nitro-group which generates a potent free radical that in turn generates reduced oxygen metabolites (such as superoxide, hydroxyl free radical and hydrogen peroxide) believed to cause death of the parasite. The specificity towards the parasite over host cells may relate to the fact that the compounds are more readily reduced by the parasite than the host cells and also because protection against oxidative damage is more restricted in trypanosomes than the host. Nitroimidazoles like benznidazole may act through reductive stress as the reduced nitro-intermediate forms of the compounds interact directly with cellular macromolecules (Docampo and Moreno, 1984).

Pathways that lead to the preferential reduction of these compounds in trypanosomes have been considered, but as yet the specific enzymes responsible for nitroreduction have yet to be identified.

Polyamine metabolism

The only drug for which a mode of action has been identified with any certainty against human African trypanosomiasis is eflornithine (difluoromethylornithine, DFMO) (Bacchi *et al.*, 1980; Müller *et al.*, 2001). The drug is a suicide inhibitor of

ornithine decarboxylase, the first enzyme of the polyamine biosynthetic pathway (Fig. 24.2). *T. b. gambiense* ornithine decarboxylase is turned over far less rapidly than its mammalian counterpart and so the parasite remains deficient in polyamine biosynthesis for prolonged periods after exposure to eflornithine. The drug acts against the form of the disease caused by *T. b. gambiense* but not that caused by *T. b. rhodesiense*. The latter parasite is innately less susceptible to the drug, possibly because it turns over ornithine decarboxylase more rapidly than *T. b. gambiense* and has higher ornithine decarboxylase activity.

Putrescine, the product of the ornithine decarboxylase reaction, is used in the building of the longer-chain polyamines spermidine and spermine and provision of high levels of spermidine can relieve loss of ornithine decarboxylase activity. Decarboxylated *S*-adenosylmethionine acts as the donor of the amino-propyl groups required for synthesis. Some inhibitors of *S*-adenosylmethionine decarboxylase, the key enzyme involved in production of these precursors, such as MDL 73811 (5′-{[(z)-4-amino-2-butenyl]methylamino}-5-deoxyadenosine) and CGP 40215, a bicyclic analogue of methylglyoxal *bis* (guanyl hydrazone) (MGBG), are also toxic to trypanosomes (Keiser *et al.*, 2001). Pharmacokinetic problems associated with getting these compounds into the cerebrospinal fluid have reduced optimism that they will become clinically useful.

T. cruzi is auxotrophic for polyamine biosynthesis, since it lacks ornithine decarboxylase and is thus naturally refractory to the effects of DFMO. However, it does require an exogenous source of polyamines, which it scavenges effectively from its host, and polyamine transporters thus represent potentially important targets in *T. cruzi*.

Nucleotide metabolism and purine salvage

Trypanosomes are auxotrophic for purines and have several purine uptake mechanisms (Carter *et al.*, 2001). They also contain multiple purine salvage enzymes. This multiplicity means that it is uncertain whether any individual enzyme will be a useful drug target itself. However, they can be exploited to activate pro-drugs. Thus the hypoxanthine guanine phosphoribosyltransferase of *T. cruzi* is capable of converting the pyrazolopyrimidine hypoxanthine analogue allopurinol to allopurinol ribotide. The ribotide then inhibits several other enzymes involved in nucleotide metabolism and also is incorporated into nucleic acid chains, leading ultimately to parasite death. Unfortunately, allopurinol has only very limited anti-trypanosomal activity when used in *T. cruzi*-infected animals because of the pharmacokinetic problems associated with delivering the drug to these intracellular parasites at levels that will lead to death. Nevertheless, its efficacy provides a convincing proof-of-concept for the approach.

Pyrimidine nucleotide biosynthesis is another potential target. It has been shown that inhibitors of CTP synthetase, including the amino-acid analogues 6-diazo-5-oxo-L-norleucine (acivicin) and α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, can clear trypanosomes from *in vivo* rodent infections (Hofer *et al.*, 2001).

Lipid and sterol metabolism; cell signalling and differentiation

Lipids play a central role in the structure of biological membranes. They also participate both directly and indirectly in cell signalling. Sterol biosynthesis has been targeted in fungi and since pathogenic trypanosomatids have similar pathways for sterol biosynthesis (Fig. 24.3), which differ from those found in mammalian cells, drugs developed against fungi may also be used against parasites (Urbina, 1997). Inhibitors of cytochrome P-450-dependent C14 α-methylase, including ketoconazole, D0870 and SCH56592, delay development of *T. cruzi*. Inhibitors of squalene oxidase (another enzyme of the same pathway), such as terbinafine, may act synergistically with the C14 α-methylase inhibitors against the parasite and improve efficacy.

Amphotericin B is a polyene antibiotic, initially developed for use against fungi. It has good efficacy against *Leishmania* and

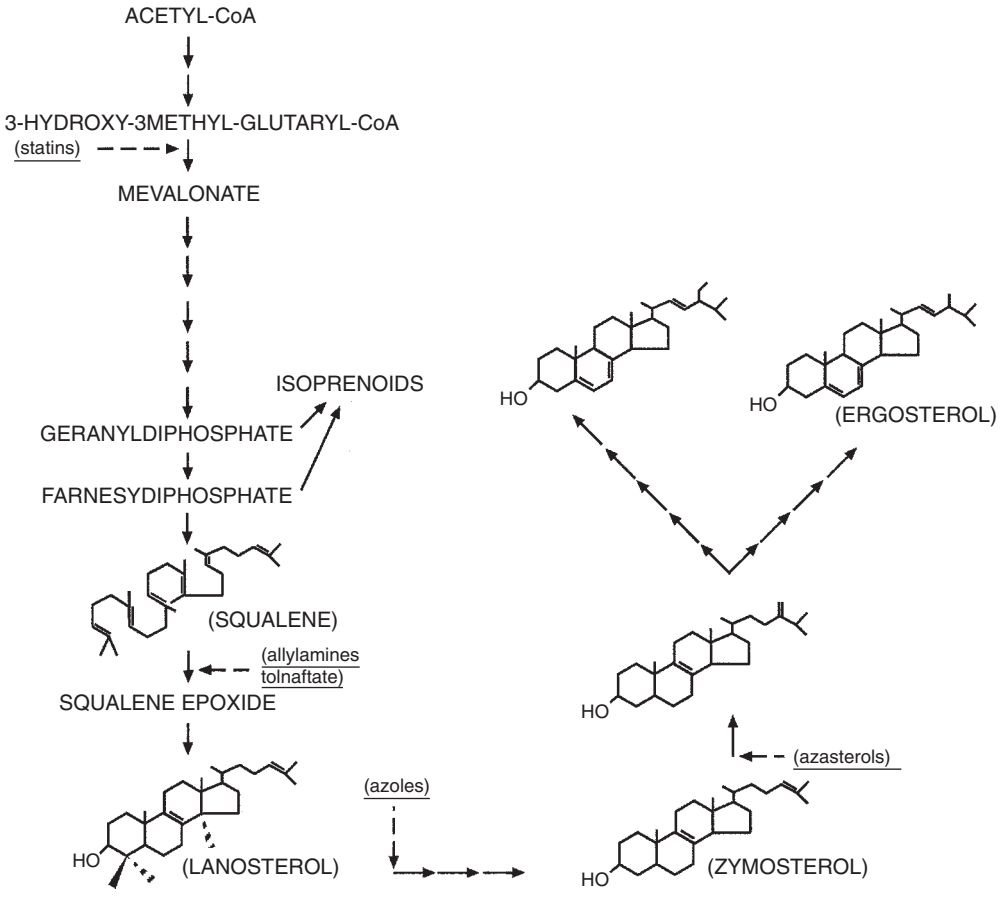

Fig. 24.3. Principal steps in the sterol biosynthetic pathway of *Trypanosoma cruzi* and fungi. Points at which inhibitors of sterol synthesis act in the pathway are shown (the inhibitors are underlined). Modified from Urbina (1997).

some activity against *T. cruzi*. Lipid-bound, or enclosed, formulations of the drug are particularly effective. Amphotericin B exerts a specific activity due to its having a much stronger affinity for ergosterol, the predominant sterol in the plasma membrane of fungi, *Leishmania* and *T. cruzi*, than for cholesterol, which is common in mammalian membranes. Binding of the drug is thought to induce the production of pits or pores that permeabilize the membranes of cells to which it binds, leading to lysis.

African trypanosomes have recently been shown to possess fatty acid biosynthetic capacity that can produce myristate for use in glycosylphosphatidylinositol anchor remodelling. Inhibition of myristate biosynthesis has attracted attention (Paul *et al.*, 2001) and thiolactomycin, an inhibitor of fatty acid biosynthesis, was recently shown to kill trypanosomes.

Trypanosomatids possess signalling pathways similar in design to some of those found in higher eukaryotes but differing in the fine details. Much effort has been exerted in the hunt for inhibitors of cell signalling pathways that may stall the growth of human cancer. There is a hope that some molecules, developed for use against neoplastically transformed cells, may find an application as trypanocidal drugs. This may be particularly applicable to compounds that

failed to inhibit mammalian signalling components but that may inhibit trypanosomatid counterparts, due to differences in structure. Cyclin-dependent kinases, small G-proteins, cyclic nucleotide-specific phosphodiesterases and protein farnesylation are among enzymatic systems under investigation.

Protein degradation and amino acid metabolism

Proteases are vital in maintaining dynamic homeostasis within cells and this class of enzyme has been extensively investigated as potential therapeutic targets in trypanosomatids (McKerrow *et al.*, 1999). Cysteine protease inhibitors have *in vitro* and *in vivo* activity against both *T. brucei* and *T. cruzi*. The turnover of proteins involving the proteasome, the crystal structure of which has been resolved for *T. brucei*, also occurs in trypanosomes and proteasome inhibitors including lactacystin are toxic.

One cysteine protease inhibitor shown to be toxic against *T. cruzi* appears to act by forcing the cysteine protease zymogen to accumulate without processing. It is this accumulation of pro-enzyme that is lethal to the cell. This observation points to a limitation with the gene-knockout approach in validating drug targets, since loss of the gene encoding the target would not have been identified as a lethal event but would lead to drug resistance.

Amino acids are important to parasitic protozoa in many ways, including roles as energy sources and pools of soluble osmolites. Methionine may be of particular importance, since it is converted to *S*-adenosylmethionine which then serves to donate methyl groups in macromolecular biosynthesis, and when decarboxylated it donates amino-propyl groups in polyamine biosynthesis (Fig. 24.2). 5-Methylthioadenosine is produced from this amino-propyl donation and then recycled via a series of steps of the methionine cycle culminating in the addition of an amino group to 2-keto-4-methylbutyrate to regenerate methionine. Methylthioadenosine analogues such as 5′-deoxy-5′-(hydroxyethylthio)adenosine have trypanocidal activity (Keiser *et al.*, 2001).

Membrane architecture, transporters and drug entry

The parasite cell surface is critical. It represents the interface between the parasite and the host and, since parasites take nutrients from their host, they have transmembrane transporters to import host metabolites. Blocking of vital transporters would kill the parasites and the trypanosomatid glucose transporters have received particular attention in this regard (Tetaud *et al.*, 1997).

Many major surface proteins of parasitic protozoa are attached via glycosylphosphatidylinositol (GPI) anchors, which were first discovered as part of the variant surface glycoprotein (VSG) coat that covers African trypanosomes, protecting them against immune attack. The GPI anchor of VSG and other proteins is constructed by enzymes differing in some respects from those in mammals (Ferguson *et al.*, 1999). The GPI anchor biosynthetic pathway has been shown to be essential and specific inhibitors against the trypanosome enzymes have been produced (Ferguson, 2000).

Specific plasma membrane transporters can also assist in selectively targeting drugs to parasites. Melamine-based arsenicals and diamidines, including berenil and pentamidine, enter *T. brucei* via an unusual adenosine transporter termed P2 (Carter and Fairlamb, 1993). Pentamidine also enters via two other transporters (De Koning, 2001). Melamine-based arsenicals may also enter via other routes, as removal of the gene *TbAT1* (Mäser *et al.*, 1999), which encodes the P2 transporter, led to only modest decreases in sensitivity of *T. brucei* to melamine-based arsenicals (Matovu *et al.*, 2003). Recognition of amino-purines and these classes of drug by the P2 transporter comes about because the transporter recognizes a motif common to 6-amino-purines and the melamine ring (Barrett and Fairlamb, 1999). The recognition motif is found on other trypanocides, including trybizine hydrochloride (SIPI 1029) and it has been attached to a variety of putative toxins for delivery to trypanosomes via this route (Hasne and Barrett, 2000).

The drawback with the P2 transporter as a means of delivering novel trypanocides is that uptake activity via this carrier can be lost, without impact on viability or virulence of the trypanosomes (Matovu *et al.*, 2003), since it is one of several purine transporters (Carter *et al.*, 2001). Loss of the transporter contributes to drug resistance (Carter and Fairlamb, 1993; Barrett and Fairlamb, 1999). Therefore other means of targeting drugs to the cells require consideration. The well-characterized hexose transporter is not suitable, since it has low affinity for highly abundant natural substrates. Other transporters, including those for nucleobases (De Koning and Jarvis, 1997) and amino acids (Hasne and Barrett, 2000), may be useful gateways for novel trypanocides.

The kinetoplast, RNA editing and regulation of gene expression

An unusual network of mitochondrial DNA, comprising a series of intercatenated circular DNA molecules, is found in trypanosomatids and termed the kinetoplast (Shapiro and Englund, 1995). The circular DNAs are of two types: maxicircles, which contain genes encoding components of the typically mitochondrial respiratory complex; and minicircles, which encode small RNA species termed guide RNAs. Guide RNAs are involved in the extraordinary process of RNA editing (Stuart *et al.*, 1997), whereby U-residues are added (or, less often, removed) from primary transcripts to yield mature translatable transcripts. This process involves a family of enzymes that work in what has been coined an 'editosome'. This mitochondrial RNA editing process is unique to trypanosomatids; hence components of the process may be good targets. It has been shown that the *TbMP52* gene that encodes a ligase crucial to the editing process is essential in bloodstream forms of *T. brucei* (Schnaufer *et al.*, 2001).

The structure of the kinetoplast itself also offers potential for attack. The complex replication pattern required to reproduce the catenated network depends on topoisomerase enzymes and inhibitors of DNA topisomerases have both trypanocidal activity and the ability to disrupt the kinetoplast (Shapiro and Englund, 1995). Several trypanocidal drugs are conspicuously cationic in nature (e.g. isometamidium, homidium, berenil and pentamidine) and there is evidence that these drugs accumulate in the mitochondrion and bind the kinetoplast of trypanosomes. Unequivocal data proving that the kinetoplast *per se* represents the target for any of these drugs, however, is lacking.

Trypanosomatids also regulate gene expression in the nucleus in a way distinguishing them from mammals (Vanhamme and Pays, 1995). Promoters that bind RNA polymerase I for expression of ribosomal genes and the genes encoding the major surface proteins (VSG and procyclic acidic repeat protein (PARP)) and RNA polymerase III for transcription of genes encoding small RNA species are known. However, typical polymerase II promoters have not been identified in trypanosomatids and large polycistronic primary transcripts, transcribed by RNA polymerase II, are processed into gene size units via the addition of a poly-A tail at the 3′-end and the addition of a common 39 nucleotide 5′ cap structure, termed the spliced leader (or mini-exon) to the 5′ end. Interference with this trans-splicing process has also attracted attention as a possible drug target. The trypanocidal reagent sinefungin, which prevents methylation of the spliced leader sequence, is a potent inhibitor of the trans-splicing process but it also inhibits many other methylation-dependent processes in trypanosomes.

Trypanocides under Consideration for Clinical Development

The orally available dicationic pro-drug DB289 (2,5-*bis* (4-amidinophenyl) furan *bis*-O-methyl amidoximine) (Rahmathullah *et al.*, 1999) is currently the only potential drug that has substantial financial backing, (through the Bill and Melinda Gates Foundation), for clinical trials against sleeping sickness. The compound is absorbed across the intestinal epithelia and is converted systemically into a trypanocidal dicationic form. Whether the pharmacokinetic

properties of the pro-drug and active metabolite will permit the drug to be used against late-stage disease is not yet known. An orally available drug that is effective against early-stage disease would be a useful addition to the armoury available against human African trypanosomiasis, since administration will be easier and less dangerous than with formulations that require injection.

Megazol is a 5-nitroimidazole that has remarkable efficacy against both *T. cruzi* and *T. brucei* (Enanga *et al.*, 2000). The mode of action of the drug is not clear but is likely to involve specific reduction of its nitro-group by a trypanosome-specific enzyme that has not yet been identified. Megazol, in combination with suramin, is effective against *T. brucei* in the cerebrospinal fluid of primates and if the drug can pass standard toxicity tests then it should be put forward for trials, possibly as part of a combination with suramin, in humans.

A triazine derivative, SIPI 1029 (trybizine hydrochloride), is used in China against *T. evansi* infections of water buffalo (Keiser *et al.*, 2001). The drug has also been shown to be active against human infective African trypanosomes, but in experimental models it could only produce cure of early- but not late-stage disease, indicating that the blood–brain barrier limits accessibility of the drug to parasites in the cerebrospinal fluid. CGP 40215 is an inhibitor of polyamine biosynthesis through its interaction with *S*-adenosylmethionine decarboxylase. As with SIPI 1029, however, activity against early-stage disease was not replicated in late-stage models, which leads to the drug being considered as less promising for further development.

New therapies for Chagas disease are also not promising (Urbina, 1999). Even where optimism had been generated that drugs developed as anti-fungals could be used in treatment of *T. cruzi* infections, compounds such as D0870 failed to progress through to full clinical development (Urbina, 1999).

At the time of writing, therefore, only DB289 is being seriously considered as a candidate for clinical development against the trypanosomiases.

Combination Chemotherapy and New Formulations of Old Drugs

With the paucity of new products close to clinical evaluation, efforts are being made to make better use of current drugs. New regimens of melarsoprol and eflornithine have recently been tested. A shorter treatment regimen of melarsoprol (Burri *et al.*, 2000) was just as effective as the traditional course and it required greatly reduced hospitalization times and resulted in far greater patient compliance. The melarsoprol short course was thus considered a success. Unfortunately, the short course of eflornithine led to unacceptable rates of relapse.

Using combinations of known trypanocides can bring benefits of reduced dosage of individual drugs, lessening side effects and extending supplies of drugs under conditions of limited availability. Eflornithine has been successfully used with melarsoprol, suramin and pentamidine in experimental animals (Jennings, 1993). Extended use of combination chemotherapy may improve the use of current drugs and this area should be pursued.

The effectiveness of combination chemotherapy has been attributed to synergistic effects resulting from inhibition of convergent biochemical pathways within the parasite. Other actions related to pharmacology within the host may also be at work. For example, eflornithine affects the blood–brain barrier's integrity and it seems to reduce inflammatory responses in the brain, which could explain its efficacy in combinations. Suramin is known to be synergistic with megazol and other nitroimidazoles in clearing parasites from late-stage infections, although neither drug is active alone. Suramin is an inhibitor and substrate of the P-glycoproteins that guard the blood–brain barrier against uptake of amphipathic xenobiotics. Therefore it may exert an action at this level, although it is also a potent inhibitor of other cell-surface receptors that may also play a role in maintaining the blood–brain barrier.

Whatever the reasons for improved therapeutic responses when using combinations of drugs, the fact that synergy is apparent means that systematic studies assessing the

efficacy of drug combinations ought to be considered a priority in research while novel reagents undergo the long road to clinical development.

In treatment of animal trypanosomiasis, the use of isometamidium complexed with carbohydrate polymers, leading to slow release from depots of drug injected subcutaneously, has improved the prophylactic life of this drug in cattle. Topical applications of some trypanocidal drugs (e.g. melarsoprol) have also been shown to cure experimental animals of *T. brucei* infections successfully. Thus alternative drug delivery systems can be effective in enhancing the efficacy of drugs currently in use.

Inhibitor Design

This chapter has focused principally on the biochemical and genetic aspects of trypanosomes that might be good targets for chemotherapy. For some of these targets, progress has been made in the design of chemical entities that selectively inhibit the trypanosomal target. However, in many cases these inhibitors have represented an end point in target validation and lead-compound identification rather than a starting point for clinical development. The major reason for this is that pharmaceutical chemists, and in particular the pharmaceutical industry, have not been prepared to take on these leads for modification and clinical development.

Ideas about the optimal approach to drug discovery have evolved significantly in recent years. Designing drugs based on specific targets remains intuitively attractive but, disappointingly, this approach has so far failed to yield new agents for use against sleeping sickness or Chagas disease. Recent advances in chemical synthesis and in particular the advent of combinatorial chemistry, together with techniques for high-throughput screening using recombinant enzymes, have brought back into favour the empirical approach for finding lead compounds such that there has been a fusing of the rational approach (target-based) with the 'nouvelle-empirical' route to drug discovery. The approach can also be applied where effective chemical entities are known, e.g. the diamidines, such that chemical modifications can be implemented to develop drugs, or pro-drugs, with improved pharmacological profiles. Combinatorial chemistry was also employed recently to make improved derivatives of lead anti-trypanosomal glyceraldehyde-3-phosphate dehydrogenase inhibitors. Moreover, as *in vitro* assays to test toxicity against bloodstream trypanosomes are now relatively simple, testing large numbers of compounds directly for activity against the parasites could yield new trypanocides more rapidly.

Production of orally available reagents

Drugs that are orally available are preferable for human medicine (but not for treating animals) since these can be administered without the need for expensive and potentially dangerous syringes. DB289 was one of a series of diamidine derivatives that were originally developed as novel drugs against the AIDS-related opportunistic infections by *Pneumocystis carinii*. The compound was rationally designed as an amphipathic pro-drug that is absorbed by the intestine and then metabolized systemically to yield a diamidine. A number of other pro-drugs, all based on the same principle of masking the positively charged groups at either end of the molecule, have also been produced. Similar chemical modifications to other trypanocidal agents allowing them to be absorbed through the intestinal epithelium should be considered in developing new drugs.

Drugs that traverse the blood–brain barrier

The blood–brain barrier has evolved to protect the central nervous system from potentially neurotoxic compounds in the blood. Most hydrophilic compounds will not cross the blood–brain barrier, which is made up of endothelial cells connected by tight junctions and reinforced with foot processes from astrocytes. Amphipathic compounds that may cross the barrier are often substrates for

P-glycoprotein pumps, which are abundant in the endothelial cells lining the barrier. There are trypanocidal drugs (for example, melarsoprol and its metabolites and eflornithine) that do accumulate in the cerebrospinal fluid to trypanocidal levels, but many others do not and therefore are valueless against late-stage African trypanosomiasis. Suramin may assist in getting other drugs across the blood–brain barrier since it inhibits P-glycoproteins and may effect blood–brain barrier permeability through other pharmacological effects. This is thought to contribute to its efficacy in combination chemotherapy. Other P-glycoprotein inhibitors may also be useful in potentiating the activity of amphipathic trypanocides against central nervous system-involved disease. Other methods for transiently opening the blood–brain barrier have been developed. For example, the bradykinin agonist, cereport (also known as RMP-7 and labradamil) (Emerich *et al.*, 2001), opens tight junctions and this increases permeability of the brain. Co-application of reagents such as these along with trypanocides may improve activity against late-stage African trypanosomiasis.

In vitro techniques to cultivate an endothelial monolayer reinforced with astrocytes present fairly good models of the blood–brain barrier (De Boer and Sutanto, 1997). Similar models of the intestinal barrier also exist and the use of these models should greatly accelerate the rate at which trypanocidal compounds can be assessed for their pharmacokinetic abilities.

Conclusions

New drug development against sleeping sickness more or less stagnated in the latter half of the 20th century. No satisfactory drugs exist for use against Chagas disease and the treatment of animal trypanosomiasis has run into problems associated with the development of drug resistance. The need for new drugs against all of the trypanosomiases is urgent. A great deal of information has been gained on potential drug targets in these organisms, but little has been done to develop any inhibitors of these targets into clinically useful drugs. The development of technologies allowing high-throughput screening of compounds against these targets and the parasites themselves should facilitate the identification of new chemical entities that can be used against the trypanosomiases. Moreover, as the pharmacological features that permit the passage of drugs through the intestinal epithelium and the blood–brain barrier become clear, drug design, from a pharmacological perspective, may also improve. However, in spite of the fact that we have unprecedented knowledge about targets in trypanosomes that are vulnerable to therapeutic intervention and the ability to make new chemical entities at a rate far greater than at any time in history, the future for chemotherapy against trypanosomiasis will remain bleak unless socio-economic barriers to developing new drugs are overcome.

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25 Trypanotolerance

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Trypanotolerance: Nature's Contribution to Improved Control of Animal African Trypanosomiasis

Entering the new millennium, there is a potent armamentarium of weapons to deal with livestock disease problems. Over the last century, major advances have been made in disease control in animals as well as in humans. A large portfolio of drugs now exists to treat and manage disease, a wide range of technologies has been developed to control vectors, and highly effective vaccines at least for some infections have been produced. Despite this progress, the world is still ravaged by diseases, both old and newly emerging.

Advances in bioengineering, biotechnology and the computing, mathematical and information sciences are likely to provide new tools for health management. However, some will give rise to ethical and safety concerns. Moreover, experience shows that estimates of time to production and of costs are often over-optimistic. On the other hand, current options for disease control are sometimes under-exploited and, if properly applied, could do much to alleviate disease constraints.

The foregoing considerations are classically exemplified by tsetse-transmitted trypanosomiasis in domestic livestock in Africa. Where the current methods of control, namely diagnosis and treatment, and/or vector control, have been properly implemented the outcome has been shown to be cost-effective and sustainable in several situations throughout Africa. These control methods are reviewed in Chapters 8 and 27. However, the overall ongoing problem at a continental level has not been significantly alleviated by the existing methods. Moreover, the prospects of a major breakthrough in terms of a new method of control, including vaccination, are not good at the present time (Chapter 2).

With this background, one of the extant options for disease control that has yet to realize its full potential is the farming of disease-resistant genotypes. It has long been recognized that certain breeds of African cattle possess the ability to survive and to be productive in tsetse-infested areas without the aid of trypanosomiasis treatment where other breeds rapidly succumb to the disease. This trait is termed trypanotolerance and is usually attributed to the *Bos taurus* breeds of cattle in West and Central Africa, particularly the N'Dama and also the West African Shorthorn. Significant differences in resistance to trypanosomiasis have also been found in *Bos indicus* breeds, although in our experience most *B. indicus* cattle in tsetse-infested areas require regular treatment, or are found only on the fringes of fly belts. Imported exotic breeds cannot be main-

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tained even in areas of low tsetse risk without intensive drug therapy. Although it will not be considered in this chapter, significant variation in susceptibility to trypanosomiasis has been observed within and between different breeds of sheep, goats, pigs, horses, donkeys and many species of wild Bovidae and Suidae, as well as among different human ethnic groups (reviewed by Murray *et al.*, 1982).

The exploitation of so-called trypanotolerant breeds, in particular the N'Dama, has been practised as a major, if not the only, option for sustainable livestock production in 19 countries in the most humid parts of West and Central Africa. However, trypanotolerance is a feature of only a third of the cattle in tsetse-infested Africa, and of no more than 10% (or 15 million) of the cattle population south of the Sahara. Failure to exploit these breeds can be attributed largely to the belief that they are not productive because of their smaller size compared with most zebu types, and to the view that their 'trypanotolerance' is effective only against local trypanosome populations.

These views have not been confirmed. In a large-scale attempt to evaluate productivity, the performance of N'Dama and West African Shorthorn cattle was compared with zebu types under different levels of tsetse-trypanosomiasis risk in 18 countries in West and Central Africa (ILCA, 1979). Several key findings were made.

- Where the tsetse-trypanosomiasis risk was low or zero the productivity of trypanotolerant breeds was equal to that of larger trypanosusceptible zebus, when assessed on a metabolic unit base.
- In many areas the level of risk was such that only trypanotolerant breeds were present.
- While trypanotolerant breeds were productive, their output fell as the level of risk increased.

Work carried out at the International Trypanotolerance Centre (ITC) in The Gambia has provided remarkable data on the productive potential of the N'Dama; for example, an analysis of nearly 700 lactation records of animals maintained under village management in The Gambia showed a milk offtake of between 400 and 600 kg over a lactation period of 14 months (Agyemang *et al.*, 1991). Such a yield is impressive for animals that have an average bodyweight of 225 kg and survive a 7-month-long dry season, often under trypanosomiasis risk. Furthermore, it was shown that when milk extracted from N'Dama cattle for human consumption was taken into consideration, their overall productivity was superior to that of zebu breeds maintained under similar traditional systems in the absence of tsetse challenge (Agyemang *et al.*, 1994).

Evidence that trypanotolerance is not solely due to acquired immunity to local trypanosome populations has been provided by both experimental and field studies. N'Dama cattle with no previous exposure to trypanosome infection have demonstrated significantly greater resistance to the effects of infection when compared with zebu *B. indicus* types, following both experimental challenge (Paling *et al.*, 1991a) and field exposure (Murray *et al.*, 1981). The criteria used to assess resistance included requirement for treatment, survival time, parasitaemia levels, severity of anaemia, oestrous cyclicity and body weight. At the same time, in several countries in West and Central Africa trypanotolerant cattle have been moved or imported successfully into high tsetse-risk areas (Mortelmans and Kageruka, 1976; ILCA, 1979). This is reflected in the fact that, between 1977 and 1985, numbers of trypanotolerant cattle in tsetse-infested areas previously unpopulated by cattle grew by 10% per year (Shaw and Hoste, 1987).

Despite the demonstration that trypanotolerant cattle are productive when assessed on a metabolic unit basis, and unequivocal confirmation that they possess significant innate resistance to trypanosomiasis, there is still a trend among livestock owners in parts of West Africa toward replacing or crossing trypanotolerant cattle with trypanosusceptible breeds (Jabbar *et al.*, 1998). This is due largely to changes in vector challenge and disease risk, but it has also been linked to the higher prices fetched at market by susceptible breeds than those fetched by tolerant breeds. Animals of sus-

ceptible breeds tend to be larger and thus more able to provide draught input into cropping; and individually they have higher milk yields than tolerant animals.

Research on trypanotolerance must therefore accomplish two aims. Firstly, it must identify how disease resistance in trypanotolerant breeds can be maintained and enhanced while retaining and reinforcing other characteristics of economic importance to farmers. Secondly, it must find ways to confer the trypanotolerance trait to susceptible animals while retaining their other traits of economic importance.

Trypanotolerance Indicators

The successful use of any criteria for identification of trypanotolerant breeds of cattle or superior animals within these breeds depends on the practicality of their measurement, on the strength of the linkage of the criteria with the economically important production traits such as viability, reproductive performance and growth, and on the associated genetic parameters.

Trypanotolerance has been defined as that property of an animal that enables it to remain productive under tsetse-trypanosomiasis challenge. The mechanisms that underlie this ability include control of parasite proliferation, limitation of the pathological effects of the parasites, and an acquired ability for better control of trypanosomiasis (Murray *et al.*, 1982; Murray and Dexter, 1988; d'Ieteren *et al.*, 2000). While a general relationship exists, control of parasite development and mitigation of anaemia, although under genetic control, do not show a strict quantitative relationship in between-animal comparisons (Paling *et al.*, 1991a). Understanding the mechanisms underlying these elements has been a major research goal over the last 30 years.

Control of anaemia

Current knowledge indicates that control of anaemia development appears to be the criterion of trypanotolerance most closely linked to overall productivity, including post-weaning growth, reproductive performance and overall cow productivity in production systems (Murray *et al.*, 1991; Trail *et al.*, 1991; d'Ieteren *et al.*, 1998, 2000).

In field studies, the degree of anaemia can be quantified easily by measuring the haematocrit or packed red cell volume (PCV) percentage, or haemoglobin concentration. Although the measurement of PCV is very accurate, the interpretation of PCV variation in the field is meaningful only if other factors affecting PCV are identified, quantified and controlled.

Control of parasitaemia

The degree of parasitaemia is not so easily measured, as quantification depends on demonstration of trypanosomes in peripheral blood by parasitological techniques. The most sensitive practical field approach has been to detect the presence of trypanosomes by the dark ground/phase contrasts buffy coat technique (BCT) and to quantify the intensity of the infection as a parasitaemia score (Paris *et al.*, 1982). This method has contributed to major progress in characterizing trypanotolerance in the field but it has two major limitations: it fails to detect a proportion of infections and it provides no indication of parasite turnover. Thus, although the direct effects of trypanosome infections on PCV and growth are obvious, a more sensitive method than BCT for reflecting parasite control is required so that individual animals can be reliably categorized for parasite control capability.

Recent work (reviewed by d'Ieteren *et al.*, 1998) explored other approaches for the characterization of parasite control, using newly developed diagnostic techniques. In particular, the use of antigen detection was assessed as a measurement for the infection control component of trypanotolerance. It was found that animals which were antigenaemic but which were able to control parasites below levels detected by BCT were significantly less anaemic and showed superior performance by comparison with BCT-positive animals. Thus, although antigen

detection tests are not yet totally reliable (Masake and Minja, 1998), their use in serial records suggests that antigen test information contributes to the more accurate definition of infection status in both aparasitaemic and parasitaemic animals residing in trypanosome endemic areas (G.D.M. d'Ieteren, Nairobi, personal communication).

Acquired ability for better control of trypanosomiasis

There is evidence that cattle which survive in a tsetse-trypanosomiasis endemic area, with or without the aid of trypanocidal drugs, acquire resistance. This has been reported for both N'Dama cattle and *B. indicus* breeds in East Africa (reviewed by Murray *et al.*, 1982). Thus, with increasing time following initial exposure to potential infection, the interval between infections increased, the interval between treatments based on detectable parasitaemia was longer, and animals were often parasitaemic without showing severe clinical effects. On this basis, it was generally assumed that animals were acquiring immunity to the local population of trypanosomes.

While this conclusion that acquired resistance might be a component of trypanotolerance was obtained in field studies that suffered a number of limitations, it was supported in subsequent and carefully controlled challenge experiments. Paling *et al.* (1991a,b) had the unique opportunity of access to N'Dama cattle produced by embryo transfer and reared from birth in a tsetse-free area, and the ability to deliver a challenge using laboratory-reared tsetse experimentally infected with well-defined clones of *Trypanosoma congolense*. It was confirmed that N'Damas possessed superior resistance on first exposure when compared with *B. indicus* Boran, as assessed by the ability to control parasitaemia, resist the development of anaemia, gain weight and continue to undergo oestrous cycles. Two years after the primary infection and immediately prior to homologous rechallenge infection, all eight N'Damas tested had neutralizing anti-metacyclic variant specific antibodies present in their sera, compared with five of the eight Borans. Following the homologous rechallenge, the prepatent period was significantly longer in the N'Damas, the level of parasitaemia was lower and there were no alterations in erythrocyte counts. In contrast, the Borans became highly parasitaemic and developed severe anaemia. These results suggested that N'Damas develop a more persistent protective response compared with Borans, and possibly possess superior immunological memory for trypanosome antigens.

Furthermore, in the N'Dama cattle, while the kinetics of parasitaemia were not affected by previous exposure to heterologous serodemes of *T. congolense*, red blood kinetics were, with the overall mean PCV values increasing significantly and progressively between the first and subsequent heterologous infections. In the same way, in the Boran cattle, there was no difference in the intensity or prevalence of parasitaemia produced by the heterologous challenge clones. However, in contrast to the N'Damas, the severity of the anaemia and the requirement for treatment in the Borans were not reduced by previous exposure. Thus, while neither the N'Dama nor the Boran cattle showed any evidence of cross-protective immunity among the heterologous serodemes of *T. congolense* tested, the N'Dama breed appeared to possess the unique characteristics not only of developing less severe anaemia to primary infections but also of having an acquired capacity to mount accelerated haemopoietic responses to subsequent challenges.

Until recently, there was evidence from field studies for the ability of N'Damas to acquire significant control of parasitaemia following *Trypanosoma vivax* infections but apparently not following *T. congolense* infections. The data also indicated that preweaner calves grazing with their dams were more resistant than post-weaners to (or were protected from) *T. vivax* and *T. congolense* infections. Further field research involving the same N'Dama cattle extended the foregoing observations by providing evidence of acquired resistance not only to *T. vivax* but also to *T. congolense*. In a series of

studies involving nearly 4500 cattle observed for varying time periods from 7.5 to 70 months of age, a steady decrease was observed in *T. vivax* prevalence with increasing age, whereas *T. congolense* prevalence increased until about 140 weeks of age and then decreased (G.D.M. d'Ieteren, Nairobi, personal communication). The higher the tsetse-trypanosomiasis risk, the larger was the increase or decrease of prevalence, pointing to the likelihood of acquired immunity to both species of trypanosome.

Correspondingly, as exposure times to natural challenge increased, the effect of both species of trypanosome on average PCV was reduced, though not dramatically so (d'Ieteren *et al.*, 1998; G.D.M. d'Ieteren, Nairobi, personal communication). A significant observation was that overall levels of PCV improved dramatically as animals aged under challenge, and that this apparent acquired capability to control the disease was not directly related to parasite control. Indeed, the PCV pattern with increasing age was observed after adjustment for level of infection and for the regression of parasite detection on PCV nested within each age class.

There is also evidence of an ability to acquire resistance and genotype dependency in *B. indicus* types in East Africa (Murray *et al.*, 1982). An example was reported at Kilifi Plantations, a dairy ranch in the coastal region of Kenya (Murray and Trail, 1986). On this ranch, the adult breeding females consisted of two types: a two-thirds Sahiwal, one-third Ayrshire; and a one-third Sahiwal, two-thirds Ayrshire. Trypanosomiasis control was based on chemotherapy (mainly Berenil, diminazene aceturate, Hoechst). Any animal with a PCV of $< 30\%$ was considered infected and was treated. It was found that the two-thirds Sahiwal required less than half the number of treatments needed by the one-third Sahiwal. This was judged as evidence of breed variation in susceptibility to trypanosomiasis. At the same time, it was found that the more times an animal had been treated, the fewer treatments it would need. Significantly, requirement for treatment was shown to be independent of age. It was concluded that these animals were becoming more immune to the local strains of trypanosomes, or possibly were acquiring resistance to the effects of the infection, i.e. anaemia.

When the interaction between genotype and previous treatments received was considered, it was found that when very few previous treatments (0 to 1) had been given, both genotypes required similar numbers of current treatments. However, as the number of previous treatments received increased, the two-thirds Sahiwal requirements for treatment became progressively less than those of the one-third Sahiwal, suggesting the greater capability of the two-thirds Sahiwal in acquiring resistance or immunity.

These studies provide evidence that previous exposure can reduce susceptibility to disease significantly, through acquired immunity to the infection and/or acquired resistance to the main pathogenic disease processes, and that it is likely that trypanotolerant breeds possess this trait (or traits) to a significantly greater extent than trypanosusceptible types.

Genetic aspects of trypanotolerance indicators

Heritability values for, and genetic and phenotypic correlations between, growth, average PCV and lowest PCV over a 3-month testing period have been reported for N'Dama cattle under moderate challenge (reviewed by Trail *et al.*, 1991). When all environmental and parasitaemia information was taken into account, the heritability of growth over the test period was 0.39 ± 0.32, which is within the expected range over this length of time. The heritabilities of PCV measures were higher than those for growth, at 0.64 ± 0.33 for average PCV over the duration of the test and 0.50 ± 0.32 for lowest PCV reached during the test. The genetic correlation between average PCV and growth was 0.70 ± 0.42, and between lowest PCV and growth 0.28 ± 0.55. High standard error reflected the small number of progeny available per sire. These values, coupled with the higher heritabilities of the PCV measures, indicate some possibility of selection on PCV values for control of

anaemia development. Evidence of genetic variation for the same components of trypanotolerance has been found in some types of *B. indicus* cattle in East Africa (Rowlands *et al.*, 1995; Mwangi *et al.*, 1998).

In subsequent studies, when antigen detection techniques were used and antigen-positive, parasite-negative animals were classified as having more ability to control parasite growth than parasitaemic animals, a significant sire effect was found suggesting that a degree of genetic control was involved. More sensitive diagnostic techniques might offer a practical possibility for selection of trypanotolerant animals based on infection criteria (d'Ieteren *et al.*, 1998).

In the field studies in N'Damas reported above, age was shown to have a major effect (G.D.M. d'Ieteren, Nairobi, personal communication). As a result, infection control 'indices' were constructed that related trypanosome infections detected at any given time to a level detected at earlier ages. Preliminary results indicated that the acquired ability to control *T. congolense* infections is under genetic control, with heritability estimates observed being as high as for anaemia control (estimated by average PCV over given periods of time). For *T. vivax* it has not as yet been possible to draw meaningful conclusions with the data processed to date. However, further analyses should provide more definite conclusions on the genetics underlying the ability to acquire better parasite control and/or better disease control and will determine whether these traits are of sufficient economic importance to justify their use in selection indices based on disease resistance.

This work reporting preliminary genetic parameters provides evidence that trypanotolerance is not only a breed characteristic but also a heritable trait within the N'Dama population. The genetic variation identified within the N'Dama breed has presented new opportunities for improved productivity through selection for trypanotolerance. New practical immunological markers (if and when available) could contribute to more comprehensive characterization of trypanotolerance in the field. Likewise, molecular markers would greatly assist and expedite selection decisions based on the resistance traits, provided that these markers were associated with a sufficiently large proportion of the genetic variation observed in the field.

Stability of Trypanotolerance

While it is generally accepted that trypanotolerance is a breed characteristic under genetic control, there is evidence that the stability of trypanotolerance can be affected by environmental factors, such as overwork, intercurrent disease and repeated bleeding, pregnancy, parturition, suckling and lactation. Probably the most important factors are nutrition and the degree of tsetse-trypanosomiasis risk.

On-farm and on-station research carried out at ITC in The Gambia provided comprehensive information on the importance of nutrition condition of N'Damas to sustain economically viable performance under disease stresses (Agyemang *et al.*, 1994; Holmes *et al.*, 2000).

In the first large-scale attempt to evaluate the effect of tsetse-trypanosomiasis risk (defined rather subjectively, but using relevant information available at that time) on performance of N'Dama and West African Shorthorn cattle at 30 different locations, research performed by the International Livestock Centre for Africa (ILCA), the Food and Agriculture Organization (FAO) and the United Nations Environment Programme demonstrated that although these breeds remain productive under tsetse-trypanosomiasis challenge, their outputs were affected by increasing risk (ILCA, 1979).

These findings have major strategic implications, in that, in higher risk situations, where tolerant livestock are maintained without the aid of trypanocidal drugs, productivity could be improved by strategic interventions, such as the use of trypanocidal drugs at critical times, vector control, improved management (nutrition) or enhancing livestock disease resistance by selective breeding. The justification for the application of such strategic additional control measures on farms with trypanotolerant livestock might be expected to decrease if livestock disease resistance were enhanced by selective breeding.

Other Attributes of Trypanotolerant Cattle

In addition to resistance to trypanosomiasis, trypanotolerant cattle (and the N'Dama breed in particular) have other advantages that contribute to their potential for use in livestock development programmes in the tropics.

These cattle are reported to possess significant resistance to several other important endemic infectious diseases, including a number of tick-borne infections such as dermatophilosis, heartwater, anaplasmosis and babesiosis. Lower tick burdens have been reported as well as lower prevalence of strongyle infections (Murray *et al.*, 1982; Mattioli *et al.*, 2000). There is an intriguing possibility that common mechanisms might underlie a single broad-based disease resistance in trypanotolerant cattle.

A further rational explanation for the fact that trypanotolerant livestock such as N'Dama cattle are more resistant to the constraints of production in tropical and subtropical zones might be superior physiological adaptation to their environment, through such factors as food utilization, heat tolerance and water conservation (reviewed by Murray *et al.*, 1982). While critical studies on trypanotolerant breeds are lacking, it is known from work on other breeds of cattle that genetically determined differences in voluntary food intake do exist. These have been ascribed to variations in fasting metabolism and maintenance energy requirements. Where food intake is marginal, as it is in many areas of Africa, breeds with an inherently low maintenance requirement will suffer less, as demonstrated by weight loss or reduced weight gains. It is believed that N'Damas can withstand higher levels of humidity than zebus (reviewed by Murray *et al.*, 1982). In addition, there is a considerable variation in the rectal temperature of N'Damas during the course of the day, with temperatures ranging from 34.4°C at dawn to 41.1°C in late afternoon in The Gambia. The teleological argument for such a phenomenon is that thermoregulation under cold conditions, i.e. below 20°C for tropical breeds of cattle, requires the use of body energy stores. Thus, when caloric intake is low, energy is conserved by allowing the body temperature to fall. Studies in East Africa have shown that the water requirements of local zebus is about half that of Herefords and is similar to those of several species of wild animals. Zebus were also better able to conserve evaporative and faecal water than Herefords. This capacity for conserving water was inherited as a dominant trait in zebu–Hereford crosses. It is possible that N'Damas have adapted to an even greater extent than zebus because trypanosome-free N'Damas have been shown to turn over less water in relation to their metabolic size (reviewed Murray *et al.*, 1982).

Biology of Trypanotolerance

The foregoing part of this chapter has reviewed knowledge of the trypanotolerance phenotype. This will now be complemented by an evaluation of current knowledge of the biological basis of the trypanotolerance trait and its underlying mechanisms.

General considerations

The overall response phenotype in any given individual undergoing infection is the result of the combined action of several distinct response mechanisms, some of which are innate and some of which are acquired. Following infection with trypanosomes, the host will, for example, respond with antibodies of different isotypes, with different specificities and affinities, with T cells with various specificities and functions, with various acute-phase reactants, and so on. Thus there may be both qualitative and quantitative differences between animals within each arm of the response. Moreover, each element of the response in a given host falls somewhere on a spectrum that at one end may be protective and, at the other, pathological. Thus, in any individual host, some elements of the response phenotype are helpful to the host in terms of survival and productivity, some are dangerous, and it is probable that there are some which have little consequence for the outcome of infection. Given

that some or all of these responses can to an extent behave independently at the population level, it follows that certain animals may be endowed with a more favourable combination of responses than others. If this is the case, a spectrum of individual components of the response can be expected within any population, ranging from beneficial (positive) to harmful (negative). A spectrum of individual host phenotypes ranging from 'resistant' to 'susceptible' can also be expected, by virtue of the particular combination of elements of response and their strengths that individuals tend to mount.

All of the elements in disease response that have been considered to this point are under genetic control. However, the effects of genes and their products are subject to environmental influences. As considered earlier, these include climatic conditions, nutritional status and workload (including the load imposed by pregnancy and lactation), as well as challenge intensity, and genetic variation in the parasite, both within and between species. Moreover, in the case of the host response in trypanosomiasis, genetic plasticity in the parasite is an 'environmental' consideration. Antigen switching is a semi-random process, which means that the range and sequence of parasite antigens to which the host is exposed may vary between individual hosts, even in the case where different individuals are challenged with the same cloned isolate. The influence of environmental factors in response to trypanosome infections is perhaps best illustrated by what happens when individuals of an inbred mouse strain are challenged with a given cloned pathogenic isolate. In this example, the host genetics are strictly controlled at the outset, and therefore if there were no environmental influences, every mouse would be expected to die at the same time following infection. This is patently not what happens and it is not even observed when all obvious environmental variables (age at infection, diet, laboratory conditions, infective dose etc.) are controlled.

So, the overall disease response phenotype is the product of a complex interplay of genetic and environmental factors, which are themselves of a complex (variable) nature. Not surprisingly, therefore, in the case of most infectious diseases, considerable variation exists in host response phenotypes. Trypanosomiasis is no exception and indeed it provides one of the most complex examples of disease response in livestock species.

In order to understand trypanotolerance it is important to appreciate that it is yet another layer of variation overlying the basic disease response process at the population level. As already inferred, some individuals in most heterogeneous populations appear to withstand infection better than others. Some do this more consistently over time, and between environments, than their peers. Some of these 'superior' animals are able to transmit their superiority to their offspring (discussed later). By definition, therefore, this robust, better-than-average response is an inherited trait and, in consequence, it can be inferred that it is at least partly genetically controlled. Trypanotolerance is this genetically controlled, above-average performance in the face of trypanosome challenge.

But how is performance measured? The pragmatic view is that the true measure is productivity, which can be determined, for example, in terms of weight gain, calving interval, milk yield and sustainable workload in draught animals, all when under challenge. Certainly as far as the farmer is concerned, this is the ultimate measure of the value of the trait. However, given that productive performance at farm level is determined by a variety of factors, of which trypanotolerance may be only one, biologists have sought simpler parameters with which to detect and quantify the trait. As already described, the best measures of response performance for the purposes of study of the basic biology of trypanotolerance appear to be PCV, the extent of parasite proliferation and, when quantified in some way, the capacity to mount effective immune responses.

Mechanisms

It is important to recognize that different mechanisms may operate in infections with different trypanosome species and that, for infections with any given parasite, different

host species may employ different resistance strategies. It is clear that the various species of African trypanosomes cause distinct disease syndromes (Chapter 16). In so far as these differences are parasite determined, they may be expected to involve different host responses; if so, protective responses may differ between infections caused by different parasites. That this may be the case is suggested by experiments in mice. For example, survival times of mice of a genetically TNF-α-deficient line, when infected with a *Trypanosoma brucei* clone, are similar to those of normal mice (Magez *et al.*, 1999). On the other hand, there is a profound effect of TNF-α deficiency on survival time of the same mouse line when challenged with a *T. congolense* clone (Iraqi *et al.*, 2001).

With respect to host-species differences in responses to defined parasite challenges, the differences in responses of domestic cattle and Cape buffalo (*Syncerus caffer*) are well documented (Dwinger *et al.*, 1986; Grootenhuis *et al.*, 1990) and serve to illustrate the point. The significance of this is that, while it is possible that such differences reflect quantitative variation in fundamentally similar host responses, they may alternatively reflect qualitative differences, i.e. different hosts may utilize different response mechanisms.

Research aimed at understanding the biological mechanisms underlying trypanotolerance has adopted two fundamentally different strategies. In the first, clues to the nature of trypanotolerance have been sought in the phenotypic differences under challenge, especially in terms of immune response, between resistant and susceptible types. Anaemia development and parasite kinetics have also been studied in some detail, as outlined above. In the second strategy, genome scanning has been adopted to search for meaningful differences in genotypes between resistant and susceptible hosts, with a view to identifying trypanotolerance genes directly.

Phenotype studies

A starting point in the identification of the mechanisms underlying trypanotolerance is to review what is known are absolute requirements in the host for it to demonstrate a resistant phenotype.

The effects of sublethal doses of γ-irradiation indicate that a functional immune system is essential for the relatively resistant C57BL/6 mouse strain to demonstrate the resistant phenotype (Morrison and Murray, 1985). Beyond this, it has recently been reported that homozygous disruption (by germline mutation) of the TNF-α gene renders relatively resistant mice fully susceptible to *T. congolense* infection (Iraqi *et al.*, 2001). The effect of the TNF-α gene in this system is apparently fully dominant, in that loss of a single copy has no effect on relative resistance.

Studies in livestock indicate that the bone marrow genotype is a significant factor in relative resistance. To demonstrate this, researchers exploited the natural tendency in domestic cattle for twins to exchange bone marrow progenitor cells in early pregnancy. The result of this exchange is bone marrow chimaerism in the twin calves. Embryos of the trypanotolerant N'Dama breed were implanted together with embryos of the susceptible Boran breed into recipient Boran cows. In every case of twinning where there was evidence of chimaerism provided by polymorphic breed-specific cell surface markers, the majority cell population in chimaeric bone marrows was of Boran origin. On challenge with *T. congolense*, N'Dama twin calves had a reduced capacity to resist the development of anaemia and to maintain growth rates. In fact, the N'Dama twins were more similar to their Boran twins than to normal N'Dama calves (A.J. Teale, Stirling, Scotland, personal communication). However, N'Damas with chimaeric bone marrow apparently controlled parasitaemia as well as non-chimaeric N'Damas. In all respects, Boran calves with chimaeric bone marrow behaved similarly to non-chimaeric Borans. As fetal bone marrow is the primary source of cells of both the immune and haematopoietic systems, the conclusion is that either or both of these systems are important for the resistance phenotype. This observation in cattle is therefore consistent with the observed

effects of γ-irradiation in mice. It is also consistent with the fact that administration of immunostimulants increases survival times of mice challenged with trypanosomes (Murray and Morrison, 1979). It should be noted that the effects of immunostimulants apply in mice of both susceptible and resistant strains and, moreover, they are associated with improved control of parasite proliferation.

These basic observations indicating the importance of the immune response in trypanosomiasis have reinforced the long-held assumption that trypanotolerance is a function of the immune system. This has led to a great deal of research comparing the immune responses of resistant and susceptible hosts.

Attention has focused on antibody responses in both mice and livestock species. In summary, in resistant mice, antibodies generally increase more quickly and achieve higher levels following infection than in susceptible mice, and these antibodies are to both trypanosome and non-trypanosome antigens. An exception is the IgG2a class of antibodies, which is produced in very large quantities in susceptible A/J mice following infection (Morrison and Murray, 1985). However, in using irradiated trypanosomes, Morrison and colleagues also made the important observation that there is no inherent difference between susceptible and resistant mouse strains in terms of their abilities to make relevant antibody responses to trypanosomes. The conclusion that can be drawn from these studies is that, important though they may be, differences in antibody responses in the murine system are a consequence of infection, rather than a primary resistance-determining factor. The results of subsequent studies of development of plasma cells in mice (Newson *et al.*, 1990) support this view.

In cattle, early immunological studies in small numbers of animals, either previously exposed or of unknown history, gave conflicting results and firm conclusions were not possible. In a definitive study in N'Dama and Boran cattle, never previously exposed to trypanosomes, there was no evidence that greater antibody responses to surface coat antigens of trypanosomes are associated with increased resistance to infection (Williams *et al.*, 1996). However, resistant N'Damas were found to make better responses to cryptic parasite antigens than Boran cattle and indeed this has been found to apply to cattle showing a better clinical response to challenge, irrespective of breed (Shapiro and Murray, 1982). Among these internal trypanosome antigens that are apparently responded to differently by resistant and susceptible cattle, the best characterized is a cysteine protease (Authié *et al.*, 1992, 1993). The response of N'Dama cattle to this antigen is predominantly in the form of IgG antibodies, and indeed, it seems that one of the clear differences between N'Dama and susceptible Boran cattle infected with trypanosomes is that N'Damas tend to mount better IgG antibody responses, irrespective of the target parasite antigen (Taylor *et al.*, 1996). It has also been observed that increases in $CD5^+$ B cells following infection in cattle are similar in resistant and susceptible types (Williams *et al.*, 1991). This B-cell subpopulation, which typically constitutes an unusually large proportion of B cells in cattle by comparison with other species, accounts for much of the proliferation in B cells following challenge (Naessens and Williams, 1992). It is also associated with the production of non-specific antibodies during trypanosome infection (Buza *et al.*, 1997).

The general conclusion from these studies is that resistant cattle types tend to mount more focused and more mature (IgG) antibody responses than susceptible cattle following infection. At a more specific level, this applies particularly to at least one and probably several internal trypanosome antigens, among which is a cysteine protease, an antigen that could conceivably contribute to the pathogenesis of the disease. However, the question of whether these differences between host types are causal or irrelevant with respect to trypanotolerance, or whether they are a consequence of better parasite control, as may be the case with antibody responses in mice, remains unanswered.

As the tools have been developed with which to define immune-cell subpopulations in livestock, they have been used to seek dif-

ferences between resistant and susceptible cattle types with respect to changes in cell populations. It appears that $CD4^+$ and γδT cells populations are reduced *in vivo* in susceptible cattle under challenge, by comparison with the cell populations in resistant animals (Williams *et al.*, 1991). However, monoclonal antibody-mediated depletion of $CD8^+$ cells in susceptible Boran cattle had no detectable effect on response to challenge (Sileghem and Naessens, 1995). Splenic B cells have also been compared in N'Dama and Boran cattle during challenge with *T. congolense* (Taylor *et al.*, 1996) and, consistent with the greater tendency to IgG antibody production in resistant animals, numbers of variant surface glycoprotein (VSG)-specific IgG-secreting cells in spleens were found to be higher in N'Damas than in Borans. Moreover, during infection, N'Dama cattle had more circulating lymphocytes that could be activated *in vitro* to undergo differentiation into IgM- and IgG-secreting cells.

With respect to cytokine production, lymph node cells (LNC) of susceptible Boran cattle collected during infection with *T. congolense* have been reported to produce higher levels of IFN-γ in response to mitogen stimulation than LNC of resistant N'Dama cattle (Lutje *et al.*, 1996). Interestingly, production of high levels of IFN-γ is a feature of the LNC of mice infected with *T. brucei* (Darji *et al.*, 1992) and is possibly associated with the suppression of the immune response that is a feature of murine trypanosomiasis.

By comparison with what is known about differences between resistant and susceptible animals with respect to aspects of the adaptive immune response, relatively little is known about differences in innate mechanisms during infection. It has been found, however, that levels of the acute phase reactant haptoglobin are markedly increased in resistant C57BL/6 mice relative to levels in susceptible mice during infection with *T. brucei* (Shapiro and Black, 1992). As far as the complement system is concerned, the ability to maintain levels of C3 and haemolytic complement activity has been found to be correlated with the level of trypanosomiasis resistance of cattle in a field challenge situation (Authié and Pobel, 1990).

Given that the ability to maintain normal blood profiles is strongly correlated with performance under trypanosome challenge in livestock, it might be expected that the haematopoietic system could reveal significant differences between types. The difficulties of studying the system, and the relative lack of tools with which to do so in livestock species, have largely limited comparisons to erythropoietin (EPO) and its receptor in infected cattle. Nevertheless, it would appear that N'Damas have a comparative advantage over Boran cattle in erythropoietic responsiveness. Thus, the transcription of cell surface EPO receptors was found to be significantly higher in the bone marrow of *T. congolense*-infected N'Damas, while the EPO response of Borans was found to be inadequate given the level of anaemia that developed (Suliman *et al.*, 1999). At the same time, transcripts of some inflammatory cytokines, which are negative regulators of erythropoiesis, are expressed at lower levels in the kidneys and bone marrow of challenged N'Damas than in Borans.

At the level of the erythrocyte itself, a clear difference between trypanotolerant N'Dama cattle and susceptible zebu animals has been described. Not only do normal uninfected N'Dama cattle have several-fold higher levels of sialic acid in the erythrocyte membrane than normal zebu cattle (Esievo *et al.*, 1986) but also these membrane components differ with respect to levels and ratios of *O*-acetyl and glycolyl groups (Shugaba *et al.*, 1994). It is conceivable that such differences may be a factor in the ability of trypanosome-infected trypanotolerant cattle to maintain circulating erythrocyte levels by comparison with animals of susceptible breeds.

The search for phenotypic differences between resistant and susceptible hosts when infected with trypanosomes has therefore been relatively productive. In summary, resistant animals make better and more focused antibody responses to trypanosome antigens, possibly reflecting a retained ability to mature the antibody response in a normal manner during the course of infections; at the same time, they possibly possess a superior immunological

memory (Paling *et al.*, 1991b; see also earlier). Resistant animals tend to maintain both erythrocyte and leucocyte profiles at more normal levels during infection than susceptible animals, and this may be a function of an important difference at the level of haemo- and leucopoiesis. If so, this would be consistent with the fact that bone marrow genotype during fetal development is influential in determining resistance/susceptibility status in later life. There is also an intriguing difference between resistant and susceptible animals with respect to erythrocyte membrane components.

The question remains as to whether these differences are meaningful in terms of trypanotolerance, because in no case has a causal relationship between a phenotypic difference and resistance/susceptibility status of the host been demonstrated. As far as aspects of immune response are concerned, while it is clear that a good immune response to trypanosome antigens is associated with the ability to survive infection, it would be surprising if this were not the case. This serves to illustrate the problems inherent in differentiating factors responsible for variation in resistance status from those that are a requirement for survival in the face of challenge, irrespective of resistance status.

Molecular genetic analysis

As far as understanding the basis of trypanotolerance is concerned, it is factors determining variation between resistant and susceptible hosts that are important. Identifying such factors is not a trivial matter but the assortment of genes, and therefore phenotypes, which occurs between generations, provides an opportunity to do so. In effect, a genetic approach in the form of linkage analysis aims to identify co-segregation of some identifiable genetic element with the trait of interest, in this case trypanotolerance. This is fundamentally different from identifying differences that exist in any single generation between groups of animals with different phenotypes. As has occurred in trypanotolerance research, when this is done many differences will usually be identified. The linkage analysis approach identifies only those differences that co-segregate (pass between generations together) with the phenotype of interest, and in this way differentiates causal and casual relationships between observed differences and the trait of interest.

The advent of easily scored molecular (DNA) markers that occur in high density in the genome brought with it the possibility of identifying regions of the genome that co-segregate with trypanotolerance. Not only does this provide the power of segregation analysis; it also points directly to the locations of trypanotolerance genes. Such information is of immediate practical use to the animal breeder, who may use genetic markers to determine which animals inherit trypanotolerance-controlling regions of the genome. It also serves to guide the molecular geneticist in the search for the specific genes concerned.

That within-breed variation in resistance to African trypanosomiasis is under a degree of genetic control in domestic cattle is well established and has been discussed. With respect to between-breed variation, the many early observations of apparent resistance in some West African Longhorn and West African Shorthorn breeds relative to the zebu were strongly indicative. However, the carefully controlled studies of responses to trypanosome challenge of N'Dama cattle reared from embryos in a trypanosomiasis-free environment at the International Laboratory for Research on Animal Diseases (ILRAD) in Nairobi (Paling *et al.*, 1991a,b) put the matter of trypanotolerance as a breed characteristic beyond question. These experiments also provided an early indication that the control of parasites and the ability to maintain PCV might not be highly correlated at the functional level. This was based on the fact that, in a series of challenges with different *T. congolense* clones, those animals which consistently showed the best parasite control did not maintain PCVs as well as some others.

The first insights into the molecular genetics of resistance to trypanosomiasis caused by *T. congolense* have come from linkage analysis studies in murine model sys-

tems, using populations produced by crossing the relatively resistant C57BL/6 strain with the susceptible A/J and BALB/c strains. Gene mapping in the two F_2 intercrosses (Kemp *et al.*, 1997) revealed three genome regions harbouring quantitative trait loci (QTL) for resistance to *T. congolense* on murine chromosomes 17, 5 and 1. These QTLs were designated *Tir1*, *2* and *3*, respectively (where *Tir* indicates 'trypanosome infection response'). Of these, the chromosome 17 QTL *Tir1*, which mapped to the vicinity of the H-2 region, has the largest effect. Further mapping in F_6 advanced intercrosses (Iraqi *et al.*, 2000) has narrowed the target regions considerably and resolved the chromosome 1 QTL into three loci, *Tir3a*, *b* and *c*. In summary, there are at least five genetic loci controlling variation to infection with *T. congolense* in crosses between the resistant C57BL/6 mouse strain and the A/J and BALB/c strains. The genes at these loci are currently unknown but research continues towards their identification. The DNA in the QTL regions is now sequenced and being analysed, and positional candidate *Tir* genes may be forthcoming in the relatively near future.

Linkage analysis in cattle has inevitably proceeded more slowly, due mainly to the relatively long generation interval in this species. Breeding of a segregating population was initiated at ILRAD in 1989 (Teale, 1993), with the pairing of four Gambian N'Dama (*B. taurus*) bulls with four Boran (*B. indicus*) cows. The bulls were derived from embryos obtained in The Gambia that were cryopreserved and transferred to ILRAD in 1983 (Jordt *et al.*, 1986). Four F_1 full-sibling families were produced by multiple ovulation and embryo transfer (MOET) into surrogate Boran cows. Subsequently, over a period of several years, seven pairs of F_1s were intercrossed to produce seven large full-sibling families by MOET comprising a total of 182 F_2 animals. F_2 animals were challenged with *T. congolense* by tsetse bite as they reached 12 months of age. During the period from a few weeks prior to challenge to 150 days after challenge, haematological parameters, levels of parasitaemia and body weights were monitored. The analysis of phenotype data in this study confirmed the positive correlation between maintenance of PCV and growth performance in trypanosomiasis, and provided an indication that extent of parasite control is also positively correlated with growth. Genotyping at microsatellite loci and data analysis have been undertaken by an international consortium of collaborating research groups, and the initial analyses have identified a number of trypanotolerance QTLs (A.J. Teale, Stirling, Scotland, personal communication). It is anticipated that the detailed results of this landmark experiment in trypanotolerance genetics will be published in the near future.

Conclusions

In general terms, therefore, there are correlations between the capacity to maintain haematopoietic and immune functions in the face of infection with trypanosomes, and the ability to survive infection and to be productive. There is also evidence of a correlation between parasite control and resistance status. However, the question remains as to whether all or any of these abilities have a primary or secondary role in trypanotolerance, or indeed whether they are merely phenotype consequences of being genetically resistant to the disease. Do they endow the host with the trypanotolerance phenotype, do they permit the resistance genotype to be expressed, and/or are they the result of trypanotolerance? There is reason to be optimistic that, provided that necessary resources are made available, the power of modern genetics will provide answers to these questions.

The Way Ahead

Clearly, in the immediate future, gains in livestock productivity in the areas of Africa under tsetse-trypanosomiasis challenge will only be made by applying better what is already available and by making better use of current knowledge. Indeed there is great scope for improvement through: (i) con-

trolled and more effective use of trypanocidal drugs; (ii) tsetse fly control; (iii) sound livestock management; (iv) optimized livestock nutrition and provision of reliable and readily available water supplies; and (v) sound national livestock policies. Rather than a lack of disease control options, inadequate funding resources and infrastructures are arguably the most serious impediment to progress.

That aside, the value and sustainability of all disease control approaches must be greatly enhanced when the livestock concerned are genetically disease resistant. In the case of trypanosomiasis, the two problems that must be overcome if disease resistance is to contribute to a major leap in livestock productivity under challenge are, firstly, the real and perceived limitations of trypanotolerant genotypes and, secondly, the relatively small numbers of trypanotolerant animals available to the breeder. The way ahead for trypanotolerance must therefore be: (i) genetic improvement of extant resistant stock; (ii) establishment of trypanotolerance in breeds with other desirable characteristics; and (iii) large-scale breeding and distribution of improved trypanotolerant stock.

For these to be achieved in a meaningful timeframe and on a significant scale, research must deliver reliable and effective selection tools and protocols, and the industry in the affected areas must adopt the very latest intensive breeding methods. There are clear signs that trypanotolerance research is delivering and that opportunities exist for its application in agricultural developments, but effective use of its products will require significant investment at all levels in the affected countries.

With the selection criteria for trypanotolerance already available, or in the process of becoming so, the design of selection programmes is becoming possible. Having established how such criteria relate to all other economically important traits for given production systems, the next step will be to compute the most appropriate relative weightings between these criteria and all economically important traits, based on economic importance, heritability and phenotypic and genetic correlations, in order to develop appropriate and relevant selection indices.

We believe that livestock production under trypanosomiasis risk should focus increasingly on integrated control strategies based on trypanotolerant livestock and on methods for increasing disease resistance, coupled with improved vector control techniques, the more strategic use of trypanocidal drugs and, where possible, improved management and nutrition. The evaluation of the interactions between the components of tsetse-trypanosomiasis risk, different disease control methods and, in general, environmental or ecosystem factors possibly influencing the host response deserves further attention in order to design sustainable strategies that are tailored more to the specific needs, constraints and opportunities of individual production systems and their relevant ecological conditions. There is no one solution that will be valid for all production systems, ecological zones, or regional or national markets.

There is increasing recognition that Africa possesses extensive and valuable animal genetic resources. It has been established that these resources can provide sustainable and environmentally sound solutions for some of the massive disease problems currently confronting Africa. Thus, the natural resistance possessed by breeds of cattle, such as the N'Dama and the West African Shorthorn, to trypanosomiasis and to several other important infectious diseases should be accepted as an important component of national and regional disease control programmes. The fact that these breeds also possess considerable production potential, and that their disease resistance traits could be exploited through crossbreeding with other cattle types, offers a real opportunity to improve livestock production sustainably in the vast areas of Africa held captive by tsetse, ticks and helminths.

As vital research continues, while we should look forward to new knowledge and to the solutions that it may bring, we should perhaps also look back occasionally to the promise that the new immunology of the 1960s and 1970s held for a trypanoso-

miasis vaccine. This is a reminder that while nature has on the one hand provided trypanotolerance, on the other hand, in tsetse-transmitted African trypanosomiasis, nature has provided one of her greatest disease challenges.

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26 Control of Blood Transfusion Transmission of American Trypanosomiasis

Helio Moraes-Souza, José Orlando Bordin and Dante Langhi Jr

Introduction

Chagas disease, an essentially rural disease when first described by the Brazilian scientist Carlos Chagas in 1909, has gradually urbanized because of social migration to large urban centres in the search for a better life and labour conditions (Dias, 1987). The urbanization of the disease occurred not only in endemic countries but also in non-endemic and developed countries located in the northern hemisphere and Australia. It was estimated that in the early 1990s more than 7 million Latin Americans were living legally in the USA, out of which 100,000 to 300,000 suffered from Chagas disease. Moreover, 248,000 Latin Americans were living in Europe, 150,000 in Japan and 80,000 in Australia (Skolnick, 1989; Dias, 1992; Milei *et al.*, 1992; Schmunis, 1994).

The lifelong evolution of Chagas disease and the fact that more than 50% of the infected individuals remain asymptomatic for many years, or for their entire life, favour the possibility of the chronic chagasic individual becoming a blood donor and transmitting the infection. The possibility of transmission of *Trypanosoma cruzi* by blood transfusion was first raised in 1936 in Argentina, but the first case of transfusion-associated Chagas disease (TA-CD) was reported in 1952, in Brazil (Mazza *et al.*, 1936; Freitas *et al.*, 1952).

With the eradication (in some countries) of the bloodsucking triatomine insects that transmit *T. cruzi*, allogeneic blood transfusions have been the main route of transmission of Chagas disease in urban areas of both endemic and non-endemic countries (Schmunis, 1991; Kirchhoff, 1993; Pays, 1998). However, the control of triatomine bugs that has taken place since the early 1980s has also contributed to a reduction in the prevalence of infected blood donors. Up to the 1970s the prevalence of infected blood donors varied between 0.3% and 25.8% (mean = 7.0%) in Brazil, and from 0.2% to 28.0% (mean = 6.0%) in the other endemic countries (Moraes-Souza, 1985) (Tables 26.1 and 26.2). In the early 1990s, a study that compiled serological screening tests for Chagas disease during the previous 10 years in endemic and non-endemic countries found 78,485 infected donors (3.71%) among 2,116,686 serological examinations. In Brazil, the prevalence rate of positive tests ranged between 0.03% and 14.6% (mean = 2.01%), whilst in other countries, including the USA, the prevalence rate of positive tests varied from 0.10% to 62.10% (mean = 4.07%) (Schmunis, 1991; Wendel and Dias, 1992; Moraes-Souza, 1999) (Tables 26.3 and 26.4).

A recent study performed in 14 Spanish-speaking endemic countries showed a sero-

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Table 26.1. Positive serological tests for *T. cruzi* in blood donors in Brazil (1949–1979).

State	City	Year[a]	Donors	Prevalence	Author[b]
Minas Gerais	B. Horizonte	1949	149	1.67	Pellegrino
Minas Gerais	B. Horizonte	1951	576	2.47	Pellegrino *et al.*
São Paulo	São Paulo	1952	1,622	2.50	Freitas *et al.*
São Paulo	São Paulo	1953	536	4.10	Passalacqua *et al.*
São Paulo	São Paulo	1954	786	5.40	Almeida *et al.*
São Paulo	Jaçanã	1958	627	2.00	Castro and Uvo
Parana	North Parana	1958	1,330	6.90	Queiroz and Pascual
São Paulo	Rib. Preto	1959	9,460	11.97	Freitas and Siqueira
Minas Gerais	Uberaba	1959	640	15.00	Jatene and Jácomo
Minas Gerais	B. Horizonte	1959	10,982	6.77	Pellegrino
São Paulo	Rib. Preto	1960	3,493	13.80	Volpn
São Paulo	São Paulo	1962	18,127	1.83	Mellone *et al.*
Rio de Janeiro	R. Janeiro	1963	756	1.83	Ferreira *et al.*
Goias	Goiania	1963	97	25.80	Oliveira *et al.*
Goias	Goiania	1964	1,474	11.00	Alexandre
São Paulo	São Paulo	1965	62,575	2.45	Mellone and Pagenotto
Rio de Janeiro	R. Janeiro	1965	941	0.30	Vegner *et al.*
Rio de Janeiro	R. Janeiro	1966	4,595	1.26	Coura *et al.*
Ceara	Fortaleza	1967	1,230	6.18	Lima *et al.*
São Paulo	São Paulo	1967	2,779	3.80	Antunes
Rio de Janeiro	R. Janeiro	1967	22,508	0.52	Gonzaga *et al.*
São Paulo	Rib. Preto	1972	4,147	5.71	Volpon *et al.*
Goias	Goiania	1975	4,372	10.43	Campos *et al.*
São Paulo	Rib. Preto	1975	110,083	12.56	Cunha
São Paulo	Rib. Preto	1975	2,000	3.80	Vichi
São Paulo	Marilia	1976	992	5.50	Castanho
São Paulo	Rib. Preto	1978	2,000	2.70	Vichi
R.G. Sul	Pelotas	1979	3,105	3.91	Baruffa
Total number of donors			271,982		
Mean prevalence				7.33	

[a]Year in with the samples were collected or the study was published.
[b]In Moraes-Souza, 1985.

logical prevalence rate that oscillated between 0.07% and 17.2% (mean = 2.37%). Amongst 1,663,857 units of blood collected in 1999 by the public Brazilian blood centres (corresponding to approximately 60% of all units of blood collected in Brazil), the positive serology rate found for *T. cruzi* antibodies was only 0.73% (Schmunis, 1999).

Individuals at Risk

Fortunately, only a small number of individuals who receive a *T. cruzi*-infected unit of blood develop the infection; however, the chance of transmission of infection by blood transfusion increases when the natural transmission of the parasite is high in the local population. Several studies indicate that the risk of a patient being infected ranges from 12% to 18% in Argentina, Brazil and Chile (countries in which the rate of natural infection has significantly decreased) but is as high as 49% in Santa Cruz de la Sierra in Bolivia, where transmission is still very active (Schmunis, 1991). In non-endemic countries, the occurrence of transmission of infection is even smaller. Twenty retrospective studies, performed by the American Red Cross on transfusion recipients who received blood products derived from *T. cruzi*-seropositive blood

Table 26.2. Positive serological tests for *T. cruzi* in blood donors in several Latin American countries (1957–1979).

State	City	Year[a]	Donors	Prevalence	Author[b]
Guatemala	–	1957	1,132	7.20	Pinto *et al.*
Venezuela	Valencia	1957	733	10.30	Maekelt
Venezuela	Valencia	1959	449	12.00	Maekelt
Guatemala	–	1959	551	11.40	Leon
Venezuela	Five cities	1960	1,659	8.20	Marquez *et al.*
Costa Rica	–	1960	221	7.69	Berrios
Ecuador	Guayaquil	1961	1,122	3.21	Rodrigues
Chile	Santiago	1962	311	3.30	Howard *et al.*
Venezuela	–	1964	17,794	6.20	Marquez
Argentina	Buenos Aires	1964	11,300	5.26	Cerizola *et al.*
Argentina	S. Del Estero	1966	1,710	20.50	Rebosolán
Argentina	Buenos Aires	1967	25,547	5.42	Cerizola and Lazzzari
Argentina	Buenos Aires	1967	1,724	1.26	Esparrach *et al.*
Peru	Arequipa	1967	500	0.20	Náquira *et al.*
Chile	Santiago	1968	505	3.20	Schenone *et al.*
Argentina	Cordoba	1969	200	24.50	Undino *et al.*
Argentina	Cordoba	1971	11,100	14.20	Damonte
El Salvador	San Salvador	1971	537	8.70	Hernandez and Cedillos
Argentina	Buenos Aires	1972	97,308	6.05	Cerizola *et al.*
Paraguay	–	1972	562	11.30	Canese
Peru	Teane	1972	393	12.90	Náquina *et al.*
Uruguay	–	1972	329	5.50	Ozimani
Honduras	Tegucigalpe	1973	50	28.00	Ponce and Zeledón
Venezuela	Caracas	1973	98,260	5.10	Maekelt
Mexico	–	1978	298	4.40	Goldsmith *et al.*
Colombia	Bogota	1971	1,012	2.70	Guhl *et al.*
Chile	Vicuna S. Coquimbo	1979	244	6.50	Lorca *et al.*
Total number of donors			275,551		
Mean prevalence				6.01	

[a]Year in which the samples were collected or the study was published.
[b]In Moraes-Souza, 1985.

donations, did not demonstrate evidence of TA-CD (Leiby *et al.*, 1997b). These results suggest that the concentration of parasites in donated blood in non-endemic areas might be too low to establish infection in most transfusion recipients.

Therefore, the risk of acquiring TA-CD depends mainly on the following factors: (i) the prevalence of the infection in blood donors; (ii) the type and number of *T. cruzi*-infected blood products transfused; (iii) the parasite concentration in the infected transfused unit; (iv) the virulence of the parasite strain; (v) the immunological status of the transfused recipient; (vi) the coverage of screening of blood donors; and (vii) the sensitivity of the serological tests (Grant *et al.*, 1989; Nickerson *et al.*, 1989; Schmunis, 1991; Schmunis and Zieker, 1998; Shulman, 1999).

It has been reported that the incubation period in cases of TA-CD ranges from 8 to 120 days (Yasuda *et al.*, 1990). Recently, it was reported that a patient with multiple myeloma, who was unintentionally transfused with a platelet concentrate from an asymptomatic blood donor who had *T. cruzi* antibodies, developed a positive ELISA for *T. cruzi* antibodies 100 days after transfusion, whilst parasitaemia was detected 40 days before seroconversion. These findings suggest that the differences between various blood components may be relevant, since four of five reported cases in the USA and Canada involved transfusion of platelet

Table 26.3. Positive serological tests for *T. cruzi* in blood donors in Brazil (1980–1989).

State	City	Year[a]	Donors	Prevalence	Author
Espirito Santo	Vitória	1980	4,108	2.36	Barros *et al.*[b]
Minas Gerais	Uberaba	1985	10,988	9.00	Moraes-Souza *et al.*[b]
Distrito Federal	Brasilia	1984	2,413	14.60	Pereira[c]
Goiás	Goiania	1989	62,814	3.30	Andrade *et al.*[c]
Minas Gerais	Several cities	1984	2,300	5.70	Dias and Brener[c]
Minas Gerais	Uberaba	1984	5,632	4.83	Moraes-Souza *et al.*[c]
Parana	Londrina	1981	3,000	7.50	Marzochi[c]
R.G. Sul	Porto Alegre	1981–1983	15,338	0.21	Liz[c]
São Paulo	São Paulo	1982	56,902	2.90	Waldmon[c]
Santa Catarina	Florianopolis	1986	3,540	0.09	Schlemper *et al.*[c]
R.G. Sul	Pelotas	1985	4,215	4.01	Baruffa[d]
Minas Gerais	B. Horizonte	1987	4,806	4.00	Dias *et al.*[d]
R.G. Sul	Porto Alegre	1988	181,266	0.93	Silva[d]
Minas Gerais	Uberaba	1989	4,923	5.0	Moraes-Souza *et al.*[d]
Rio de Janeiro	R. Janeiro	1989	9,828	0.03	Azevedo[d]
Total number of donors			372,073		
Mean prevalence				2.18	

[a]Year in which the samples were collected or the study was published.
[b]Moraes-Souza (1985); [c]Schmunis (1991);[d]Wendel and Dias (1992).

concentrates (Leiby *et al.*, 1999). Temperatures at which blood components are stored have been shown not to inactivate *T. cruzi*: frozen parasites remain infective for long periods of time and patients might be infected after being transfused with fresh or thawed plasma, or cryoprecipitate (Filardi and Brener, 1975).

Although most recipients of *T. cruzi*-infected blood do not develop acute-phase Chagas disease, when it does occur the acute phase presents as a fever unresponsive to antibiotics, with lymphadenopathy and hepatosplenomegaly. Untreated patients may recover spontaneously after 6–8 weeks and enter the chronic phase of infection. However, cardiac failure, neurological complications including encephalitis, meningitis and death may occur during the acute phase of the disease in severely immunocompromised patients (Rocha, 1992).

The predominance of asymptomatic or oligosymptomatic infections, as occurs with vectorial transmission, explains the small number of fewer than 300 well-documented cases of TA-CD in the literature (Wendel and Dias, 1992; Teixeira, 1997). Epidemiological studies performed in the 1970s in Brazil, prior to the implementation of both vector control and blood donor screening, estimated that 20,000 new cases resulted from 4 million blood transfusions per year (Dias, 1979). In the 1980s, with the implementation of control strategies for vectorial and blood-borne transmission, the incidence in Brazil dropped dramatically (Schmunis, 1999). As a result of the National Program for Blood and Blood Derivatives of the Health Ministry of Brazil (Gerência Geral de Sangue e Hemoderivados da Agência Nacional de Vigilância Sanitária do Ministério da Saúde), the number of individuals infected by blood transfusion is thought to be fewer than 100 per year. A recent study conducted by the Pan American Health Organization (PAHO) measured the risk of transfusion-transmitted infectious diseases in 12 countries of Central and South America. It was concluded that the probability of getting a transfusion-transmitted *T. cruzi* infection ranges from 2×10^4, in Ecuador, to 219×10^4, in Bolivia (Schmunis and Ziecker, 1998). Despite efforts over the last decade, by almost all countries in Central and South America, to eliminate domestic transmission of *T. cruzi*, control of TA-CD depends on implementation of strategies that assure reliable screening of blood donors (Dias and Schofield, 1998).

Table 26.4. Positive serological tests for *T. cruzi* in blood donors in several Latin America countries (1980–1990).

State	Province or City	Year[a]	Donors	Prevalence	Author
Argentina	Cordoba	1983	283,962	7.60	Bergoglio
Argentina	13 Provinces	1981	95,904	8.70	Schmunis[b]
Argentina	13 Provinces	1981	95,904	8.70	Schmunis[b]
Argentina	11 Provinces	1982	102,857	8.80	Schmunis[b]
Argentina	Rioja	1986	588	20.40	Schindler[b]
Argentina	20 Provinces	1987	153,018	8.62	Perez and Segura[b]
Bolivia	Santa Cruz	1982–1983	280	62.10	Zuna *et al.*[b]
Bolivia	7 Provinces	1988–1989	1,197	22.47	Carrasco *et al.*[b]
Chile	Santiago	1980–1985	5,442	1.45	Eight references[b]
Chile	Region I	1982–1983	2,342	2.02	Two references[b]
Chile	Region II	1981–1984	2,212	4.07	Two references[b]
Chile	Region III	1982–1983	1,144	6.50	Four references[b]
Chile	Region IV	1981–1985	2,057	6.90	Four references[b]
Chile	Region V	1983–1984	1,995	3.16	Villablanca *et al.*[b]
Chile	Region VI	1983–1989	2,041	1.27	Valenzuela *et al.*[b]
Colombia	Santander	1987	491	7.50	Guhl *et al.*[c]
Costa Rica	3 Cities	1983–1985	2,574	1.01	Urbina *et al.*[b]
Ecuador	9 Provinces	1986–1990	26,167	0.28	Red Cross of Ecuador[b]
Guatemala	Several cities	1987	1,260	5.00	Caceres *et al.*[b]
Honduras	Several cities	1987	2,225	11.60	Ponce and Ponce[b]
Mexico	Puebla	1986	200	17.50	Velasco-Castrajón *et al.*[b]
Paraguay	Assuncion	1986	1,000	6.80	Schmunis[c]
Uruguay	6 Provinces	1989	2,566	2.88	Calegari[b]
Uruguay	Montevideo	1986	40,774	0.92	Franca[c]
Uruguay	Montevideo	1986	39,691	1.03	Arago[c]
USA	California	1988	1,027	0.10	Kendt[b]
Venezuela	–	1985–1989	972,599	2.70	Brito[b]
Total number of donors			1,841,517		
Mean prevalence				4.85	

[a]Year in which the samples were collected or the study was published.
[b]Schmunis (1991); [c]Wendel and Dias (1992).

Strategies to Prevent Transfusion-associated Chagas Disease

Current strategies to prevent transfusion-associated Chagas disease include: (i) general approaches, such as donor education and the identification of the putatively high-risk infectious blood donors by their history; (ii) laboratory investigation of the donor blood; (iii) treatment of the collected blood with gentian violet in areas of high endemicity; and (iv) alternative prophylactic measures such as filtration and irradiation of blood products (Moraes-Souza and Bordin, 1996).

General approaches

Because *T. cruzi* infection is lifelong and most infected people are asymptomatic, the education of the donor population by recruiters and pamphlets should be encouraged. In addition, a pre-donation questionnaire, to identify individuals who might be infected with *T. cruzi*, may be useful in selecting safer donor populations. Using a pre-donation questionnaire, it may be possible to exclude blood donors who emigrated from endemic areas; however, such an approach may significantly reduce the blood supply in certain geographical areas (Appleman *et al.*, 1993).

The use of questionnaires designed to detect risk of infection may be particularly relevant in non-endemic countries but may have limited usefulness in endemic areas. In a non-endemic area, using a questionnaire, blood donors should be considered to be at risk for *T. cruzi* infection if they lived in an endemic area for more than 1 year in a dwelling with mud walls or thatched roof, or if they received a transfusion in an endemic area, or if they have a history of Chagas disease (Galel and Kirchhoff, 1992). In one study on the use of a questionnaire for detecting risk factors for Chagas disease in an endemic country (Salles *et al.*, 1996), affirmative answers to two questions were demonstrated to be epidemiological evidence for previous exposure to *T. cruzi*: 'Does someone in your family have Chagas disease?' and 'Have you ever lived in a house where the Chagas bug was present?' In another study, the sensitivity of a pre-donation questionnaire to identify possible *T. cruzi*-infected blood donors ranged from 50% to 55%, including questions like: 'Have you ever lived in a house where the Chagas bug was present?'; or 'Are you familiar with the Chagas bug?'

The use of a questionnaire as a policy to screen donors of a non-endemic area for the risk of exposure to the parasite, such as from birth, from extended stay, or from transfusion in an endemic area, has been shown to be effective in detecting *T. cruzi*-seropositive and parasitaemic blood donors (Leiby *et al.*, 1997a,b). Recently, a study suggested the possibility of vertical transmission of Chagas disease in two blood donors seropositive for *T. cruzi* infection, with no other known risk factor (Leiby *et al.*, 1999). In that study, the donors had a family medical history of cardiac ailments, especially arrhythmias and death related to enlarged hearts, as commonly associated with Chagas disease (Hagar and Rahimtoola, 1991).

Pre-donation questionnaires can be practical and may identify persons at high risk of infection, but may not be as sensitive as other policies, such as serological testing, to prevent transfusion-transmitted Chagas disease. Furthermore, such a questionnaire policy may not be specific enough to avoid loss of excessive numbers of healthy individuals. Studies in a non-endemic country showed that a screen of donors for *T. cruzi* risk factors might exclude only 75% of *T. cruzi*-seropositive (and presumably parasitaemic) donors from donating blood. Yet such a policy could result in the loss of up to 8% of healthy donors, which could impact negatively on blood availability (Leiby *et al.*, 1997b).

Laboratory investigation

The diagnosis of *T. cruzi* infection can be made by detecting either parasites, parasite antigens, or anti-*T. cruzi* antibodies. Active circulating forms of *T. cruzi* (trypomastigote forms) may be detected directly in acute infection by microscopical examination of blood or the buffy coat; however, this method lacks sensitivity, especially in chronic infection, because of the low number of circulating parasites (Woo, 1971). Parasitological diagnosis of chronic *T. cruzi* infection can also be made by indirect methods, including xenodiagnosis and blood culture (see also Chapter 12).

Blood culture

One blood culture method consists of adding 1 ml of possibly *T. cruzi*-infected donor blood to 3 ml of liver infusion tryptose (LIT) medium. Microscopical examination for *T. cruzi* in the culture is performed after 30 days; negative samples are further evaluated after 60 days. Modifications in the blood culture technique such as triplicate cultures and 120 days of culture, increase sensitivity from $< 50\%$ to between 79% and 94% (Luz *et al.*, 1994) (see also Chapter 12).

Xenodiagnosis

This is a laborious procedure involving feeding blood from a potentially infected donor to 40 third-instar laboratory-reared triatomine nymphs, and subsequent dissection and microscopical examination of the triatomine bug intestinal contents for *T. cruzi*. Xenodiagnosis is highly specific but, like

blood culture, has a sensitivity of about 50% (Chiari, 1992). An important limitation of blood culture and xenodiagnosis for screening blood donors is the long time required to obtain results.

Serological screening

As parasitological methods have limitations, serological screening has become an accepted alternative means of preventing transfusion-associated Chagas disease. Serology detects specific antibodies to *T. cruzi* antigens in silent, chronic carriers of *T. cruzi* infection. A policy to test donors for the presence of specific antibodies to *T. cruzi* can be effective in preventing transfusion of parasitaemic blood. Studies have shown that nearly two-thirds of *T. cruzi*-seropositive blood donors may be parasitaemic at the time of blood donation and may therefore be capable of transmitting Chagas disease (Tibbals *et al.*, 1998), though only 12–49% develop *T. cruzi* infection (Schmunis, 1991).

Methods available for the serodiagnosis of *T. cruzi* infection include: (i) complement fixation (CF); (ii) indirect haemagglutination (IHA); (iii) indirect immunofluorescence (IIF or IFAT); and (iv) ELISA. Generally, these assays are sensitive, but the serological diagnosis of Chagas disease is complex, yielding both false-positive and false-negative results (Camargo, 1992).

- The *CF test* (Machado-Guerreiro test), although still used in certain areas of South America, is a difficult test to standardize and has thus been replaced by other techniques (Camargo, 1992). Its sensitivity with extracts of *T. cruzi* as antigen ranges from 35%, in acute infections, to 95% in chronic infections (Ferreira, 1992).
- The *IHA test* has been used widely as a serological screening test for *T. cruzi* infection because it is easy to perform and requires no special equipment. In general the IHA is a sensitive test (Camargo *et al.*, 1973).
- The *IIF test* was considered for many years to be the reference test for detecting IgG antibodies in chronically infected individuals and IgM antibodies in acutely infected patients. However, the final result is based on the subjective interpretation of the reaction (Camargo *et al.*, 1984).
- The *ELISA test* has allowed automation in the diagnosis of *T. cruzi* infection and it does not rely on the subjective interpretation of the result.

The overall agreement between ELISA and IHA is about 97%, between ELISA and IIF 90%, and between ELISA and a combination of IHA and IIF about 98%. The reported sensitivity and specificity of ELISA range from 78% to 99% and 97% to 99%, respectively (Camargo, 1992). Another recent comparative study showed that sensitivity of ELISA was 99.3% and of IHA 99.8% and the specificity was 100% and 74.9%, respectively (Blejer *et al.*, 1999). Cross-reactions have been observed with serum containing antibodies against *Leishmania* spp. and *Mycobacterium tuberculosis* and with sera from autoimmune disease (Camargo, 1992; Langhi *et al.*, 1996). *Trypanosoma rangeli* has a partially overlapping geographical distribution with *T. cruzi* and occasionally affects *T. cruzi* serology (Guhl, 1990; Ramirez *et al.*, 1998). The serological diagnosis of *T. cruzi* infection has been limited by poor knowledge of the composition and immunogenicity of the various parasite antigens. The ideal *T. cruzi* antigens have been defined as those present in different endemic areas, absent in other infectious agents, and not reactive with autoantibodies (Stolf, 1992). Absence of antibodies to *T. cruzi* does not preclude possibility of a very recent infection, because the antibodies are not readily detected during the early acute phase.

In a comparison between four laboratories (Bolivia, Chile, Colombia and Panama), four different tests (CF, IHA, IIF and ELISA) varied considerably in efficiency to discriminate sera from truly infected and non-infected individuals (Camargo, 1992). In a further study, three commercially available serological tests (IHA, IIF and ELISA) varied in sensitivity from 91.1% for IHA to 100% for IIF and ELISA when evaluated against sera from individuals with positive xenodiagnosis. However, specificity ranged from 25.4% for IIF to 91.3% for IHA, and 96.1% for ELISA when evaluated with sera from cases of leishmaniasis (Langhi

et al., 1996). Lack of specificity would result in too many false healthy donors, causing unnecessary alarm and anxiety (Carvalho *et al.*, 1993). An ELISA test based on recombinant antigens showed no cross-reactivity with sera that were positive for other parasitic diseases (Carvalho *et al.*, 1993).

In the light of these discrepancies, in some countries, such as those of the MERCOSUL block (Argentina, Brazil, Paraguay and Uruguay), it is mandatory that units of blood are screened for *T. cruzi* antibodies by at least two distinct serological techniques.

ELISA with an antigen derived from *T. cruzi*, coated on to polystyrene beads, for detection of antibodies to *T. cruzi* in the sera of blood donors in USA, showed a sensitivity of 100% in samples positive by either xenodiagnosis or consensus (reactive in ELISA, IHA and IIF) and specificity was 99.98% with negative samples (Brashear *et al.*, 1995).

WESTERN BLOT (WB) The WB method for confirmation of seroreactivity has not been completely successful because sodium dodecyl sulphate (SDS) detergent used may denature conformational epitopes, exposing epitopes that are cross-reactive with sera from patients with other diseases (Wong *et al.*, 1986). Nevertheless, it has been reported that WB may recognize distinct patterns of antibody response in some patients with *T. cruzi* infection, leishmaniasis and double infection (Chiller *et al.*, 1990; Reiche *et al.*, 1998). A more recent study used a recombinant and synthetic peptide antigen-line immunoassay for serological confirmation of Chagas disease in blood donors who reacted in at least one of three serological screening assays, assuming as a gold standard the consensus results obtained by the three different screening assays. The test showed a sensitivity of 99.4% and a specificity of 98.1%, for positive and negative sera; for samples reacting in one and two screening tests, 3.4% and 1.9% were negative, respectively (Saez-Alquézar *et al.*, 2000).

RADIOIMMUNOPRECIPITATION ASSAY (RIPA) RIPA has been proposed as a confirmatory test for serological screening. To be considered positive, the RIPA needs to show characteristic bands at 32, 34 and 90 kDa. In one evaluation with blood the RIPA showed a specificity of 100% in ELISA non-reactive samples and a sensitivity of 100% with sera from xenodiagnosis-positive patients (Brashear *et al.*, 1995). However, in our experience, RIPA showed a sensitivity of 91% when tested with sera from xenodiagnosis-positive patients, and a specificity of only 26.6% with samples from leishmaniasis (non-chagasic) patients (Langhi *et al.*, 1996).

POLYMERASE CHAIN REACTION (PCR) ASSAY Preliminary results with PCR indicate that it is possible to detect one parasite in 20 ml of blood (Diaz *et al.*, 1992; Avila *et al.*, 1993). Nevertheless, false-negative PCR has been reported to occur in clinical cases with positive xenodiagnosis (Sandler, 1989).

LATENT CLASS EVALUATION Seroprevalence of antibodies to *T. cruzi* has been investigated in many countries. In areas with high prevalence of infection, positive serological screening tests have a high predictive value for infection; in areas of low prevalence, positive tests have a low predictive value because false-positive results may predominate. In Brazil it is mandatory that units of blood should be screened for *T. cruzi* antibodies by at least two distinct serological techniques. When there is no perfect serological gold standard a statistical model called the latent class model may be used to evaluate the sensitivity, specificity and positive predictive value of serological tests (Szatmari *et al.*, 1995). Latent class evaluation of ELISA, IHA and IIF with 1951 Brazilian blood donors gave an estimated sensitivity of 100% for ELISA and IIF but only 60% for IHA. The estimated specificity was 100% for IHA, 99.69% for IIF and 99.95% for ELISA. According to this evaluation, the possibility of being a *T. cruzi*-infected blood donor was 0% when only one of the tests was positive. In contrast, when both ELISA and IHA were positive, the probability of being a true infected blood donor was 85.71%, and when positivity with both ELISA and IIF occurred, the probability of a true infection was 99.92% (D.M. Langhi, *et al.*, 2002).

Alternative prophylactic strategies

Filtration of blood products

It has been suggested that the filtration of blood components may result in decreased risk of *T. cruzi* infection. Leucocyte depletion reduced the number of *T. cruzi* in infected blood but some remained (Moraes-Souza *et al.*, 1995; Moraes-Souza and Bordin, 1996; Fabron *et al.*, 1999).

Plasma lyophilization

The various methods of lyophilization to prepare plasma or derivatives such as albumin, gamma globulin and clotting concentrates have been shown to effectively inactivate *T. cruzi* (Amato-Neto *et al.*, 1966).

Overview and Conclusions

Despite all the preventive measures discussed above, blood recipients are still being infected with *T. cruzi*. Therefore, definitive control of TA-CD in endemic countries depends on the commitment of governments and all professionals directly or indirectly involved in blood transfusion medicine. In the late 1980s, when assessment of the status of Chagas disease in the Southern Cone countries (Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay) showed a dramatic picture, the governments of these countries decided to implement an ample control initiative, which included elimination of *T. cruzi* transmission by blood transfusion, through the improvement of the blood transfusion services and the serological control of the blood donors (OPS, 1992; Dias and Schofield, 1998). In Brazil, since 1991, in addition to the US$135 million for controlling the insect vector, US$32 million was employed to improve the government blood centre facilities. Furthermore, the Brazilian Minister of Health developed, in 1998, a National Global Goal to reach the highest quality and safety in the whole blood preparation process by 2003 and allocated approximately US$200 million to this programme (OPS, 1999; H. Moraes-Souza, Brasilia, 1999, personal communication).

From only three countries having legislation to regulate blood transfusion, after 8 years all Latin American countries with the exception of El Salvador and Nicaragua have created laws, decrees or regulations that regulate the collection and use of blood components (Schmunis, 1999). However, only three of 15 countries were performing complete serological screening, suggesting that, although the risk is decreasing, TA-CD probably still occurs throughout the region (Schmunis and Ziecker, 1998; Moncayo, 1999).

The definitive control of TA-CD will only be guaranteed in endemic countries when: (i) governments enforce compliance with the laws; (ii) all donors are volunteers; (iii) collection, processing and use of blood complies with quality control; (iv) reagents used in serological screening of donors are of good quality; and (v) use of blood and blood components is judicious (Schmunis, 1999). In conclusion, besides the recommendations described above, the current control of TA-CD depends mainly on: (i) serological screening of donors using two techniques with different principles; (ii) use of gentian violet in highly endemic regions; and (iii) clinical screening of blood donors in non-endemic countries to eliminate those who present risk factors for *T. cruzi* infection.

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27 Insecticidal Control of Tsetse

Reg Allsopp and Brian H. Hursey

Insecticides Used for Tsetse Control

Chemical compounds used to control tsetse flies

Tsetse flies (*Glossina* spp.) are susceptible to most insecticides but their biology is unique in many ways and only chemicals with specific properties have proved suitable. A wide range of natural and synthetic insecticides is available and although many offer effective levels of toxicity, relatively few have been widely used. Toxicity to the insect is obviously important but has not been a major determinant. Low toxicity to the operators has been a key factor, particularly where tsetse control was carried out over large areas and those applying the insecticides under arduous African bush conditions could be subjected to prolonged exposure. However, the tsetse life cycle, with a period of juvenile development several centimetres below ground, has largely determined the characteristics required, the formulation and the method by which insecticide is delivered.

Only two chemical groups have enjoyed wide success in the control of tsetse flies. These are the organochlorines, notably DDT and dieldrin, plus endosulfan (which is in the cyclodiene subgroup), and more recently the synthetic pyrethroids.

DDT (dichlorodiphenyltrichloroethane) was first synthesized by O. Zeidler in 1874 (West and Campbell, 1950) but its insecticidal properties were not demonstrated by the Swiss scientist Paul Muller until the 1930s (Muller, 1939). A Swiss patent was issued in 1942 and it was tested in the USA the same year. It proved highly effective, with a low mammalian toxicity, and initially the entire production was used by the military to protect troops involved in the Second World War against insect vectors carrying such diseases as typhus and malaria. It was released for non-military use in 1945. Its low production costs together with long persistence (half-life of 10–35 years, which is particularly variable under tropical conditions), insolubility in water and high contact toxicity made it cost-effective and a great commercial success. By 1945 DDT and another class of chlorinated hydrocarbon compounds produced by the Diels-Alder reaction (e.g. dieldrin and endosulfan) were widely used in agriculture and by 1972 some 3 million tonnes of DDT had been produced (WWF, 1998). Muller was awarded the Nobel Prize in 1948.

The organochlorine insecticides became the cornerstone of tsetse and trypanosomiasis control throughout Africa for some 40 years. Treatment of resting and breeding sites with residual deposits made them lethal to tsetse for several months – long enough to ensure that they remained available and toxic to young tsetse emerging from months of pupal development underground. In time, the very characteristic which made these compounds so commercially successful in developed countries, i.e. their persistence,

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led to restrictions on their use and ultimately to international bans.

As a result of increasing concerns surrounding the use of persistent insecticides, techniques were devised to reduce large-scale bioaccumulation. Endosulfan (hexachlorohexahydro-methano-benzo dioxathie-pin-oxide) breaks down more easily than the other organochlorine insecticides and does not have the same potential for bioaccumulation. Being an effective insecticide that can be dispersed in light oil solvents, and widely available at reasonable cost, endosulfan was a suitable choice for non-residual aerosol applications from aircraft.

The toxicity to tsetse of other insecticidal compounds such as the organophosphates and carbamates was assessed in the laboratory and in the field but none proved to be suitable alternatives to the organochlorines until the discovery the synthetic pyrethroids. The insecticidal properties of natural pyrethrum from *Chrysanthemum* flowers was recognized by the Chinese 2000 years ago but the first documented research on the insecticidal properties of pyrethrum was reported by Staudinger and Ruzicka (1924). Synthetic analogues of pyrethrin with high insecticidal activity but low mammalian toxicity were produced as early as the 1940s. These early compounds, the allethrins, underwent further development and the potent insecticide resmethrin was reported by Elliott *et al.* (1967). Marketed as Bioresmethrin, this was a potent insecticide yet readily metabolized by mammals. It was, however, unstable in sunlight, which was a fundamental disadvantage of the pyrethroids to that date and a severe impediment to their extensive agricultural use.

The problem of photodegradation was finally solved with the modification of resmethrin to permethrin. A series of photostable, synthetic pyrethroids followed, culminating in the production of deltamethrin (Elliott *et al.*, 1974; Roussel-Uclaf, 1982), previously known as NRDC 161 and decamethrin. This most powerful insecticide is orders of magnitude more toxic than the organochlorines to tsetse flies (Table 27.1); it has a broad invertebrate control spectrum yet is readily degraded by many vertebrates.

Delivery methods

Chemical control depends upon sufficient contact between the tsetse fly and the insecticide for the fly to pick up a lethal dose. One stage in the tsetse life cycle, a period of pupal development lasting from 1 to 3 months, takes place underground and protects this juvenile proportion of the population from direct chemical attack. Delivery methods therefore had to be designed to ensure that the entire target population contacts the insecticide at some point and this requires either long-lasting, carefully placed residual deposits or repeated space sprays spanning the full tsetse life cycle. Based on a wealth of ecological and behavioural knowledge, this requirement was engineered in a number of ways, such as: (i) by depositing residual insecticide, either selectively on to places such as resting or breeding sites that tsetse will visit (e.g. by ground spraying), or by treating the entire habitat with residual insecticide from the air; (ii) by repeatedly spraying non-residual insecticide aerosols on to tsetse flies, either over large areas using aircraft (e.g. by the sequential aerosol technique), or in more localized areas using hand-held or vehicle-mounted fogging machines; and (iii) by attracting tsetse to artefacts that are impregnated with persistent insecticide (e.g the artificial and live-bait techniques).

Ground spraying has been the most widely used method for delivering residual insecticides and the sequential application of low-dosage aerosols from fixed-wing aircraft (sequential aerosol technique, or SAT) for non-residuals.

Residual techniques

Various methods have been developed for treating tsetse habitats with residual deposits of insecticide. Vehicle-mounted spraying machines have been used – for instance, on all-terrain-vehicles such as the Mercedes Unimog in Botswana and Zambia. Helicopters were used in Nigeria and Cameroon where high insecticide dosages, such as ultra-low volume (ulv) formulations of endosulfan at 1kg/ha, were needed to provide a residual effect. Similar operations

Table 27.1. Relative toxicity of some insecticides to tsetse flies (based on topical applications to *Glossina austeni* and *G. morsitans*) and to mammals (oral LD_{50} to rats) taking DDT as the standard. Also showing oral and contact toxicity to honey bees.

Chemical group	Insecticide	Chemical formula	Relative toxicity		Oral LD_{50}		Contact LD_{50}
			To tsetse flies	To mammals	To rats[a] (mg/kg)	Honey bees (ng/bee)	Honey bees (ng/bee)
Organochlorine	DDT	$C_{10}H_{22}O$	1	1	113	Non-toxic[b] (27,000)	Non-toxic
	Dieldrin	$C_{12}H_8Cl_6O$	9	3.05	37	Hazardous	
	Endosulfan	$C_9H_6C_{16}O_3S$	12	1.41	80	Moderately toxic[b]	Harmless[c]
Organophosphate	Malathion	$C_{10}H_{19}O_6PS_2$	0.5	0.05	2100	Highly toxic[b]	(710[c])
	Dimethoate	$C_5H_{12}NO_3PS_2$	2	0.75	150	Highly toxic[c]	Toxic (120[b])
Carbamate	Carbaryl	$C_{12}H_{11}NO_2$	2	0.38	300	Lethal[b]	
Natural pyrethrum	Pyrethrin(s)	$C_{21}H_{28}O_3$	7	0.15	500–1000	Toxic[c] (150)	(120–190[c])
Synthetic pyrethroids	Permethrin	$C_{21}H_{20}C_{12}O_3$	29	0.23	500	Extremely toxic[b]	
	Cypermethrin	$C_{22}H_{19}C_{12}NO_3$	117	0.45	250	Highly toxic[b]	No risk from field applications[c]
	Cyfluthrin	$C_{22}H_{18}C_{12}FNO_3$	387	0.45	250	Highly toxic[b] (37)	Harmful[c]
	Lambda-cyhalothrin	$C_{23}H_{19}ClF_3NO_3$	560	0.78	144	Highly toxic[c] (38)	(909[c])
	Deltamethrin	$C_{22}H_{19}Br_2NO_3$	1100	0.84	135	Toxic[b] (79)	(500[b])

Information based on unpublished data from the UK Natural Resources Institute; [a]WHO (1990); [b]EXTOXNET website; [c]*The Pesticide Manual*.

were carried out in a number of West African countries with lower dosages of endosulfan and with synthetic pyrethroids but the indiscriminate nature of these methods caused environmental contamination and the technique has been largely discontinued. Ground spraying (see Plate 8) using knapsack machines with long spray lances did prove highly effective for applying insecticide in a selective and controlled manner and for many years it was adopted by control authorities virtually throughout Africa.

When DDT became commercially available in 1945, its suitability for tsetse control was immediately apparent. Being highly toxic to tsetse but not to the operators, miscible with water when formulated and extremely persistent, even when exposed to Africa's climatic extremes, it could be applied to the tsetse habitat in such a way that it remained toxic to an entire generation of tsetse flies. It was also inexpensive.

Ground spraying initially involved the treatment of all vegetation within reach of an operator's spray lance. It subsequently became 'discriminative' or partial spraying, where only the vegetation most favoured by tsetse within the wider habitat was treated. It was then further refined to 'selective' spraying, being restricted to resting and breeding sites such as tree trunks, lower branches, rot holes and holes in the ground (Davies, 1967).

The technique is best suited for application in those regions, such as southern Africa, that have a prolonged dry season. At this time of year, tsetse flies – particularly savannah species – are already under stress from temperature extremes, limited favourable habitat and reduced host availability. During the dry season water is scarce, deciduous vegetation is normally leafless and tsetse populations are at their lowest density. They seek refuge in shadier sites and spraying can be specifically targeted so that only some 20% of the habitat requires treatment. The lack of any significant rainfall ensures that a single application of insecticide will persist at a lethal level well beyond the pupal period. The main impediment to success under these conditions is the occurrence of bush fires which are common towards the end of the dry season and can destroy insecticide deposits before they have achieved their required effect. This can be minimized by controlled, pre-spray burning, but may be incompatible with some forms of land use.

In moister areas where rainfall is more frequent, residual insecticide deposits can be washed away. This may require more frequently repeated spraying or more persistent – and generally more expensive – formulations. Bioassays of the treated vegetation indicate where additional effort is required.

To be effective, ground spraying requires meticulous planning and careful implementation. Operations in Nigeria and Uganda in the 1960s were carried out with almost military precision. It is highly labour intensive and while the planning must ensure good coverage it must also minimize the distances over which water and insecticide have to be carried. Access roads, usually cut by hand, need only be temporary but they frequently become permanent with local use and can open up wilderness areas for unlawful hunting pursuits etc.

Non-residual techniques

To overcome the problems posed by underground pupal development, while reducing the dependence upon residual insecticides, SAT was designed to deliver a series of low-dosage, non-residual insecticide aerosols which would drift through the target area to kill all the adult tsetse flies. This has to be repeated while juveniles continue to emerge from underground for the duration of the pupal period. To minimize environmental impact and cost, the inter-spray period must be timed to ensure that newly emerged females do not mate and deposit new larvae before the next application, as this would prolong the duration of underground development and thus the number of treatments. The pupal period and first larval period are temperature dependent and suitable timing intervals can be calculated from simple formulae (Glasgow, 1963) supported by entomological monitoring such as ovarian ageing (Saunders, 1962). Most successful SAT operations have employed five treatments at intervals of 15–20 days.

Aerial spraying for tsetse control developed from crop-spraying techniques. Insecticides such as DDT and benzene hexachloride (BHC) dissolved in diesel oil were initially applied through the thermal exhausts of fixed-wing aircraft in South Africa (du Toit and Klug, 1949). The method was progressively improved, notably at the Tropical Pesticides Research Institute in Tanzania, until large-scale trials were carried out in Zambia (Park *et al.*, 1972). Thermal exhaust systems produced relatively coarse aerosols, therefore less drift and some residual effect. Specialized rotary atomizing equipment was subsequently developed to produce smaller droplets and these were used to apply more readily degradable formulations of endosulfan, with very encouraging results in Botswana (Kendrick and Alsop, 1974) (see Plate 9).

The drift of low-dosage aerosols and penetration through the tree canopy to the primary tsetse habitat is dependent upon droplet size. The aerosol must consist of droplets with diameters in the range of 10–80 μm, ideally with a volume median diameter (VMD) of 20–30 μm (Hadaway and Barlow, 1965). However, the terminal velocity of these minute droplets is very low; for instance, a 20 μm droplet will fall through still air at only 0.012 m/s (Matthews, 1979) and any upward air movement or general turbulence in excess of this prevents its descent.

Stable (but not completely still) air conditions are therefore essential for SAT and these are characterized by the presence of a temperature inversion. During normal daylight hours there is a lapse rate between the warmer ground and the air above, i.e. air temperature decreases with height. At night, the ground loses heat in the form of long-wave radiation and the layer of air immediately above it warms up, inverting the temperature gradient so that temperature increases with height. This creates an inversion layer that can last throughout the night and can vary in depth from ground level to a few metres above the canopy or to several thousand metres. As long as it is present to at least a few metres above the canopy and the aircraft can spray into the inversion layer, the aerosol droplets are able to descend to the target location.

SAT spray planes therefore fly low-level, i.e. close to the tree canopy, at night. Indeed, the pilots prefer to remain within visual contact of the canopy or the ground and for this, plus other night-time manoeuvres, the aircraft must be fitted with powerful lights. Also, the insecticidal properties of SAT aerosols are short-lived and they must reach the entire target population in a short period of time; thus it is a 'blanket' technique. To ensure that the target area is accurately and comprehensively treated, the aircraft must be capable of accurate, high-resolution navigation with flight intervals some 250 m apart. This can now be inexpensively achieved using global positioning systems (GPS).

A total area in excess of 140,000 km^2, mainly in southern Africa, was treated with low-dosage applications from fixed-wing aircraft between 1968 and 1991 (Table 27.2). Botswana reintroduced aerial spraying in 2001 and 2002.

Thermal fogging

Hot fogging is ground based but delivery of the insecticide is similar in principle to the early thermal exhaust methods used for SAT in South Africa and Tanzania. The fogging machine injects insecticide under pressure into the exhaust of a small engine, where it evaporates before being forced out through a nozzle and condenses into small droplets (see Plate 10). The resultant non-residual aerosol, like that produced by aerial spraying, only affects the adult tsetse population and requires a sequence of applications to have any significant effect on the target population.

Fogging machines can be vehicle mounted but, with vehicle access being restricted in most tsetse habitats, they are generally carried by operators on foot. As with SAT, the aerosols they produce require stable air conditions to prevent the small droplets being blown away from the tsetse habitat. Night-time or twilight operations are therefore favoured but the former is virtually impossible without a network of suitable access roads. The method is used during daylight hours in the early morning and late evening.

Table 27.2. Areas treated by fixed-wing aerial spraying to control tsetse flies, 1968–2001.

Year	Botswana	Côte d'Ivoire	Kenya	Nigeria	Somalia	Tanzania	Uganda	Zambia	Zimbabwe
1968								1600	
1969									
1970								1535	
1971								3055	
1972								3700	
1973	1150							2970	
1974	1300								210 (trial)
1975	2700							3400	484 (trial)
1976	2500			2000				1100	
1977	4000			2240				2000	
1978	2500							2000	
1979	3000	1700				1700			
1980	1800						626		
1981	5500		300						
1982	6000			1600				1000	2400
1983	9500				1000			2000	2100
1984	7350		300		2635				1700
1985	6000								1700
1986	5800								3200
1987	4000							4500	5000
1988					3800				2000
1989	6820								
1990	4000								
1991	4000								
2000									
2001	7180								
2002	8700								

The fogging machine is heavy, it generates considerable heat and, with drifting spray in relatively still air conditions, it is uncomfortable to use. An operator can only treat about 20–30 ha/day and cannot achieve the same blanket coverage that is possible from parallel aerial swathes. As single treatments have minimal, short-term effect, the method is limited in both range and applicability. It is best suited to the provision of temporary relief – for instance, where tsetse are a nuisance.

Formulations and dosages

Ground spraying

Wettable powder (w.p.) formulations of the organochlorides have been the most widely used for ground spraying. Being relatively insoluble and therefore difficult to formulate, DDT is normally dispensed as an aqueous suspension. Although this results in a loss in the efficiency with which deposits adhere to the treated vegetation, the persistence obtained is adequate for the purpose. Wettable powders are, however, generally less expensive than the emulsifiable concentrate (e.c.) or ulv formulations required for some alternative insecticides. In areas where the dry season is short or where intermittent rain is frequent, it can be necessary to revert to an oil-based formulation of DDT or to consider repeating the spraying at intervals calculated to ensure that the full pupal period is effectively covered.

DDT used specifically for ground spraying in Zimbabwe was normally supplied as a 75% w.p. in convenient 20 kg packs. Two such packs mixed with 375 l of water would provide 750 l of 4% aqueous suspension – sufficient for a day's work for a team of 20 operators (typically comprising one supervisor, eight sprayers working in shifts of four, eight porters, one distance recorder, one driver and one driver's assistant).

Ground spraying is mostly carried out using pressurized knapsack sprayers with a capacity of about 12 l, pre-set to dispense the insecticide at a constant pressure of about 30 p.s.i. The greater the pressure, the finer is the spray and residual deposits require a coarse spray with droplet diameters in the range of 500–2000 μm. The insecticide is directed through a spray lance fitted with nozzle producing a fairly narrow (< 60°) and usually cone-shaped spray (Davies, 1967).

Spray operators become well experienced in where to place the insecticide and how much to apply (see Plate 11). They normally spray to run-off, which provides a high residual dosage at the site but, through selective spraying, this equates to an overall dosage of about 200 g/ha for DDT. The more expensive pyrethroids such as deltamethrin have been used in place of DDT but, with a much higher toxicity to tsetse (Table 27.1), the application rate need only be about 12 g/ha.

Ground-spraying campaigns did not always achieve the required level of tsetse reduction in a single season and retreatment was common. To minimize the effects of reinvasion between seasonal campaigns, each operation had to penetrate deep into the tsetse infestation and this was well illustrated in Zimbabwe where ground-spraying barriers were used for many years to protect agricultural activities south of the fly belt. These barriers extended some 30–40 km in depth annually and some areas were sprayed up to 13 times over a period of 20 years (Douthwaite and Tingle, 1994). Nevertheless, when sufficient resources became available to increase this effort following independence in 1980, it resulted in the eradication of the entire tsetse population up to the natural barrier provided by Lake Kariba.

Wooff (1965) reported that spraying teams treated 22 acres (8.9 ha) per sprayer per day in Uganda and each team in Zimbabwe treated in the region of 50 square miles (128 km^2) per month (Robertson and Kluge, 1968); thus the larger operations, extending to several thousand square kilometres, required many teams. Each team had transport and at least one senior officer supervising. During the 1970s and 1980s, Zimbabwe operated over 70 such teams and treated almost 150,000 km^2 (Fig. 27.1).

These campaigns required meticulous planning, preparation and implementation. Each was a major logistical exercise but they did achieve success in Nigeria, Zimbabwe, Uganda and Kenya. Ground spraying almost certainly removed tsetse flies from a greater area of Africa than any other single technique, with some 400,000 km^2 treated from the 1950s to the 1980s. However, as worldwide environmental concern increasingly censured the use of residual insecticides and financial constraints curtailed the use of logistically demanding, labour-intensive, public-sector activities, ground spraying became almost universally discontinued.

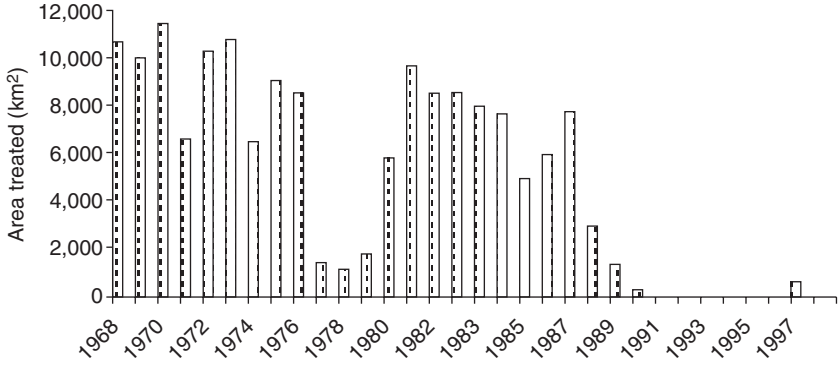

Fig. 27.1. Areas ground sprayed in Zimbabwe, 1968–1997.

Sequential aerosol technique

SAT is a non-residual technique and although the water-based w.p. formulations used for ground spraying are inexpensive, they are not suitable. Emulsifiable concentrate and ulv formulations are readily atomized into tiny droplets and have proved most effective for aerial spraying. Endosulfan e.c. has been most widely used and the active ingredient (a.i.) is initially dissolved in a solvent such as xylene, then diluted in a light oil carrier similar to kerosene (paraffin). Large volumes of chemical are required to ensure dispersal of the a.i. and penetration into the tsetse habitat; thus some 90,000 l of formulated endosulfan is required to treat an area of 2000 km^2 (see Plate 12). The bulk of this is the solvent, which evaporates almost immediately after dispersing the 20–30% of a.i. as tiny, drifting droplets and within hours the insecticide cannot be detected. Five applications over a period of 2–3 months add up to less than 10 kg of endosulfan a.i./km^2.

Endosulfan has been used at a range of dosages, largely determined by the target species and environmental considerations. In the environmentally sensitive Okavango Delta of Botswana, where the only species is the relatively small *Glossina morsitans centralis*, dosage rates were used as low as 6–12 g/ha in the 1970s. These achieved some control success (Davies and Bowles, 1979) but did leave survivors. *G. morsitans morsitans* was eliminated from some areas in Zimbabwe using 14 g/ha but there were indications some time after spraying that small numbers of the larger species, *Glossina pallidipes*, had survived (Hursey and Allsopp, 1983). In subsequent years the dosage rate in Zimbabwe was raised to 20 g/ha for *G. morsitans* and 22–24 g/ha for *G. pallidipes*.

Old pregnant females are more tolerant to insecticide than the young newly emerged flies and require the highest dose rate (Kwan *et al.*, 1982). After the first cycle, only newly-emerged flies, including nonparous females, should be present and subsequent applications can therefore be reduced from, say, 20 to 16 g/ha (Allsopp, 1990). Another factor consistent with lower endosulfan dosages in later cycles is its positive temperature coefficient of toxicity (TCT). As the seasonal temperature increases towards the end of the winter and towards the end of the spraying operation, the insecticide actually becomes more toxic. Deltamethrin has a negative TCT and is most effective at the height of winter.

G. pallidipes is a substantially larger fly than *G. m. morsitans* or *G. m. centralis* and successful control requires a higher dosage. From topical application studies, Fenn (1992) concluded that endosulfan applied at < 30 g/ha may leave survivors. A dose rate of 28 g/ha was used against *G. pallidipes* in Somalia in the 1980s when meteorological conditions made aerial spraying extremely difficult. The dosage was raised to 34 g/ha when survivors were discovered between cycles but the end result, confirmed by independent assessors, was eradication from almost 4000 km^2 (Clarke, 1990).

Dosage is not the only critical factor for aerial spraying; the number and speed of droplets drifting through the habitat are also important. To maximize the former, the volume application rate should be kept high. Thus when the dosage is dropped from, say, 20 g/ha to 16 g/ha it is advisable to decrease the formulation concentration from 30% to 20%. Higher concentrations than 30% have shown a tendency to crystallize in the atomizer and are generally avoided. Droplets falling through still air by sedimentation only have no lateral velocity and are unlikely to impact on tsetse flies. This was one possible reason for the failure to eradicate *G. pallidipes* from some deep river valleys in hilly terrain in Zimbabwe where sumps of cold, static air were observed during night-time spraying.

The pyrethroids have had a chequered history when applied by the SAT. Natural pyrethrum was used for an aerial spraying operation in Kenya in 1986 but achieved limited success. The results were, however, compromised by high flying and by spraying during daylight hours when the temperature inversion had broken down. Similar operational procedures had also reduced the effectiveness of an operation using deltamethrin in Uganda in 1980 (Sserunjoji, 1981). In the

1980s, deltamethrin applied at 0.25 g/ha successfully removed *G. morsitans* from some areas of western Zimbabwe but left *G. pallidipes* survivors. Partly as a result of an indication (later refuted) that insecticide mixtures can have a synergistic effect (Harris and Williams, 1981), cocktails of pyrethroids with endosulfan (e.g. 6 g endosulfan/ha + 0.1 g alphacypermethrin/ha) were used in Botswana. These reduced the *G. m. centralis* population substantially but did not achieve eradication.

After an unsuccessful attempt to eradicate tsetse from the Okavango delta with targets between 1991 and 2000, the Botswana Government reintroduced aerial spraying in 2001 and achieved excellent results with deltamethrin applied as a ulv at 0.26 g/ha to 0.3 g/ha. Extensive post-spray surveys continued into 2004 without capturing a single tsetse fly.

Environmental Impact

Concern about the accumulation of pesticides in food chains was sparked by Rachel Carson's *Silent Spring* (1962) and exacerbated by speculative but scientifically unsubstantiated criticism from well-known opponents of insecticidal control of pests (particularly tsetse) (Ormerod, 1976; Linear, 1982, 1985).

Tsetse control has attracted an inordinate amount of environmental criticism, frequently from sources without practical experience of the African situation. As this concern has grown, operations, particularly SAT, have been increasingly subjected to ecotechnical and biodiversity monitoring. Since its adoption for routine tsetse control in the 1970s, some 50 person-years (py) have been spent studying the impact of SAT on non-target organisms and the physical environment (20 py of these devoted to Botswana alone). Consequently, the development of environmentally sensitive operating procedures, new tsetse-control technologies and the sciences of environmental impact and risk assessment in this context have all accelerated. In turn, undesirable impacts have been reduced and so too have the costs of monitoring and assessment.

Unfortunately, there has been no corresponding reduction in the distinction between real and imagined risks. Decision making can therefore be misguided and stakeholder confidence in control technologies can be undermined by political viewpoints, fears based on misinformation and poor understanding of the real environmental issues (Grant, 2001). Some impacts of tsetse control are as follows.

Ground spraying

Indiscriminate ground spraying, i.e. to the entire habitat, has caused severe acute effects on non-target animals, including reptiles, small mammals, fish, birds and insects.

A pilot study in discriminative ground-sprayed areas of Zimbabwe (Matthiesson, 1985) did indicate that DDT residues were accumulating in some wildlife species and appeared to substantiate claims by local environmentalists that DDT was causing eggshell thinning in fish eagles and other raptors. DDT residues were certainly widespread in Zimbabwe but this was not entirely due to tsetse control, since DDT was more widely used for public health and crop protection.

A 5-year UK-funded research programme provided the definitive assessment of DDT bioaccumulation in Zimbabwe. One lizard and four bird populations had accumulated DDT residues considered sufficient to put them at risk in periods of extreme stress, e.g. drought. Two bird species declined by 90% after 2–3 years in DDT-sprayed areas. Several other bird and terrestrial invertebrate populations were scarcer in areas sprayed with DDT than in those not sprayed. The overall conclusions were that these effects were reversible, that there were no significant effects on fish populations or soil processes and that DDT accumulation caused less environmental damage than either human settlement or elephants (Douthwaite and Tingle, 1994).

Ground spraying, often in remote wilderness or hilly areas, is arduous and can lead to potentially dangerous contact with wild animals. There is also a high risk of operators being exposed to the spray. The health hazard with DDT, for instance, is low but pyrethroids can cause irritation when in contact with the skin. This effect can be reduced by wearing

protective clothing but this is not always practised when operators are working in thick bush and at very high ambient temperatures.

Ground spraying often requires retreatment over a number of years but in spite of this the non-target effects, with DDT at least, are less than might be expected and bioaccumulation is less pronounced than in the temperate climates of Europe or North America.

Control operations using alternative insecticides such as deltamethrin have been successful, but at a cost (Daniels, 1995).

Sequential aerosol technique

The sensitivity of tsetse to insecticides such as endosulfan or deltamethrin, and the carefully calculated sequence of applications timed to match the fly's almost unique life cycle, confer a degree of specificity on this technique. Almost immediately after spraying, a few hours at most, the insecticide is not detectable in the terrestrial environment, though it can last for several days in still water. At the population level, non-target species are consequently less seriously affected than with the residual techniques. There will inevitably be some acute mortality amongst non-target insects of comparable sensitivity but their different life cycle and the lack of insecticidal persistence allows them to recover between applications.

Endosulfan has been rigorously monitored for non-target effects in Botswana (Douthwaite *et al.*, 1981), Zambia and Zimbabwe (Nagel, 1995) and Somalia (Fallows, 1988). These studies revealed a temporary depression of non-target aquatic and terrestrial invertebrate populations but fish were the main concern, because of their particular susceptibility to this insecticide.

Fish mortalities did occur in shallow, static water but the most significant effects were caused by overdosing associated with navigational errors, equipment malfunction or accidental spillage. On average, mortality was 0–4% of the sampled fish populations per spray cycle but up to 60% were killed where there was over-spraying. There was evidence of physiological damage to liver and brain tissues and tilapia nests were reduced by 25% in sprayed areas. Recovery from the physiological damage was demonstrated and it was concluded that non-target effects are minimal at the population level and in the short term, provided that there is no continuous retreatment.

The impact on honey bees was monitored in Somalia. Those kept in conventional hives and at ground level were unaffected and there was no evidence of mortality, changes in flight activity, migration from the treatment area or reduced honey production. No evidence of impacts on wild bees have been recorded.

Post-spray studies 8 years after aerial spraying in Zimbabwe showed no evidence of any species having been exterminated.

The synthetic pyrethroids are less toxic to fish than endosulfan and thus considered more suitable for spraying wetlands. They are, however, acutely toxic to crustaceans and monitoring in Zimbabwe did show that where rivers were still carrying water when sprayed (many dry out during the spraying period) aquatic invertebrates were severely affected. At a dosage of 0.25 g/ha, deltamethrin caused catastrophic drift of benthic organisms in rivers for up to 48 h, though standing populations of the same benthos species were not significantly affected.

The Okavango is the world's largest inland delta and an important Ramsar site. While carrying out their SAT operations in 2001 and 2002, the Botswana Government commissioned the University of Botswana's Harry Oppenheimer Okavango Research Centre to assess the impact on non-target species of five deltamethrin applications at dosages up to 0.30 g/ha. Long term studies continue but initial indications, based largely on the biotrack® system, a product developed at Macquarie University, Australia, suggest that acute effects on insect abundance and species composition are significant but short lived, with recovery within 1 year.

Residual aerial spraying

Residual spraying of organochlorine insecticides from helicopters and trucks was monitored in West Africa and showed mortality among the same groups as were affected by

indiscriminate ground spraying (see Plate 13). In addition, amphibia, monkeys and fruit bats were killed (Koeman *et al.*, 1971). It resulted in the disappearance of some bird and arthropod species from the treatment area for up to a year. In Cameroon, some butterfly species and a common shrew only reappeared 3–4 years after spraying (Douthwaite, 1992). However, even with the very high dosages used to give long-term persistence, the effects on non-target populations were not considered to be irreversible.

Thermal fogging

The dosage rates are similar to those used for aerial spraying. Thus, in Botswana, endosulfan e.c. with diesoline as a solvent is applied at approximately 12 g/ha.

The environmental effects have not been assessed but the technique has limited penetration and is only suitable for treating fringing riverine woodland. Unfortunately, such ecotones support a relatively high faunal biomass and must be considered sensitive to such specifically targeted insecticidal treatment.

Comparative Cost of Insecticide Delivery Methods

In order to compare costs over time, the following estimates are adjusted from the local currency (in the early years of insecticidal tsetse control this was mostly UK£) to US$ at the exchange rate applicable at the time. (Historical exchange rates were provided by the University of British Columbia, Pacific Exchange Rate Service.)

The cost of ground spraying 900 square miles in Kenya in the late 1950s was given as £38,100 (Glover *et al.*, 1960). Adjusting with the applicable exchange rate, this equate to about US$48/km^2. Ground-spraying costs in Nigeria during the 1950s and 1960s were given by Davies (1964, 1971) and over a period of 14 years an area of some 23,300 km^2 was treated at an average cost of about US$56/km^2 (Table 27.3).

The cost of ground spraying in Uganda in 1963/64 was somewhat higher and Wooff (1965) calculated the cost of treating 644 square miles to be £233/mile^2 or US£$270/km^2. Conversely, the cost in Zimbabwe between 1962 and 1966 (Robertson and Kluge, 1968) was significantly lower at the equivalent of US$25/km^2 (Table 27.4).

Aerial spraying was the favoured control method in Botswana and Zambia during the 1970s and 1980s. In the former, low dosages (12 g/endosulfan/ha or equivalent cocktails with pyrethroids), wide swathes (up to 370 m) and sometimes only four applications were used and this was reflected in the cost, which averaged US$100/km^2 between 1973 and 1980. One-off operations in the denser vegetation types of Nigeria and the Côte

Table 27.3. Costs of ground spraying in Nigeria, 1955–1969.

Year	Cost (£/mile^2)	Area (mile^2)	Cost (US$/km^2)	Year	Cost (£/mile^2)	Area (mile^2)	Cost (US$/km^2)
1955/56	100	7	117.19	1964/65a	59	256	64.53
1956/57	102	55	119.53	1964/65b	7	537	7.66
1957/58	91	69	106.64	1965/66a	14	590	15.31
1958/59	103	115	120.70	1965/66b	56	110	61.25
1959/60	104	122	121.88	1966/67a	4	432	4.38
1960/61	82	113	96.09	1966/67b	21	1165	22.97
1961/62	38	245	44.53	1967/68	20	1350	21.88
1962/63	38	196	44.53	1968/69	19	620	20.78
1963/64a	36	125	42.19	1968/69	18	1490	19.69
1963/64b	30	165	35.16	1968/69	23	470	25.16
1963/64c	45	190	49.22				
1963/64d	6	710	6.56				

Table 27.4. Costs of ground spraying in Zimbabwe, 1962–1966.

Year	Area (km^2)	Cost US$/$km^2$
1962	896	28.66
1963	512	25.70
1964	1280	23.08
1965	2048	19.82
1966	1510	28.78

d'Ivoire required higher dosages (20 g endosulfan/ha) and narrow swathes (200 m) and the costs in 1977 and 1979 were US$304 and US$244/km^2 respectively (Lee *et al.*, 1982).

SAT was used annually in Zambia from 1968 to 1978 and the costs were summarized by Evinson and Kathuria (1984). They remained relatively constant until 1976 when the kwacha weakened against the US$; however, the higher cost per unit area between 1976 and 1978 could also have been related to the smaller area treated, as cost is generally inversely proportional to the size of area treated (Table 27.5).

Ground-spraying operations continued in Zimbabwe until the late 1980s and between 1982 and 1985 the government also introduced SAT – providing an opportunity for direct cost comparisons (Table 27.6). In a series of integrated chemical control operations, the ground-spraying costs were higher than they had been 20 years earlier but were still competitive at approximately half the cost of aerial spraying per unit, though the ground-spraying areas did require overlapping and retreatment.

The cost of ground spraying in southern Africa remained relatively competitive for many years and although it had increased by the 1980s it was actually dropping when abandoned through international pressure to ban the use of DDT, accelerated by the introduction of EU funding for tsetse control in the region. Replacing DDT with a persistent synthetic pyrethroid was an option but it roughly doubled the cost and so, with bait techniques increasingly available and cost-effective, ground spraying became redundant.

Tsetse control costs do vary substantially according to operational parameters and caution is clearly required when comparing costs for strategic purposes. The costs of the various control techniques used in southern Africa in the 1980s were compared by

Table 27.5. Costs of aerial spraying (SAT) in Zambia, 1968–1978.

Year	Area (km^2)	Cost (ZKw/km^2)	Cost (US$/$km^2$)
1968	1600	117.19	165.06
1970	1535	150.14	211.46
1971	3055	101.64	143.15
1972	3700	92.96	130.93
1973	2970	104.28	160.43
1975	3400	118.52	185.19
1976	1100	282.77	403.96
1977	2000	265.00	335.44
1978	2000	288.40	360.50

Table 27.6. Costs of ground spraying compared with SAT in Zimbabwe, 1982–1985.

Year	SAT areas (km^2)	SAT costs (US$/$km^2$)	Ground-sprayed areas (km^2)	Ground-spraying costs (US$/$km^2$)
1982	2400	289.37	8324	174.2
1983	2100	261.57	5222	128.5
1984	1700	254.48	7359	116.25
1985	1681	242.19	4812	109.57

Table 27.7. Comparative costs (Z$/km^2) of ground spraying, aerial spraying, targets and insecticide-treated cattle at 1990 prices (Daniels, 1995).

Technique	Basic scenario (Z$)	Pessimistic scenario (Z$)	Optimistic scenario (Z$)
Ground spraying (DDT)	1781	2135	1430
Ground spraying (deltamethrin)	3100	3800	2500
Aerial spraying with endosulfan	2700	3700	2000
Targets (deltamethrin)	1500	2100	1100
Dipping (deltamethrin)	361	723	181
Pour-on	703	1406	352

Barrett (1992) and by Daniels (1995), who produced high and low estimates (Table 27.7) based on operational variations.

Thus: (i) the cost of ground spraying increased with operational difficulty and rugged terrain; (ii) aerial spraying costs are inversely proportional to the size of area treated (because fixed and capital costs remain virtually the same irrespective of the area treated); and (iii) targets costs increased if deployment and re-servicing rates were reduced by operator efficiency and vehicle breakdowns etc.

Ground spraying with deltamethrin was considered by Daniels to be the most expensive option, irrespective of extenuating factors, and both ground spraying with DDT and targets could be more expensive than aerial spraying if the former involved logistical difficulties but the latter did not.

The effect of external factors on control costs was also clearly illustrated in Botswana when aerial spraying was replaced by the artificial bait technique in 1992. Tsetse control in Botswana is a government activity and public-sector labour costs are high. The switch to targets was not accompanied by a reduction in expenditure on tsetse control but resulted in an increasing annual budget requirement – while the area treated decreased. Based on the annual budget allocation to the Tsetse Control Division and the area treated, the cost of using SAT in 1991 was Pula 681.19 (US$175.68)/km^2. This increased to Pula 6028.19 (US$1763.85)/km^2 in 1992, when only 374 km^2 was treated (with targets) as the Division embraced new working practices after 20 years of dependence on aerial spraying contracted out to the private sector. By 1996 the cost of control with targets had reduced to Pula 1144.29 (US$219.47)/km^2 (Mullins *et al.*, 1998).

Consistent with the Daniels' interpretation (Table 27.7), the high cost of deploying targets in Botswana was a reflection of the Division's low productivity. This was mostly caused by the lack of suitable government transport as and when required – a factor that had also affected ground spraying efficiency in Uganda some 34 years previously (Wooff, 1965). The speed at which targets were deployed improved tenfold in 1998/99 when the Botswana government employed a private contractor with reliable vehicles and less expensive, casual labour. A contract for 10,000 targets was awarded in 1999 and the deployment cost, inclusive of all materials, odours and insecticide provided by the Division, was Pula 479.0 (US$95.8)/km^2. Retreatment of these targets, twice at 6-monthly intervals, with some 60% damaged by wind and wildlife, increased the overall cost to Pula 1142.0 (US$228.4)/km^2. Treatment of the entire Okavango infestation, almost 16,000 km^2, with SAT in 2001 and 2002 was achieved at a cost (aerial spraying contract and insecticide) of approximately US$270/km^2.

The cost of tsetse control can also vary according to the ultimate objective and thus the intensity with which the technique is applied. Barrett (1997) put the case for control or suppression rather than eradication, which would reduce the operational costs though not the recurrent cost. Thus, targets used to suppress a population might not require four or more targets per square kilometre but could perhaps achieve the objective with one per square kilometre. Similarly, with aerial spraying: eradication

normally requires five applications, narrow flight intervals and relative high dosages. All could be adjusted to achieve control only, as was illustrated in Botswana during the 1970s and 1980s, and could reduce the overall cost by some 30–50%.

Future for Insecticidal Control

Tsetse control has been a front-line option for reducing the impact of trypanosomiasis since the vector was recognized at the turn of the 20th century and for more than 50 years control strategies have been dependent on the use of insecticides. During that time, chemical control methods were used to treat some 500,000 km^2, albeit in a widely separated and uncoordinated way. In spite of this, epidemic sleeping sickness persists in countries such as Angola, Zaire and Sudan (Smith *et al.*, 1998); bovine trypanosomiasis continues to reduce the off-take of meat and milk from livestock by some 50%.

There has been little long-term control success; tsetse flies still infest enormous tracts of Africa and the situation is deteriorating. Huge areas cleared by 20 years of ground spraying in Nigeria are being reinvaded. Keeping areas free of tsetse following 18 years of helicopter spraying in Cameroon is proving most difficult. Zimbabwe struggles to prevent reinvasion into 40,000 km^2 cleared of tsetse with ground spraying, aerial spraying and targets over a period of 40 years. Botswana has seen the return of trypanosomiasis in the Okavango Delta after an absence of 17 years achieved by 30 years of tsetse control with SAT and targets.

Unlike some other pest management situations involving insecticides, this lack of success is not the result of chemical resistance, nor is that likely to become a problem. Experience shows that tsetse always were, and continue to be, easily killed with insecticides. The inability to sustain control achievements is more likely to be a problem of management or commitment than an inherent technical deficiency. The lack of significant progress has certainly affected strategic thinking amongst control authorities and donors. In recent years, large-scale insecticidal campaigns, specifically targeted at reducing tsetse numbers, have been largely replaced by localized, community-owned operations using 'appropriate' technologies in a broader agricultural context. This has further slowed the impetus of tsetse control and in turn rural development. In contrast, financial independence, a commitment to tsetse control as the primary means of combating trypanosomiasis plus a readiness to build on successful elements of a long and chequered operational history without prejudice undoubtedly contributed to the success of Botswana's 2001/2002 control strategy; a strategy which already appears to have created a tsetse free zone in northern Botswana and may, in time, be confirmed as having eradicated tsetse flies from the Okavango delta.

For instance, between 1986 and 1999 the EU funded a Regional Tsetse and Trypanosomiasis Control Programme for southern Africa (RTTCP). It was a major and important initiative for the region which aimed to use various control techniques to eliminate tsetse flies from a 320,000 km^2 common-fly belt spanning four countries, thus releasing land for settlement and agricultural development. The concept was ambitious but it was clearly focused and technically sound. Unfortunately, issues of cost, environmental impact and to some extent European perceptions about the importance of community participation resulted in the demise of ground spraying and aerial spraying, leaving the region with very limited technical options. As the donor and, consequently, the programme progressively diverged from the primary objective, the focus that gave the four participating countries a common purpose was lost. It fragmented into national development projects, the regional vision became obscured and tsetse control was reduced to open-ended holding operations. After 14 years and substantial expenditure, the programme not only failed to achieve its original objective but it left the four countries without the technical capability, the resources or a coherent strategy to cope with the tsetse problem and its continuing effect on rural development.

The reasons most frequently given for de-emphasizing large-scale chemical control are

environmental impact and cost but neither can be legitimately used to contraindicate the use of techniques such as ground spraying or low-dosage sequential aerial spraying. A considerable body of evidence shows clearly that the direct effects of insecticide applications for controlling tsetse flies in tropical Africa are minimal and short-term. Unit costs have increased as materials and delivery methods have become more sophisticated but, considering that US$1 in 1950 would have an equivalent value of US$20 in 2001, they have remained remarkably competitive. Furthermore, generalizations should be avoided when comparing control costs for strategic purposes. In some situations the supposed high-cost options (aerial spraying, for instance) might not be far removed from the low-cost 'appropriate' options such as the deployment of traps or targets etc.

So what lies ahead? Africa is still in the grip of trypanosomiasis and its resourceful vector, the tsetse fly. Meeting in Lomé, Togo, in June 2000, the Organization of African Unity (OAU) Heads of State recognized that this is untenable. They declared 2001 to be the year in which the continent would embark upon a campaign of tsetse eradication and accorded it the highest OAU priority for reducing disease and improving livelihoods amongst member states. The FAO/IAEA/OAU/WHO Programme Against African Trypanosomiasis (PAAT) endorsed this historic declaration and recognized the need to embrace area-wide control techniques to achieve this formidable objective.

A programme to eradicate tsetse flies from some 9 million km^2 of Africa is highly ambitious. It will be complex, take many years and possibly cost some US$20 billion (Budd, 1999); but Sachs (1999) advocated the mobilization of global science by rich countries to address the problems of world poverty and the UK prime minister, Tony Blair (Labour Party Conference, October 2001), challenged the affluent democracies of the developed world to do more to eliminate the miseries of Africa. It will also have environmental consequences, but these too must be considered within the context of the current welfare and future prosperity of 260 million of the world's poorest, most disadvantaged people.

Ultimate success will depend upon many things, including: (i) collaboration within the African Union (AU) to set and achieve realistic milestones based on rational judgements; (ii) international support for the AU priorities, acceptance of the technical feasibility of their proposal and commitment to alleviating poverty through disease management; and (iii) decision makers taking account of lessons learned from past successes and failures, remaining open-minded about control methods and then applying them with care and single-minded determination.

Within this complex political, technical and humanitarian scenario, is there a role for chemical control? The AU recommends the pivotal use of the sterile insect technique (SIT). This technique has eradicated tsetse from Unguja Island, Zanzibar, and, being environmentally benign, it has appeal within the international community. It is certainly suitable as a component of large-scale area-wide control but it cannot stand alone and will need to be used in conjunction with at least some of the chemical control delivery methods mentioned above. Realistically, residual aerial spraying is a non-starter and thermal fogging would only have limited application, but discriminative ground spraying and, particularly, aerial spraying could be key elements, used where appropriate, in large-scale integrated control campaigns.

There is a caveat. The problems caused by African trypanosomiasis are desperate and they must be addressed urgently. However, if large-scale chemical control campaigns were to re-emerge as options within the AU campaign to eradicate tsetse, whilst this might be propitious, it would be neither a new beginning nor a panacea. For the foreseeable future, tsetse control will continue to depend on insecticides, because there is no real alternative, but this should not be prolonged unnecessarily. As the AU campaign gathers momentum there should be no complacency or relaxation in the search for new ways of eliminating tsetse, curing the disease or breaking the transmission cycle.

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28 Development of Bait Technology to Control Tsetse

Glyn A. Vale and Stephen J. Torr

Situation in the 1960s

In the late 1960s, when *The African Trypanosomiases* (Mulligan, 1970) was being written, it seemed unnecessary for the book to devote a chapter to bait technology. This paucity of attention reflected the disappointing indications then available for the general usefulness of baits as control tools, despite the fact that in the first half of the 19th century it had sometimes seemed that baits could contribute greatly to control operations. By 1910 Maldonado had used sticky screens on his workmen to assist the removal of *Glossina palpalis* from the island of Principe. In the 1920s Harris had designed a trap that had helped to deal with *Glossina pallidipes* in KwaZulu/Natal, and in the 1940s Whiteside had shown that DDT applied to cattle could reduce the abundance of *G. pallidipes* in Tanzania. However, none of these baits appeared suitably cost-effective in comparison with the alternative methods of control that involved the destruction of wild hosts, bush clearing and the widespread application of insecticides. Hence, the general view that had developed by the 1960s was that baits were useful only as survey devices and research tools.

Notwithstanding this seemingly dismal outlook for bait control, there was concern about the alternative methods. For example, game destruction, bush-clearing and much use of persistent insecticides were ecologically damaging. The spraying campaigns were technically demanding and required tight planning to meet seasonal deadlines. Moreover, the seasonal application of spraying measures meant that tsetse were free to invade the cleared areas for much of the year. The concept of bait control was appealing by comparison. If it were possible to improve baits substantially, they might offer a relatively simple method of control that had minimal ecological impact, and which could be used not only to eradicate the flies but also to form a year-round barrier to invasion. Furthermore, any improvement in baits was likely to enhance the effectiveness of surveys.

Thus, even while Mulligan (1970) was being written, a new look at baits for tsetse had started. There were two main sorts of baits to examine: live and artificial. The live baits were mainly humans or cattle and were either mobile or stationary. Flies visiting them were usually caught by hand-nets and the catches, though often large, consisted mostly of males of a few tsetse species such as *Glossina morsitans*. The artificial baits were mainly various types of stationary trap, which were cheaper to operate than the live baits. Although the catches were usually much

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smaller than those from live animals, they did contain high percentages of females of all species. Moreover, trap catches contained high proportions of those species such as *G. pallidipes* that were poorly represented in catches from most live baits. Hence, the goal of the renewed research was to combine the best features of each of these two types of bait, to get the large catches of the live baits while also preserving the economy and the broader compositions of trap catches.

The research strategy was first to elucidate the basic responses of tsetse to baits, then use the knowledge produced to design cheap and effective baits for routine deployment, and finally study the movement and population dynamics of tsetse to understand the optimum density, siting and management of baits.

Basic Studies

Studies of the relative abundance of various hosts in the bloodmeal identifications of tsetse and in the population of potential hosts had shown that the majority of male and female tsetse visit a few thinly spread animals in the few days of the tsetse hunger cycle (Robertson, 1983). Hence, research had to start with an explanation for the 'gap' between what it was known must occur in the attraction of tsetse to wild baits and what the catch data seemed to show about the numbers and composition of samples of tsetse attracted to live hosts and traps. Essentially it seemed that the mass of catch data (Buxton, 1955) involved little real information.

Initiatives

Two closely linked initiatives helped to bridge the gap (Vale, 1993). Firstly, there were purely intellectual developments, such as recognizing more rigorously that the overall response to baits involves a sequence of many separate types of response, few of which were measured by catch data then available. Moreover, there was the fuller appreciation that the baits studied were not actually the baits that exist in nature. For example, cattle 'hosts' investigated were not just the cattle themselves but the combination of cattle and humans.

The second initiative was the refinement of research techniques. Partly, this involved the use of devices such as drop cages, observation pits, electrocuting devices and video recordings that provided a more objective and quantitatively reliable indication of the individual types of response to baits. There were also techniques that allowed a clearer separation of the effective stimuli from baits. For example, ventilated pits (Vale, 1974) distinguished between visual and olfactory attractants. Green (1988) separated the various wavelengths of light responsible for the distinctive performance of baits made from different fabrics. Hall *et al.* (1984) used a variety of chemical and electrophysiological techniques to isolate and identify odour attractants.

Basic questions in bait development

Following improvements in the theoretical and practical bases for research, there was a steady improvement in the understanding of bait-oriented behaviour from the late 1960s. Some of the important milestones in this were the answers to the following questions.

Why were catches from live baits so biased in composition?

Ironically, it emerged that the biases were largely 'artefacts' produced by the hand-net catching that had been used for so long. One of the problems was that some of the olfactory and visual stimuli produced by humans were potent repellents, especially for females, mature flies and certain tsetse species such as *G. pallidipes* (Vale, 1974). The failure to recognize this repellence earlier seems to be due to the fact that humans also produce attractants, such as the visual stimulus of motion. Hence, with the interplay of mixed signals, it was still possible for tsetse to be seen near humans. A further problem

was that hand-net catching was an inefficient means of capturing the relatively few tsetse that did appear near humans, or baits accompanied by humans.

The most disturbing biases in catches from live baits were no longer evident when relatively efficient electrocuting devices were used to catch flies visiting animals that had no humans nearby. The number of flies caught increased by about five to 20 times, depending on the sex and species of the flies, to give samples that represented more closely the composition of the population at large. At last it could be credited that what was measured near the research baits was a fair indication of normal behaviour.

Why were catches from live baits so large?

It was quickly confirmed that the visual stimuli from animals are important, especially the stimulus of translocatory movement. It was also found that host odour played a greater part than had previously been supposed. About 90% of the tsetse arriving at a stationary host did so in response to the host's odour (Vale, 1974) and tenfold increases in the dose of odour resulted in two- to threefold increases in the numbers of tsetse attracted to the source (Hargrove *et al.*, 1995). Odour was shown to be important in encouraging flies to alight on hosts, whether the hosts were stationary or mobile. However, it was only with stationary baits that odours seemed to increase attraction from many metres.

Why were the catches from traps so small?

In part, trap catches had been small because the traps were stationary and had been used mostly without odours. However, even when the numbers of tsetse near the traps were enhanced by odours or movement, the catches were still disappointing, often representing no more than 5% of the flies arriving within a metre of the trap (Hargrove, 1977). It emerged that the main problem was that many flies would not pass through the entrance of the traps; most of those flies that did enter were retained.

Host-oriented responses

The answers to the above questions made it possible to design a wide variety of critical experiments to elucidate basic aspects of host-oriented behaviour, and so understand more clearly the opportunities for bait development. As a result, more is probably known about the host-oriented responses of tsetse than any other haematophagous insect (Gibson and Torr, 1999).

Attraction from many metres

For all but a few minutes of each day, tsetse rest on vegetation or in natural 'refuges' such as antbear holes. While a resting fly will take off in response to the visual stimulus of a passing host, host location generally commences with the fly taking off in response to a circadian rhythm of spontaneous activity (Brady, 1988) to 'range' in search of a host. Work performed mainly on *G. pallidipes* and *G. morsitans* in Kenya and Zimbabwe indicates that the long-range detection of stationary hosts relies on host odour. The odour plume from a single ox is effective up to about 90 m, and that from a large herd of cattle is effective at several times this distance. Tsetse detecting odour exhibit optomotor upwind anemotaxis and fly upwind at ~6 m/s and ~0.5 m above ground. If the flies move out of the meandering plume they make a reverse turn, bringing them back into contact with odour, and then they continue their upwind flight. In addition to this anemotactic mechanism, there is some evidence that tsetse employ orthokinetic and klinokinetic responses to changes in odour concentration (Warnes, 1990). Using these strategies, tsetse can locate a stationary host efficiently, usually within 20 s of starting the upwind flight (Griffiths *et al.*, 1995).

There are large interspecific variations in the ability of host odour to attract flies from a distance. In general, the *morsitans* and *fusca* groups appear highly responsive to host odour (Vale, 1974; Makumi *et al.*, 1996), so that the odour from a single ox increases the number of flies attracted to the animal by four to ten times. On the other hand, ox

odour merely doubles attraction for tsetse of the *palpalis* group (Merot *et al.*, 1986), and for *G. austeni* within the *morsitans* group (Kappmeier and Nevill, 1999).

Attraction at close range

Although odour is important in bringing flies to within a few metres of a stationary host, the closer approach depends on a variety of visual cues. Blue light reflected from the bait increases attraction whereas ultraviolet and green-yellow light decrease it. Size, shape and patterning of the bait are also important, with small oblongs being less effective than large squares and circles. These visual stimuli bring the fly to within about half a metre of the host. The final alighting on a potential host depends on various olfactory and visual stimuli (Torr, 1989). Landing is enhanced by the perception of carbon dioxide and is greatest on large, black horizontal oblongs. Upright oblongs are particularly poor stimulants for the alighting response. Such simple responses mean that tsetse are far more likely to alight on a dark compact object, such as a warthog, than on a tree bole.

Feeding

Almost all tsetse visiting certain animals, such as goats and impala, fail to obtain a bloodmeal, according with the low proportion of such animals in the bloodmeal identifications of tsetse. However, even when the host species is one that forms a large proportion of tsetse diet, the feeding success can still be remarkably low (Vale, 1977). For example, only about 20% of tsetse feed after approaching a warthog and while the proportion that feed after arriving near cattle can be greater, this is still only around 40–50%. The age of the host seems as important as host species, the feeding rate being only about 10% on calves and young warthogs (Torr, 1994). Such age-related differences in feeding success mean that tsetse attracted to a herd of cattle take most of their meals from the older animals within the herd.

Low feeding success is due largely to the host's defensive responses, which intensify with increasing densities of tsetse (Vale, 1977; Torr, 1994). The low feeding rates probably reflect the low reproductive rate of tsetse and the concomitant fact that female tsetse are unable to sustain a daily mortality of $> 3\%$/day. A fly feeding on a host is at risk of being killed by the host's defensive behaviour and thus tsetse have evolved a strategy to reduce feeding-related mortality (Hargrove and Williams, 1995).

The studies of host-oriented behaviour indicated that male and female tsetse must visit baits several times during the few days of the hunger cycle, thus enhancing the potential effectiveness of baits for surveys and control. The studies also showed that any stationary bait must incorporate odour attractants if it is to be fully effective. Hence, it was essential to identify the active components of host odour.

Odour attractants and repellents

A number of attractants were identified, including the following, which are listed with the approximate rate at which they are released in the odour of a large ox: carbon dioxide at 2 l/min, acetone at 5 mg/h, butanone at 0.5 mg/h, 1-octen-3-ol (octenol) at 0.05 mg/h, 4-methyl phenol at 0.05 mg/h, and 3-*n*-propyl phenol at 0.005 mg/h. While searching for attractants, several naturally occurring repellents were also discovered, such as pentanoic acid and 2-methoxy phenol.

Carbon dioxide was dispensed from a pressurized cylinder. All other attractants could be dispensed from semi-permeable containers, such as sachets made from low-density polyethylene. Acetone and butanone could be dispensed most conveniently from open bottles. When all of the identified attractants were released artificially at natural doses they were only half as effective as the natural odour, suggesting that at least one important attractant remains to be identified (Hargrove *et al.*, 1995).

Despite the fact that the identified range of attractants is incomplete, and carbon dioxide is too costly and inconvenient to dis-

pense in routine surveys and control campaigns, the combination of the other identified attractants released at ten to 100 times their natural dose is cost-effective for *G. pallidipes* and *G. morsitans*. The combination increased the number of tsetse visiting traps up to about ten times, and doubled the strength of trap-entering responses, so that trap catches were increased up to about 20-fold. Currently, the doses of odour recommended for routine use with baits for *G. pallidipes* are: butanone at 150 mg/h, octenol at 0.5 mg/h, 4-methyl phenol 1.0 mg/h, and 3-*n*-propyl phenol at 0.1 mg/h. For *G. brevipalpis* the 3-*n*-propyl phenol can be omitted; for *G. morsitans* no phenols need be used.

Baits for Surveys and Control

The first of the new baits developed in the 1970s arose largely from empirical work with tsetse of the *palpalis* group in West Africa. Some of the most effective devices were the biconical and pyramidal traps (Laveissière *et al.*, 1990), which have been widely used for surveys ever since. However, these traps were not sufficiently cost-effective for use on their own in large-scale control operations. Moreover, they performed poorly against the *morsitans* group. These problems were overcome from the late 1970s onwards by a more analytical approach to bait development.

Traps

Analyses involved studying the effect of various visual and olfactory stimuli on different types of response, e.g. arrival beside the trap, alighting on it, entering it and moving within it. As a result of the studies it was possible to design economical traps, such as the F3 and Epsilon (Laveissière *et al.*, 1990). These presented a roughly 1 × 1 m expanse of phthalogen-blue cloth when viewed from outside but had some black cloth inside, clearly visible through the trap's basal entrance. The blue outside had the advantage of attracting tsetse without causing them to alight, so encouraging them to move to the black alighting surfaces inside the trap. Such traps caught about 30% of the *G. pallidipes* and 15% of the *G. morsitans* that visited them, i.e. efficiency was improved about tenfold. The Epsilon and F3 traps were poorly effective against *G. brevipalpis* and *G. austeni* but efficiency could be improved for *G. brevipalpis* by moving the netting cone of the traps, to become lateral instead of apical.

The relative efficacy of the various types of trap varied according to the tsetse species to be sampled (FAO, 1992). It is more surprising that the relative efficacy also depended on the geographical location. Thus, for *G. pallidipes* in Kenya and the Eastern Province of Zambia, the Ngu trap proved about twice as effective as the Epsilon. Against the same species in Zimbabwe, the relative performance was reversed.

Targets

Unfortunately, traps are complex, three-dimensional structures that need to be inspected every few days if they are to be kept in good order. Moreover, it is difficult to induce some tsetse, especially *G. morsitans* and *G. austeni*, to pass irreversibly through the trap's entrance; if the entrance is enlarged to let more flies in, the flies also move out more readily. However, if the intention is simply to kill tsetse instead of catching them for survey purposes, then it is not necessary to rely on trapping *per se*. It is more cost-effective to replace the trap with a simple visual target that is coated with insecticide. Moreover, even for survey purposes it proved beneficial to use a target coated with sticky deposit to sample *G. austeni* – albeit that the sticky deposit was inconveniently messy and was poor for retaining other larger species of tsetse.

Most interest attached to targets that were treated with insecticide and used for control. Hence, there were two main topics to address: the insecticide to use and the design of the target.

Insecticides

In the late 1970s and early 1980s a variety of insecticides was tested for use on the targets, including DDT and dieldrin wettable powder formulations. The wettable powders were soon abandoned, because they were readily washed away by rain and so demanded the costly provision of a roofed target to protect the insecticide deposits. By the mid-1980s the pyrethroids had become the universally recommended insecticides. For tsetse of the *palpalis* group an emulsifiable concentrate was shown to be a suitable formulation. For flies of the *morsitans* group a suspension concentrate formulation was better, since these tsetse often touched targets only briefly, so requiring a particulate insecticide that can be picked up rapidly.

Although tsetse were knocked down within about half an hour of contacting pyrethroids, the insects eventually recovered if held in the laboratory. However, if a knocked-down fly were exposed in the field it was usually removed quickly by ants, so that in bioassays of pyrethroid deposits the knockdown was usually equated with death. On this basis the eventual assessment was that a cloth target thoroughly soaked in a suspension concentrate containing 0.1% w/v deltamethrin remained effective for about 2 months in the wet season and about 4 months in the dry season. A soaking in a 0.6% suspension ensured that the deposit was effective for at least a year. Other pyrethroids were also suitable provided that their concentrations were adjusted in proportion to their toxicity relative to deltamethrin (Mangwiro *et al.*, 1999). For example, alphacypermethrin should be used at 1% to last a year.

Target design

The first targets used in West Africa were simple Phthalogen-blue screens a metre square. In southern Africa the first targets were more complex, even after the abandonment of the roof. For example, the roofless target initially consisted of a black cloth panel, 1.0 m tall and 0.7 m wide, with panels of fine black net 1.0 m tall and 0.5 m wide on each side (Laveissiere *et al.*, 1990). The central, visually effective panel was not large enough to encourage all tsetse near it to alight, but those that did not alight collided with insecticide deposited on the net. Eventually the targets in West Africa became a little more complex, and those in southern Africa more simple, with the recommendation that the targets in both places be made of a combination of black and blue cloth. The blue was important as a visual attractant but the black formed the main alighting surface, so that insecticide needed to be deposited only on the black. The black component formed that part of the target on which tsetse tended to alight naturally. Hence, for *G. morsitans* and *G. pallidipes* the black usually occurred in the centre, with blue side panels. For *G. palpalis* the black formed the side panels and the blue was central (Laveissière *et al.*, 1990).

Most of the targets recommended for the *morsitans* group were fixed to a wire frame that was mounted eccentrically on a steel post, to permit the screen to swivel like a wind vane, about 15 cm off the ground. The movement was not to enhance attraction to the bait – indeed the swivelling had no effect on attraction. Instead, the swivelling reduced the area that the screen presented to the wind, so preventing wind damage and minimizing the support needed.

Whereas the targets described above represent the main stream of target development, the research involved has demonstrated enough of the basic principles of target-oriented behaviour to allow a variety of effective targets to be designed to suit local supplies of raw materials, and to predict the efficacy of these targets. Much was also done to see how natural objects, such as the trunks and stumps of trees and fallen logs, could be adapted to become targets. Suitable natural objects could be identified by persons with a detailed understanding of the stimuli offered by each type of object in its particular surroundings. However, the principles were too complex to be adopted reliably in control operations.

Overall, the substitution of targets for traps has been cost-effective in all parts of Africa and with all species of tsetse – the general principle being that an effective trap can be readily converted into a target that is

twice as effective at about half the cost. Nevertheless, in some cases a hybrid policy is preferable: using a trap so that the flies which enter are caught for survey purposes, and treating the trap with insecticide so that the flies which touch the trap without entering are killed.

Sterilizing baits

Another approach to bait improvement was the use of chemo-sterilant to substitute for insecticide or removal trapping. The idea was to expose the flies to the sterilant and then allow them to return to the population. The sterilized females would be effectively dead and the sterilized males would interfere with the reproduction of those females that had not visited the baits, so roughly doubling the effectiveness of control campaigns (Langley and Weidhass, 1986).

Special traps that exposed tsetse to the sterilant metepa were highly effective, but were too costly and complex for widespread use and the sterilant was too great a risk to human health. Insect growth regulators are safe alternatives to sterilants and can have much the same doubling effect, provided that the treated males pick up enough chemical to transfer to females during mating. However, it could not be shown convincingly that males can transfer effective quantities of the regulators for more than a few days after treatment. Moreover, tsetse did not die as a result of exposure to baits with sterilants or growth regulators – the flies would still be able to transmit trypanosomiasis and move through invasion barriers made of such baits. Growth regulators seem to be of little benefit now but are options to hold in reserve against the day when resistance might develop against the pyrethroid insecticides.

Insecticide-treated cattle

In the late 1960s a wide range of insecticides was tested on cattle in Zimbabwe, but none killed the flies or knocked them down for more than about a week after the cattle were treated, confirming the earlier indications that insecticide treatment of cattle was unlikely to be cost-effective. However, in the mid-1980s when deltamethrin had been shown to be effective on targets, it was also tested on cattle (Thomson, 1987), leading to large-scale field trials in the late 1980s that confirmed its effectiveness. Despite the subsequent use of insecticide-treated cattle in many thousands of square kilometres of Zimbabwe, little was done immediately in the country to study the technique further. This was because the governments in southern Africa were considering removing tsetse from large uninhabited areas where cattle were absent and could not be introduced, so emphasizing the need to concentrate research on artificial baits. Hence, much of the development of the cattle technique continued elsewhere, especially in West Africa (Bauer *et al.*, 1992). The outcome of research in the various places is that the most cost-effective means of applying the insecticide is as a dip. Where dip tanks are not available, a pour-on formulation is recommended. Several pyrethroids can be used as substitutes for deltamethrin, including alphacypermethrin and cyfluthrin, but their recommended dose is higher than for deltamethrin, to allow for their lower toxicity.

Bait Deployment and Management

Having designed suitably cheap and effective baits it was necessary to determine where to put them, and in what pattern and density to eradicate the flies and prevent invasions. Answers to these matters required a fuller understanding of the movement, distribution and population dynamics of tsetse. Fortunately, the new sampling devices arising from the basic studies of tsetse behaviour allowed the detection of some serious errors in earlier understandings. For example, information concerning tsetse movement had referred mainly to male *G. morsitans* and indicated that the flies moved about 150 m per day. When the new sampling devices allowed the capture of large numbers of other tsetse it was realized that females move much more rapidly – about 1 km/day for female *G. pallidipes*.

Required density of baits

Predictions for the optimal density of baits depended on studies of the population dynamics and movement of tsetse, as detailed in Chapter 7. The full mathematics of the predictions are complex, but fortunately they can be reduced to a simple 'rule of thumb' applicable in places not subject to invasion, i.e. in isolated pockets, or at least 8 km inside a large area treated with baits. Then, the level of population reduction after a year of operation of well-maintained baits is roughly predictable from the equation:

$$N_{365} = N_0/10^X$$

where N_0 is the number of tsetse before bait deployment (day 0), N_{365} is the number after a year (day 365), and X is the percentage of the adult tsetse population removed per day on the first day or so of bait deployment.

For example, if each bait removes 1% of the population per square kilometre per day, the number removed by four baits per square kilometre would be about 4%. This would mean that the population declines by a divisor of 10,000 (10^4). Hence, if the starting population density of tsetse were 1000/km^2, the population after a year would be only 0.1/km^2, i.e. below the self-sustaining level of about 1/km^2, so that the population would be exterminated. The value of X is defined as applying to the first few days of deployment, since the removal rate with stationary baits declines once the baits have been operating for a week or so. This is because the mean age of the tsetse population decreases and the younger flies are less readily available to stationary baits.

The approximate percentages per trap per day to use for calculating X are shown in Table 28.1, which covers situations in which the traps and targets are used with the recommended odour (see above). It is also assumed that the traps and targets are optimally sited and that the cattle graze in tsetse-infested habitat, at a time of day when the flies are at peak activity, i.e. usually in the few hours after sunrise and before sunset. The values shown for cattle in Table 28.1 are first approximations, since (as indicated later) the responses to cattle have not been measured fully. Baits to form barriers to tsetse invasion are most effective when deployed in bands 8–10 km wide, at the sort of densities required to eradicate a dense, isolated population in a year, e.g. odour-baited blue-black targets at about 4/km^2 for *G. morsitans* (Hargrove, 1993).

Optimal siting of stationary baits

It is safest to spread the baits as evenly as possible, especially in an invasion barrier, but quite large departures from evenness are permissible when the baits are deployed to eradicate tsetse from large areas in a year. For example, if targets are to be deployed at 4/km^2 it is acceptable to put them at intervals of approximately 150 m in lines 1.5–2 km apart, so that no part of the habitat is more than about 1 km from a target. Spacing that is less even than this is not recommended, especially when higher densities

Table 28.1. Approximate percentages of the population removed per bait per day, when various baits are deployed at 1/km^2, under conditions specified in the text[a].

Bait	*G. morsitans*	*G. pallidipes*
F3 or Epsilon trap	0.1	1.0
Black target, 1.0 × 1.0 m	0.7	1.5
Blue-black target, 1.0 × 1.7 m	1.0	2.0
Ox, *c.* 400 kg	3.0	3.0

[a]The value of X for N baits/km^2 is $1 - (1 - P)^N$, where P is the indicated percentage in the table, and X and P are expressed as their proportional equivalents (i.e. 1% becomes 0.01).

of targets are being deployed to achieve faster control – it cannot be guaranteed that all of the flies will diffuse to targets more than about a kilometre away in much less than a year.

Studies with a variety of natural and artificial features at sites showed that dense leafy bushes and shady trees can limit the ability of *G. morsitans* and *G. pallidipes* to perceive the bait, navigate towards it and get caught after arrival. In general, catches were usually greatest at sunny sites, on paths or in clearings a few metres in radius. Catches of asymmetrical traps such as the Epsilon increased by about 50% when their entrances faced downwind. There was no evidence that leaving a bait at one site caused a merely local reduction in densities of *G. morsitans* and *G. pallidipes*, suggesting that there is no need to relocate baits continuously in order to enhance catches.

Adoption of New Bait Techniques

Putting together the whole sequence of bait development considered above, there was an enormous improvement in the cost-effectiveness of baits for use in all parts of Africa in the three decades after 1970. This is illustrated for *G. morsitans* and *G. pallidipes* in Zimbabwe by the cost per fly caught or killed at stationary baits (Fig. 28.1). By using the blue-black target baited with attractants, the cost-effectiveness of control was increased about 100–1000 times relative to the odourless traps used in the early 1970s. Insecticide-treated cattle are even more cost-effective than targets as control tools, provided that the cattle are already present and being dipped, so that their costs are counted only as the marginal expense of substituting pyrethroid for the usually cheaper chemicals used against ticks. Odour-baited traps have improved the cost-effectiveness of surveys by 10–100 times, the increase being greatest with *G. pallidipes*.

The new traps for tsetse surveys were adopted rapidly in all parts of Africa but acceptance of the bait methods of control was slower, reflecting the need to perform extensive field trials. Nevertheless, starting in the late 1970s in West Africa and in the mid-1980s elsewhere, bait methods eventu-

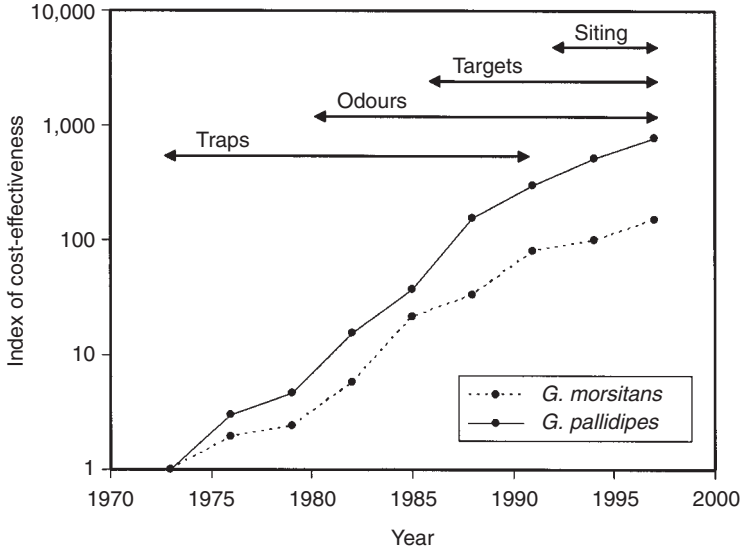

Fig. 28.1. Index of the cost-effectiveness of the recommended types of stationary bait, during phases (covered by horizontal lines) in which research concentrated mainly on the indicated topics. The index is the number of female tsetse caught or killed per unit cost at today's prices, adjusted to an index of 1.0 for 1974 (referring to an odourless Morris trap).

ally replaced almost entirely the various spraying measures. Sometimes the control baits were traps used without insecticide (Dransfield *et al.*, 1990) but it was usual to employ insecticide-treated traps or targets in the larger operations (Laveissière *et al.*, 1990). The combination of insecticide-treated cattle and artificial baits was common (Bauer *et al.*, 1999; Warnes *et al.*, 1999).

The speed and effectiveness of transfer to bait control is illustrated by the case of Zimbabwe (Fig. 28.2). During the transfer in southern Africa, the baits and various spraying techniques were used in a variety of situations in the northeast and west of Zimbabwe, in the west and east of Zambia, in the Caprivi Strip of Namibia, and in Malawi. This allowed an assessment of the relative merits of the techniques. In making the comparisons it is crucial to distinguish between the various types of objective of the control operations: (i) the first attack on the flies in an eradication campaign; (ii) the mop-up of any residual pockets of infestation left after the first attack; and (iii) the prevention of invasion into the cleared area.

The comparisons are summarized in Table 28.2. All of the indicated methods of control are suitable for the first attack, subject to certain limitations. For example, cattle cannot be used where they do not occur, targets cannot be used where they will be stolen, aerial spraying cannot be performed satisfactorily among mountains, and ground spraying with DDT cannot be used where ecological considerations prevent it. Bait methods of control, especially the insecticide treatment of cattle, can be the most satisfactory means of mopping up small residual foci found when the short spraying season is over – the baits can be deployed promptly before the foci spread. The baits are better than spraying measures in the prevention of invasions, because the baits work all year round.

It is in the production of invasion barriers that one of the main benefits of bait development has been felt. For example, from the late 1980s the stabilized fly-front in Zimbabwe was held effectively by the use of targets and treated cattle, despite occasional lapses in bait maintenance, as when there were delays in obtaining target

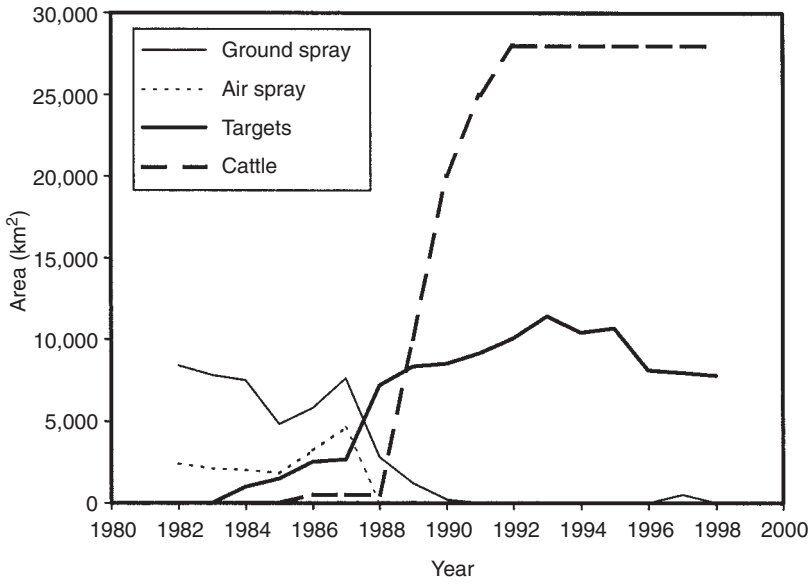

Fig. 28.2. Areas of Zimbabwe treated annually by various methods of tsetse control (from data supplied by W. Shereni, Harare, 1998, personal communication).

Table 28.2. Technical feasibility of different methods of tsetse control to deal with various control objectives (* poor, *** good). Feasibility is subject to limitations described in the text.

Method	First attack	Mop up	Invasion barrier
Aerial spraying	***	*	*
Ground spraying	***	*	**
Targets	***	**	***
Treated cattle	***	***	**

materials and when droughts made it difficult to fill dip tanks. If the baits were not available as barriers, it would have been necessary to rely largely on ground spraying to clear the annual reinfestations, involving the ecological impact of residual insecticide applied to the same areas for many years in succession.

Present Recommendations

The technical details of the baits themselves are covered by a number of publications (e.g. FAO, 1992) and manufacturers' recommendations, and Chapter 29 considers implementation policies. Hence, it is pertinent now to consider only some of the potential pitfalls arising from the apparent simplicity of the technology. The general danger is that the low technology of bait control encourages the idea that the normal standards of planning and execution can be lowered with impunity. Certainly, the bait systems can be more forgiving than some of the other methods of control, but all too frequently bait programmes are adopted with poor understanding and commitment.

Size and shape of treated area

One problem is that the ability of baits to counter invasion has sometimes led to the notion that baits can be used effectively in any area, irrespective of the invasion risk. However, it should be recognized that the flies must enter the treated area to be killed and if the invasion pressure is strong the invading flies will travel up to about 5 km into the bait placements. Accordingly, it is necessary to consider carefully how the invasion problem applies in the particular size and shape of area to be treated. When dealing with the highly mobile species of the *morsitans* group it is not recommended to attempt to tackle an area much less than 1000 km^2 unless the invasion pressure is weak or is made so by additional baits in the invasion source.

Heterogeneity

The use of bait technology by local communities to control (rather than eradicate) tsetse means that several ecological and social heterogeneities assume huge importance. Firstly, the distribution and abundance of tsetse varies between operational areas. Consequently the control measures can have a variable impact on the incidence of trypanosomiasis. For example, a 95% reduction in tsetse abundance would be more likely to affect the incidence in areas where cattle were originally bitten once a week, not 100 times a day.

Secondly, the patchy distribution of kraals, watering points and pastures means that it can be difficult to ensure an even distribution of insecticide-treated cattle. Furthermore, the distribution of targets can also be patchy since farmers may find it difficult to deploy the baits far from home. The uneven distribution of baits reduces their capacity to counter invasion (Hargrove *et al.*, 2003).

Thirdly, the costs, benefits and ability to contribute towards community-based action vary according to such matters as the num-

bers of cattle owned, the type of cattle and the reasons for keeping them. For instance, near Tanga in Tanzania some of the cattle owners keep just one or two 'improved' dairy cattle that they zero-graze, whereas the traditional Maasai pastoralists there each own about 100 zebu cattle. While both of these groups are affected by trypanomosis, the costs and benefits of tsetse control differ greatly between them.

Compared with most other methods of control, bait systems can be more readily tailored to suit the wide variation in local requirements. Moreover, the baits offer more time for appraisal and modification while the control programme is in operation, so allowing the tailoring to be refined while the operation proceeds. Hence, the full benefit of bait control is not achieved by deploying the baits in a presumed 'standard' system, and then forgetting them.

Further Research and Development

Some of the further research required involves solutions to problems long outstanding. One such matter is the identification of the unknown attractant(s) in ox odour – success should be able to double the effectiveness of traps and targets, reducing by half the number that need to be deployed in most situations. Another matter is the need to improve trapping efficiency for *G. morsitans* and *G. austeni*, thus making surveys more reliable and convenient with these species. The greater number of topics for further study arise from the change of strategy in tsetse control – the idea that we should now learn to 'live with tsetse', relying on the local community to control the flies on a small scale with a minimum of government input. This shift away from the concept of large-scale operations was made possible primarily by the development of the bait techniques which, much more than spraying measures, seem usable by the farmers themselves. The new strategy raises some important issues that have been addressed only cursorily and these matters are considered below.

Insecticide-treated cattle

The application of insecticide to cattle is by far the most appealing technique for use by farmers, involving simple procedures that require no special purchase of baits. However, there are several questions to answer now that the technique is being considered more as the mainstay of tsetse control for many decades, not just as a temporary assistance to large-scale eradication that is to sweep on beyond the farming district.

Some of the questions are veterinary. For example, why does the incidence of trypanosomiasis decline when the cattle treatments produce only a slight reduction in tsetse abundance, and how does this impact on the recommended treatment tactics? How can it be ensured that the insecticide treatment for tsetse control will not aggravate tick resistance to acaricides or upset the natural immunity to tick-borne diseases? In the socio-economic context: how can a farmer be convinced to take his herd into vegetation types where tsetse are most abundant, i.e. the places hitherto avoided? How is cattle distribution governed by various aspects of livestock management practices, particularly those relating to grazing patterns and the distribution of watering points?

Many of the other questions are entomological. For example, although much is known about the responses of tsetse to stationary, artificial baits, relatively little is known about the responses to mobile animals, so that it is more difficult to predict the optimal treatments of animals in various circumstances. However, what little is known suggests that the samples of flies visiting mobile animals and stationary baits can differ in many important respects, such as their age structure, physiological state, sex ratio and species balance (Vale, 1974). Moreover, the different species and ages of live baits and the different herd structures can elicit different responses. Can repellents be used to assist the insecticide treatments, possibly encouraging flies to avoid animals not treated with insecticide and concentrate on those that are?

In the Busia District of Western Kenya, farmers reduced the risk of tsetse biting their zero-grazed cattle by surrounding their herds with a 1.5 m high barrier of insecticide-treated netting. The owners reported that this intervention reduced disease incidence and improved herd health and productivity (B. Bauer, personal communication, 2003, Nairobi). Recent research in Zimbabwe has demonstrated that some tsetse will fly over such barriers and thus the risk of disease is not eliminated completely. However, the protective effect of the netting barrier can be further improved by using wood smoke to repel tsetse, and the combination of these simple technologies can reduce the biting rate by ~95% (S. Torr, unpublished data).

Further important matters concern the insecticides. The first work in Zimbabwe and West Africa contributed to the generally accepted view that the pyrethroids are effective on cattle for 2–3 months. Recent work in Zimbabwe suggested that the persistence periods averaged only 1 month, and could be as short as 5 days in hot weather (Vale *et al.*, 1999). This is worrying, since it implies that treatment intervals might sometimes need shortening, thus increasing costs which are already daunting to poorer farmers. Moreover, the dung from insecticide-treated animals killed many dung beetles during the wet season, in numbers sufficient to reduce substantially the population density of at least some species (Vale and Grant, 2002), thus raising concerns that the insecticide treatments could interfere with the incorporation of manure into the soil.

Current work with *G. pallidipes* in Zimbabwe suggests that, even with an increased frequency of insecticide application, the overall costs of cattle treatment can be reduced substantially by restricting the application of insecticide to those hosts and body regions where most tsetse feed, such as the front legs and belly of adult cattle (Thomson, 1987; Vale *et al.*, 1999; Torr *et al.*, 2001). Furthermore, such restricted application appears to reduce to insignificant levels the insecticide contamination of dung.

Reduction in scale

With the new strategy of small-scale operations we need to understand more about the routes and rates of tsetse invasion in order to combat the invasion more economically and effectively. Conventional studies of movement, based largely on recaptures of flies marked and released, are unlikely to furnish the detailed information required. A more promising prospect is the use of a harmonic radar system for tracking individual tsetse – the 'ultimate' tool for studying tsetse movement. This system has been developed in the UK, primarily to track tsestse, but so far it has been applied in earnest only to other insects, allowing them to be 'watched' continuously at ranges of up to 1 km (Riley *et al.*, 1997). The radar technique should be used with tsetse to supplement and to help interpret the movement data produced by conventional means.

Overview

By 1990 the programmes of bait development in various parts of Africa had achieved in large measure the two things they had set out to do in the late 1960s. Firstly, they had improved the cost-effectiveness of tsetse surveys. Secondly, they had designed economical baits that can eradicate tsetse on a large scale and can form invasion barriers to hold the front while the tsetse clearance is consolidated and the further advance of control operations is prepared. Partly encouraged by the successful use of baits in the 1990s, the strategy for tsetse control now involves small-scale operations, with virtually permanent barriers to invasion. The optimal implementation of this new strategy requires research on a variety of topics, covering the use of insecticide-treated cattle and the prevention of invasion into small blocks. Meanwhile, one or a combination of bait types can tackle tsetse effectively in most circumstances, provided that the tactics are designed with understanding and implemented with commitment.

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29 The Application of Bait Technology to Control Tsetse

Peter Van den Bossche and Reginald De Deken

Baits have been used since the early days of tsetse control. In Zululand, for example, traps were used to control *Glossina pallidipes* (Harris, 1932), and DDT-treated oxen were effective tools to combat tsetse in Tanzania (Whiteside, 1949). Despite these initial successes, it took several decades before baits were recognized as an integral part of the arsenal of tsetse control methods. This was because the original baits were not sufficiently practical and cost-effective, and results were often not reproducible. The situation changed drastically after systematic research into tsetse's behaviour added a scientific backing to the principles governing tsetse's responses towards baits (Gouteux *et al.*, 1981; Vale, 1993). Since then, baits have become increasingly effective, simple, more user-friendly and less damaging for the environment. Furthermore, the development of persistent pyrethroid insecticides that are highly effective against tsetse offered the possibility of using baits that were toxic for long periods, so avoiding the need for frequent re-treatments. As a result of the better understanding of bait technology, baits have successfully controlled a wide range of tsetse species. However, sometimes the basic principles of bait technology are ignored, with ensuing disappointing results. This chapter describes the factors determining the effectiveness of bait control under various epidemiological circumstances.

Effectiveness of Baits to Control Tsetse

Several factors contribute to the effectiveness. Some, such as bait size and odour, are features of the baits themselves and have been considered in Chapter 28. Other factors are external, including matters such as the distribution of the tsetse population in relation to the position of the baits, the tsetse's preference for the bait as against wildlife, and the invasion pressure of tsetse. A bait intervention will only be effective when both the inherent and external factors have been taken into account. This may be easy with inherent factors. External considerations, on the other hand, are not always well understood and applied.

The interaction between tsetse and baits can be optimized by adjusting the distribution and density of baits according to the dynamics of the local tsetse population (Chapter 7). This means that stationary baits are options to consider in most areas as long as their numbers, distribution and upkeep can be managed. The situation is different when mobile baits are used, because the distribution and density of cattle is hard to dictate. Grazing patterns of cattle vary between livestock management practices, which may in turn change according to the season (D'Amico *et al.*, 1995; Scoones, 1995; de la Rocque *et al.*, 2001). Usually, cattle are

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kraaled and herded away from cropped areas during the rainy season. After crops have been gathered, cattle are allowed to roam free and feed unsupervised, mainly on crop residues. As the dry season progresses the cattle are forced to move further afield and to find diverse foods.

General Patterns in Effectiveness of Baits

It is possible to recognize some general patterns in the effectiveness of the different types of baits under the various epidemiological situations. For many years, the rapidly expanding human populations have, together with their livestock, encroached on areas where game is the main food source of tsetse. This encroachment has resulted in significant changes in the interaction between tsetse and livestock and has had important repercussions on the local epidemiology of animal trypanosomiasis, as detailed below.

The local epidemiology of trypanosomiasis usually follows a sequence commencing with the introduction of people and livestock into a tsetse-infested area and the subsequent progressive clearing of natural vegetation for cultivation. The epidemiological changes are a direct consequence of the gradual reduction in the number of wild hosts and the increase in the density of livestock and humans, so that tsetse usually become increasingly dependent on livestock as food. Progressive clearing of natural vegetation makes an area less suitable for the fly. Finally, the savannah species of tsetse will be confined to marginal, often protected, areas where wildlife and suitable habitat are still abundant, e.g. national parks, game management areas or forest reserves. Game animals will again constitute an important host. The interaction between tsetse and livestock will be restricted to the interfaces where the distributions of livestock and tsetse overlap, such as the edges of protected wildlife zones or watering points. Riverine species of tsetse, on the other hand, seem to adapt better to changes in the environment and often survive in the remaining sparse vegetation along rivers or lakes, in residual forests (*fôrets sacrés*) and in plantations, feeding on domestic animals and humans (Gouteux and Laveissière, 1982; Mwangelwa *et al.*, 1990).

Although these modifications in the tsetse's environment and the resulting changes in the interaction between tsetse and livestock are gradual processes, it is possible to distinguish within it four stages that affect the choice of a bait method (Table 29.1 and Fig. 29.1).

Game areas (Fig. 29.1a)

When pressure for land is high, tsetse may be controlled in game areas to make them suitable for livestock production, or a barrier may be erected to prevent tsetse from invading into adjacent settlements. Traps or targets are the only possible bait methods to employ, since cattle are absent, or present at very low density. The effectiveness of stationary baits in such circumstances has been proved on various occasions (Green, 1994). In southern Africa, vast areas of uninhabited flat and hilly terrain suitable for livestock production have, for example, been cleared of savannah species of tsetse using various modifications of the target technology (Lovemore, 1999).

Areas recently reclaimed with low densities of cattle (Fig. 29.1b)

Where cattle are present at low density and trypanocidal drugs offer insufficient protection, baits can be used to control tsetse and reduce the incidence of trypanosomiasis, without necessarily eliminating the flies. The effectiveness of insecticide-treated cattle may well be limited in these situations. This is because treated cattle will have to compete with many other baits, such as warthogs and bushpigs, the most important wild hosts of the *morsitans* group of tsetse in eastern and southern Africa (Weitz, 1963; Robertson, 1983). Although tsetse do feed on domestic animals, an increase in the number of cattle does not necessarily decrease the proportion of feeds taken from wild hosts. For example, a survey conducted

Table 29.1. Expected effects of the introduction of cattle and vegetation clearing in an area infested with savannah species of tsetse on the epidemiology of trypanosomiasis and effectiveness of bait method.

		Population density			Host preference		Effectiveness of bait method	
Epidemiological situation	Figure	Tsetse	Cattle	Game	Game	Cattle	Stationary	Mobile
Game areas	29.1a	+++	(+)	+++	+++		+++	–
Areas recently reclaimed with low densities of cattle	29.1b	+++	+	++	++	+	+++	–
Cultivated areas with high densities of cattle	29.1c	++	++(+)	+	+	++	+++	+++
Cattle/tsetse interface	29.1d	+	+++	+	+++	+	+++	–

(+) = importance between – and +
++(+) = importance between ++ and +++

Fig. 29.1. The consequence of gradual encroachment of people and livestock into an area infested with savannah species of tsetse on the epidemiology of bovine trypanosomiasis. Reprinted from Van den Bossche, P. *Some General Aspects of the Distribution and Epidemiology of Bovine Trypanosomiasis in Southern Africa*; copyright (2001), with permission of Elsevier Science.

in Mkwaja Ranch in Tanzania, where cattle formed 75% of the animal biomass, revealed that they only provided 5–6% of the total bloodmeals, while *c.* 75% were from warthogs and bushpigs (Gates and Williamson, 1984). Reliance on insecticide-treated cattle in those circumstances is thus unlikely to be satisfactory and stationary baits are probably the most effective control method (Table 29.1).

Cultivated areas with high densities of cattle (Fig. 29.1c)

The effectiveness of insecticide-treated cattle rises with increasing density and wider distribution of cattle and with the concomitant decrease in the density of wild hosts. Tsetse are then obliged to take a higher proportion of bloodmeals on cattle. For example, because of the scarcity of game animals in the densely cultivated areas of the eastern plateau of Zambia, up to 75% of bloodmeals were taken from cattle (Van den Bossche and Staak, 1997). Similar observations have been made elsewhere (Robertson, 1983; de la Rocque *et al.*, 2001). In such circumstances, insecticide-treated cattle are usually an effective alternative to stationary baits against savannah species of tsetse, or could be used in combination with traps or targets to mop up residual tsetse foci. In eastern Zimbabwe, deltamethrin treatments of cattle dealt effectively with bovine trypanosomiasis in a highly cultivated area of *c.* 2500 km^2 along the border with Mozambique (Thomson and Wilson, 1992). Similarly, in the pastoral zone of Yalé in Burkina Faso, the application of a pyrethroid pour-on to cattle caused a spectacular decline in the incidence of trypanosomiasis (Bauer *et al.*, 1999).

Cattle/tsetse interface (Fig. 29.1d)

After the extensive clearing of vegetation has made an area unsuitable for permanent occupation by a self-sustaining tsetse population, tsetse control operations aim to prevent occasional or seasonal invasions from the adjacent, tsetse-infested areas. This may require an invasion barrier at the livestock/tsetse interface. Although cultivation usually results in a steep decline and often eradication of tsetse, this is usually not the case for the riverine species. Such species can persist in patches of often sparse vegetation along rivers, feeding on various hosts, including cattle. Interaction between cattle and tsetse can be high along the interfaces of this fragmented habitat, especially during the dry season when cattle are watered along the rivers. In this case, insecticide-treated cattle may be an effective tool to control flies. However, the interaction may be too short and too irregular to eradicate the tsetse population. A combination of mobile and stationary baits, or stationary baits on their own, may be more appropriate for this purpose.

Effectiveness of Baits to Prevent Invasion by Tsetse

Reinvasion by tsetse is a serious threat to the success of any tsetse control operation. The tsetse's potential to reinvade areas is demonstrated by considering the speed with which the tsetse invasion front advanced after the southern African rinderpest epizootic at the end of the 19th century (Jack, 1914). In areas where the rate of tsetse invasion is high, the continuous replenishment of the tsetse population may make the population's growth rate higher than the mortality rate, even in the presence of baits. This is likely to occur when mobile savannah species of tsetse are controlled in a small area surrounded by tsetse-infested land (Hargrove, 2000). Under such circumstances, control can be achieved after the operational area has been extended to reach either a natural barrier to invasion, or an area of manageable invasion pressure such as the edge of the fly belt. Alternatively, artificial barriers to invasion can be put in place. Artificial barriers may also be required to protect areas once tsetse have been cleared, or to minimize the interaction between tsetse and livestock at the interface of a tsetse-infested protected zone and a cultivated area unsuitable for tsetse (Fig. 29.1d). The current trend

towards tsetse control in relatively small areas and the need to protect such areas, once tsetse have been cleared, means that artificial barriers to tsetse invasion are becoming increasingly important.

An artificial barrier must limit penetration by tsetse to such an extent that a self-sustaining population cannot be established behind the barrier, or cause a significant level of challenge at the edge of a cultivated area. An effective barrier to the invasion of *Glossina mortisans morsitans* and *G. pallidipes* can be obtained by deploying targets in a band 6–8 km wide along the invasion front with target density required for control (Hargrove, 1993). Such target barriers have been used extensively in Zimbabwe to prevent tsetse from reinvading from the wildlife areas in the Zambezi Valley and neighbouring Mozambique (Lovemore, 1999). In Malawi, target barriers have been used effectively to reduce the challenge of cattle along the edges of tsetse-infested game parks (Van den Bossche *et al.*, 2000). In the north of Cameroon, targets were effective barriers against the massive invasion pressure of *G. m. submorsitans* (Cuisance and Boutrais, 1995). Traps can also act as effective barriers to invasion of riverine species of tsetse. For example, the deployment of traps not treated with insecticide at 100 m intervals along 10 km of a riverine system in Burkina Faso protected previously cleared areas (Politzar and Cuisance, 1983).

Hence for savannah species of tsetse, targets deployed in any 8 km wide section of a control operation can, if necessary, act as a barrier without the need to adjust the deployment pattern. In areas that are cleared progressively, target barriers can be moved forward gradually and act as a rolling carpet of baits until the planned area has been cleared completely or a natural barrier to invasion has been reached. The question remains as to whether insecticide-treated cattle constitute an effective barrier to such reinvasion. This depends largely on the probability that invading flies contact a treated herd, which in turn depends on the distribution of treated herds. In a commercial farming system the distribution of herds can, to a certain extent, be altered according to the requirements. Under communal conditions, however, cattle distribution undergoes substantial seasonal changes. Because of this seasonality it is almost impossible to ensure an even distribution of insecticide-treated cattle throughout the year. This was demonstrated clearly in eastern Zimbabwe (Warnes *et al.*, 1999) and the Ghibe Valley of Ethiopia (Rowlands *et al.*, 2000), where insecticide-treated communal cattle were unable to prevent tsetse from moving into tsetse-free areas. Furthermore, flies that visit the treated cattle may die afterwards but not before many of them feed, and so treated cattle near an invasion front will be challenged continuously (Baylis *et al.*, 1994; Vale *et al.*, 1999). Stationary baits, on the other hand, can be deployed in accordance with the distribution of suitable tsetse habitat, and thus constitute effective barriers.

Sustainability of a Bait Intervention

Throughout the long history of tsetse control, large areas have been cleared using a variety of tsetse control methods. Unfortunately, many of the areas that once were fly-free have been reinvaded. For the most part, the failure of governments to sustain the clearance has been due to the inability to meet the recurrent costs of invasion barriers or suppression operations. In the 1990s, guided by the redefinition of a government's responsibility for controlling animal diseases and supported by the availability of low-technology bait methods, a range of measures has been proposed that were expected to contribute to the sustainability of tsetse control. Broadly speaking, this came down to shifting most of the responsibility for tsetse control on to the local communities. However, few of the bait campaigns to control animal trypanosomiasis have been successful when relying heavily on the support of local communities

There are numerous definitions of sustainability. In the context of tsetse control by stakeholders, a campaign could be considered sustainable when it produces benefits valued sufficiently by the stakeholders to ensure the allocation of adequate internal

and/or external resources to continue activities with long-term benefits. The main stakeholders are the cattle owners in the area under control, including those who visit the area temporarily such as transhumant pastoralists.

The evaluation of benefits and the willingness to contribute to community efforts are largely dependent on the way the benefits are perceived. Willingness to contribute is usually high in epidemic areas, where trypanosomiasis-related mortality in livestock is high, or in an endemic situation where highly valued livestock (oxen and cows) are at risk. In areas where tsetse cannot be eradicated, the benefits accruing from fly suppression are perceived mostly in the beginning of the campaign, when the effects of a declining challenge on animal health are often spectacular (Bauer *et al.*, 1992; Knols *et al.*, 1993). Once the tsetse population has been reduced substantially and challenge is low, the willingness to allocate sufficient resources to sustain the campaign usually declines (Cuisance *et al.*, 1991; Barrett and Okali, 1998). Hence, bait campaigns supported by beneficiaries are often considered to be unsustainable. It is, however, plausible that the decision made by the stakeholders to continue or halt a control campaign is based on a careful consideration of the perceived benefits of the campaign and the amount of often scarce resources required. A decision to abandon a control operation may be taken when the tsetse population has been suppressed sufficiently to reduce challenge to an acceptable level. On the other hand, initiation or resurrection of control is possible when challenge increases and the benefits perceived from alternative and often cheaper control methods (e.g. trypanocidal drugs) are insufficient. Hence, the sustainability of bait campaigns may be related to the capability of the stakeholders to implement an operation when required, rather than the capability to maintain an ongoing operation indefinitely. If this is the case, the sustainability of such 'strategic' tsetse control interventions will depend on the availability of the necessary inputs and the organizational capacity of the stakeholders. The latter may require involvement of the public or private sector. The availability of inputs is affected by the socio-economic environment in which bait operations are conducted. Availability of foreign exchange may, for example, be an important determinant in the acquisition of certain inputs, such as insecticides and some odour attractants.

To ensure optimal effectiveness of a reinvasion barrier, the maintenance of the baits requires careful planning, coordination and sometimes coercion. Often the local communities cannot meet these requirements. Besides, people near the invasion front must play the most crucial role in stopping invasion, and these are the people who may see least benefit from the effort. Hence, it is unlikely that stakeholders living on or near the front would be willing to participate in ventures of this kind for long, particularly if they have to pay for the inputs at unsubsidized prices. The maintenance of a reinvasion barrier is thus best sustained technically and financially by the public sector, possibly assisted by the private sector. In western Zambia, for example, the government issues private tenders for the maintenance of a target barrier along the Zambezi River.

Continued control of tsetse may have side-effects that reduce the perceived benefits accruing from the campaign and jeopardize sustainability. The side-effects seem most serious with insecticide-treated cattle. The intervals at which cattle are treated with insecticide for tsetse control are usually too long for complete control of ticks, but the prolonged application of pyrethroids to a large number of cattle may affect tick numbers, especially with the one-host types such as *Boophilus* spp. This will reduce the transmission of *Babesia* spp., the parasites responsible for redwater, and so prevent the development and maintenance of enzootic stability for the disease. Reduced immunity against *Babesia bigemina* has been observed in large parts of eastern Zimbabwe where insecticide-treated cattle have been used for tsetse control (Van den Bossche and Mudenge, 1999). A further problem is that ticks may become resistant to the pyrethroids used for tsetse control, so removing these pyrethroids from the list of chemicals that can be used on those occasions when tick control is needed. The development of behavioural resistance in

tsetse after prolonged use of insecticide-treated cattle may also form a potential threat to the sustainability of this method. Finally there is the risk that the insecticide will affect dung fauna, and hence the effectiveness and rate at which manure is recycled.

In general, the side effects on ticks, tsetse and dung fauna are insufficient to threaten seriously the sustainability of insecticide-treated cattle. Nevertheless, research must be undertaken to recognize and minimize the extent of these possible problems.

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30 Community Participation in Tsetse Control: the Principles, Potential and Practice

Robert D. Dransfield and Robert Brightwell

Why Bother with 'Participation'?

In recent years, there has been much discussion about the merits and demerits of different approaches towards cost-effective, sustainable tsetse and trypanosomiasis control. Opinions have become somewhat polarized between two approaches. These are described by the Programme Against African Trypanosomiasis (PAAT) (1999) as the 'area-wide interventions' approach and the 'farmer/community-based' approach. The former 'traditional' approach may be broadly described as a 'technological fix' or top-down intervention at national or regional level. This approach is advocated both for tsetse control and eradication. The farmer/community-based approach, on the other hand, promotes community participation, and the integration of tsetse and trypanosomiasis control activities into general rural development activities. The rationale for this approach is that chemotherapy will continue to be the main way to control the disease in low-challenge areas but that vector control by farmers should be increasingly utilized in high-challenge areas. This chapter focuses on this approach to tsetse control. The principles and practicalities of involving farmers and communities in tsetse control are considered below and achievements and constraints are highlighted.

Since the involvement of farmers or communities in tsetse control is a fairly recent idea, a few moments should be taken to understand why it achieved its current status. The biennial meeting reports of the International Scientific Council for Trypanosomiasis Research and Control (ISCTRC) illustrate how ideas have changed. In the late 1980s, participation by livestock farmers was first welcomed as a means to reduce the ever-rising cost of tsetse control. For example, in 1989 the Kenyan Minister for Livestock Development commented that:

> it is because [previous] techniques are highly technical, expensive, toxic and specialized that the Kenya Government took over fully responsibility of tsetse control often with little if any involvement of local farmers. This is why the recent work done … in developing the use of cheap and simple smell-bait targets and trapping technology is being highly commended (OAU/STRC, 1991).

Two years later, the ISCTRC Council recommended that 'efforts be made to educate and train peasants, at reasonable cost, in the utilization of new techniques … for vector control' (OAU/STRC, 1993).

By 1993, community participation was being linked to the concept of sustainability. The Vice President of Uganda stressed the importance of active community participation for sustainability in his opening speech

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at the Kampala meeting (OAU/STRC, 1995). In the Banjul meeting 2 years later, participants recommended incorporating existing control techniques into rural development programmes, and investigating their potential for uptake by local communities (OAU/STRC, 1997). At Maputo in 1997 (OAU/STRC, 1999a), recommendations were made to popularize vector control as a routine activity and, in order to ensure sustainability of tsetse control operations, to give more attention to enhancing capacity of community groups and other partners.

Despite the feelings of optimism suggested by these recommendations, the sleeping sickness and animal trypanosomiasis situation continent-wide did not improve over this period. In 1985 the Director of the Organization of African Unity/Inter-African Bureau of Animal Resources (OAU/IBAR) expressed concern that, 'despite sustained efforts by member governments and donors for a decade, there has been no appreciable break-through in control of trypanosomiasis' (OAU/STRC, 1986). In 1999, the Mombasa conference concluded that 'the sleeping sickness situation in Africa is gloomy. About 60 million people are at risk, and more than 10 million km^2 of fertile land are infested by tsetse. The infection rate in humans is back to where it was in the 1930s (OAU/STRC, 1999b).

Why is this? Has there been a real change on the ground to apply the farmer/community-based approach, which has then failed to come up with the goods? Hursey (1999) argued that this is the case, at least in Zimbabwe:

> Donor pressure (EC mainly) applied from 1987 successfully suppressed this effort by forcing a change to communities and bait techniques, to the extent that virtually no progress has been recorded in the subsequent 10 years or more despite annual expenditures remaining the same.

As shall be seen, such views are not supported by what is happening on the ground.

The overriding reason for the deteriorating situation is probably the general economic decline of many African countries over the last 30 years. Leonard (1993) described how, during the 'great third world depression' of the 1980s and 1990s, many cash-strapped African governments cut operating expenses to veterinary departments. In some countries this was exacerbated by insecurity, or civil breakdown. Surveillance and treatment programmes for control of sleeping sickness collapsed. Those countries carrying out some tsetse control, mainly in southern Africa, became unable to afford the high costs of aerial spraying. During this period, decision making on tsetse control passed to externally funded programmes, such as the Regional Tsetse and Trypanosomiasis Control Programme (RTTCP).

It is true that such donor involvement in decision making resulted in a growing pressure for community involvement to be written into proposals. Such pressure for change is not only in relation to control of tsetse flies. It derives rather from a much broader analysis of development efforts over the last 50 years. There is a growing consensus that a strictly top-down development process simply does not provide the goods. The outcome of such policies in the past has been a 'waste of money and a bloody mess' – quoted in Chambers (1997) from an official of a multilateral agency on integrated rural development projects. Most development specialists would now agree that working with and, more importantly, learning from communities is the only way to promote sustainable rural development. This, in short, is why we need to bother with participation – not as a minor adjunct to established tsetse control methodologies, but as a *potentially* more effective approach to rural development.

What is 'Community Participation'?

The term 'community participation' means many things to many people. As White (1996) put it, participation has become a 'Hurrah' word – one that brings a warm glow to its users but is often little more than a façade of good intentions. If its value is to be usefully assessed, there needs to be much more precision in the meaning of the term.

Barrett and Okali (1998) specified several levels of community participation in tsetse

control, ranging from people tolerating the technology in an area, through provision of labour or financial contribution towards traps and targets, up to making decisions on the control methods, their implementation and coordination of all activities. However, these levels focus upon the technology of control by the community, rather than the determination of communities' needs and priorities. This is an important omission because, if those needs and priorities are not served, control will not be maintained.

A more useful definition of four levels of participation is given by White (1996), who described how, for each level, the objectives of participation differ between the development providers (or from the 'top down') and its recipients (that is from the 'bottom up'):

1. A minimal or 'nominal' participation, in which participants give only token support, is used by organizations to make a project appear more legitimate, whereas members of the community participate for essentially social reasons, such as 'inclusion' or 'display'.
2. An 'instrumental' participation, in which participants contribute services or financial contributions, is used by development providers to reduce costs, and by the participants to gain access to a service, such as a dip.
3. 'Representative' participation, in which participants form representative organizations for management purposes, is used by development providers to promote sustainability, with the 'target group' participating in order to obtain leverage or control.
4. 'Transformative' participation, in which participants determine their own needs and priorities and take collective action to achieve these, is seen by either party as a means of empowerment.

The latter two levels, of representative and transformative participation, may be seen as moving towards another misused term: 'community-based'. This was coined to describe a situation in which development was fully initiated, resourced and managed by the community (Laleman and Annays, 1989), without any inputs from a development provider. Swallow and Bromley (1994) proposed the term 'co-management' when expertise is provided externally. Since very few communities are completely isolated from the outside world, this seems an unnecessary complication to an already overburdened terminology; most 'development' takes place as an interaction between the established and the new. It is hard for a community to initiate a novel practice or project if its members are unaware of what is possible, and what is involved. Providing information and training does not (or, at least, *should* not) necessitate taking over the project management.

Chambers (1994) advocated a system in which the 'community takes the lead in determining their needs while the [development] practitioner assumes the role of facilitator, convenor, catalyst, consultant, searcher and supplier for the community'. He identified the crucial element as whether the community determines its own needs, and considered real development to be led by local demand rather than external supply. This is the key criterion of whether a programme is community-based or not – whether the direction and pace of development are under the control of the community. Chambers noted that changes in the present attitudes, behaviour and methods of most professionals are essential for such an approach to be facilitated.

In practice, 'community participation' and 'community based' are at opposite ends of the participation spectrum described by White (1996). This is not to say that a community-based initiative cannot use similar methods to externally run projects. However, their aims and objectives are very different. Community participation and community-based initiatives tend to be mutually exclusive because externally run programmes prevent goal ownership, by either assuming responsibility or generating dependency. The disparity between community participation and community-based becomes explicit when participatory projects end. It is obviously unreasonable to blame a community for a project's failure when they had no say in its planning, have not controlled its management and regard it as coming from outside.

Consideration should also be given to what is meant by the word 'community'. This issue comes to the fore when it has to be decided who can speak, act, or take decisions on the community's behalf. Many local government officials consider themselves to have this role, even if they are appointed by central government and come from a different area and ethnic group. In such cases, the level of consultation may be nominal, little information may be passed on to ordinary people, and involvement (not surprisingly) is minimal.

More importantly, communities are never homogeneous – even if all come from the same ethnic group. For example, where trypanosomiasis only affects livestock, those most immediately concerned with livestock are likely to show the strongest interest and hence participation. For other members of the community, the involvement may be nominal. If it is completely lacking, there are likely to be serious damage or theft problems. This is especially the case if there is a history of conflict between cattle-owners and farmers, when it will be difficult to obtain much participation from the latter group. Equally, if other 'stake holders', such as local government or elders do not feel involved, there may be a serious conflict of interests.

Promoting Participation

Many potential 'tools' or inputs are available to encourage participation. Those most widely used are summarized below. These methods vary in terms of the level of participation for which they are suited, their effectiveness and their potential risks. The options that are available for tsetse and trypanosomiasis control and are suitable for educating and mobilizing communities will be considered first

- Public meetings have long been favoured for promoting public awareness, 'sensitizing' people to important issues and encouraging participation. Most commonly, these consist of speeches by officials and 'experts', followed by endorsements from senior members of the community. Unfortunately, public meetings are rather poor at transferring information (Simbowo and Campbell, 1992) and, because they discourage discussion, they generate little involvement or 'goal ownership'. Because public meetings are regarded as 'political', they may be hijacked by local government officers or politicians for their own agendas.
- A related method is to distribute leaflets, posters or other publicity material. Illiteracy can constrain the effect of printed materials and care is required if illustrations are to be understood correctly. Inputs such as these can serve to inform, especially if used in conjunction with other methods, but their passive nature makes them inefficient at changing attitudes. Community meetings and posters have been used to promote instrumental participation in tsetse control in several countries, including Kenya, Uganda, Zambia and Burkina Faso.
- Farm visits by extension workers were long considered the primary method for encouraging farmers and communities to take up new methods and participate in development activities. They remain one of the most effective means of communicating information and can also be effective in changing attitudes. However, as governmental ability to deliver services has decreased, and donors have switched to funding through non-governmental organizations (NGOs), extension visits have become more patchy and keyed to short-term initiatives. One risk here is that, if the 'message' is not under government control, government officers may regard extension workers with suspicion and even hostility. For example, paraveterinary training programmes may be considered as challenging veterinary regulatory authorities.
- Participatory Rural Appraisal (PRA) and Rapid Rural Appraisal (RRA) encompass a family of participatory techniques that have been used both to gather and to disseminate information, and to promote community action. Techniques include semi-structured interviews, transect walks, focus groups, mapping by local people, time lines and seasonal calendars

(for a review of these, see Chambers, 1997). Used properly, PRA encourages involvement and can be a powerful tool for empowerment. The main constraints tend to be bad practice in its application. Whilst PRA should be regarded as an approach for facilitators working with a community, it is often now regarded as a one-off event, sometimes to be completed in a single day. PRA is conducted with a small number of selected 'opinion leaders' and 'key informants', who are then expected to pass on information, organize, and involve the rest of the community. In practice, this may not occur.

- Drama is one technique (sometimes included within the PRA 'family') that is potentially very effective in reaching the less privileged members of society, and in promoting involvement by stimulating discussion. Theatre has made its greatest impact in South America, where 'revolutionary theatre' gained prominence. Its forms range from scripted plays, through role-plays in workshops, to community theatre – where ordinary people devise the topic and scripts (Mavrocordatos, 1998). Plays broadcast by radio have the potential to reach farmers over a very wide area. In South America, revolutionary theatre is considered an important means for communities to discuss and become involved in development. In Africa, theatre has primarily been used in its 'didactic' form, to put over a specific message, probably most successfully in AIDS awareness education, but also for tsetse control (Okoth, 1998). Where theatre is only used to disseminate messages, rather than to stimulate discussion, its effect on participation is limited. Participatory theatre designed to stimulate discussion has, however, been used in Kenya for community mobilization in relation to tsetse control (Brightwell *et al.*, 2001).

Community education and mobilization are the most important tools for involving a community in development activity. Whilst public meetings and workshops may suffice for nominal and instrumental participation, they are unlikely to be adequate for higher levels of community involvement. For these, farm visits, various PRA techniques and the use of drama are more effective. Other ways to promote participation include the following.

- Training of community members is likely at some stage to be a component at most levels of participation, especially if novel technologies are being utilized. This is usually the case in tsetse control programmes. On-the-job training is usually much more effective than formal courses or workshops held far from the normal working environment.
- Employment of members of the community is sometimes considered an element of instrumental participation. In tsetse control this may include cutting access tracks, building crushes or dips, clearing trap or target sites, erecting or assembling traps or targets, trap maintenance and monitoring of fly numbers.
- Rather than paying a salary, labour may form a 'community contribution' to the project. This will only work if the opportunity costs are low. It is most frequently done for trap security, where community members take on responsibility for ensuring that traps are not damaged or stolen. Sometimes members of the community are encouraged to put their names on traps or targets they have made, or to put them on their own land, so as to heighten the sense of ownership and subsequent care of the trap. Without this contribution, theft can render control with traps or targets completely ineffective – for example, in Somalia in a British-funded tsetse control project with no community participation, only 27 out of 1600 screens remained in the field after 1 month (H. Politzar, Nairobi, 1992, personal communication). Free labour is sometimes available for trap making, especially from kits, which can assembled in the homestead at whatever time is most convenient. Labour contributions have proved less successful for trap maintenance and are seldom worthwhile for cutting access tracks, which usually requires a team of people to work

together. The technique of 'contingent valuation' has been used to estimate potential levels of community contribution, but with only limited success.

- Financial inputs, materials and subsidies are often used to initiate and support development activities. In tsetse control projects, outside agencies have donated traps, targets and pour-on, and funding for transport and workshops. Such inputs may be provided to meet all costs, on a cost-sharing basis, or even with full cost recovery. The primary risk is dependency amongst its recipients. There are now a few instances where inputs are provided by the private veterinarians, presumably with an appropriate profit margin: this provides a much more viable possibility of long-term sustainability (see Bastiaensen *et al.*, 1999).
- Encouragement of group formation and subsequent organizational strengthening are sometimes used to facilitate representative participation. This type of facilitation can have a positive effect if groups form naturally, and are small and cohesive. There is a considerable risk of dependency if outside parties are involved in the early stages of group formation. A second risk is diversion from the group's original aims towards those of the donor organization.

Some of these methods are mutually incompatible, depending on the level of participation being promoted. Inputs that promote participation in tsetse control, especially provision of employment and free or heavily subsidized inputs, may well compromise any chances of control becoming community-based, and hence sustainable in the long term. Experience from many projects suggests that externally initiated control always requires some continued input. Unfortunately, in practice, irrespective of how small these requirements are, such inputs eventually cease, which then jeopardizes continued control.

Comment should be made here on research projects that are set up specifically with the purpose of encouraging or engineering community participation, so that it can then be studied. Such projects were seen as a means of improving the practice of participation. In practice, the objectives of the social scientists and the farmers tend to be very different (Williams *et al.*, 1995) and farmers have little influence on how the project is set up or run. Mwangi (1996) reported on differences between a community and a research organization in Busia, Kenya. These were related to how funds were raised for the project, and approaches to time management. A further difficulty is that the act of carrying out the research may significantly affect the behaviour of community members, and hence the outcome of the project, so rendering any research conclusions suspect.

Consequences of Participation

Firstly, consider participation at the nominal and instrumental levels – that is, where the programme is planned and managed from outside, but where some effort is made to involve local people. Such participation can have both positive and negative effects.

Smith (1998) identified some ways in which even nominal participation can be beneficial. Communities can be mobilized to improve take-up of services for specific sectors, such as veterinary checks or treatment schemes. Such improved access can put people in a better position socially and economically, and therefore politically. Community contributions mobilize underutilized resources and can serve to redistribute resources to poorer members of society. Reduced costs to government can make a programme more cost-effective, and therefore more sustainable from limited government funds. Enlistment of individuals to become project workers can raise the consciousness of those individuals, who may become involved in other areas of community development. Cooperation and consultation can have a positive impact on project management and future development policies.

However, nominal participation has also attracted criticism as being tokenist, and even repressive. Smith (1998) noted that

consultation with the community may simply be a means of legitimizing the values and priorities of the planners, rather than those of the community. It may also be used to place responsibility on those least able to bear it, becoming part of the justification for a reduced level of social expenditure. Nominal participation can result in a kind of forced labour, where people, already with insufficient time or resources to make an adequate living, are forced to utilize some of their scarce resources on something of no immediate benefit to them.

In general, most nominal and instrumental participation has a mixture of these positive and negative impacts, but where nominal or instrumental participation can become wholly negative is when the development 'partner' is purely transient. This is the case with a research project or donor programme, or in any activity that government no longer views as its responsibility, or is only undertaking when donor funds are available. As pointed out earlier, this is very much the situation that prevails in tsetse and trypanosomiasis control. In this situation, communities are left with nothing with which to participate. It is then very difficult, if not impossible, to switch from the dependency situation of nominal participation to a real empowerment situation, where communities can carry out activities without the involvement of a partner.

The consequences of representative and/or transformative participation are to enable the community to continue (and intensify) the development activity, without continued outside input. It should thus enhance sustainability. However, this process may also bring conflict. White (1996) commented '[Participation] will challenge power relations both within any individual project and within wider society. The absence of conflict in many supposedly participatory programmes should raise our suspicions.' Such conflict has indeed taken place in a few participatory tsetse control programmes, but is seldom documented. An exception to this is the demise of the Olkiramatian and Shompole Community Development Project in Kenya, which was described by Williams *et al.* (1995). In this project, unequal power relations between the community and an international research organization, and within the community between the traditional leadership and the younger more educated members, eventually destroyed the project. Such an outcome is fortunately not inevitable and can hopefully be avoided in less high profile projects.

Achievements

Let us start by looking at government policy towards community involvement in tsetse control in those countries affected with the problem. If community involvement does not figure in policy statements, it cannot realistically be expected to have been given any sort of priority in the field. The information available on this comes from the most recent available country reports from the OAU/STRC meetings, together with data given in the PAAT country profiles.

In terms of national policy, only four countries in Africa (Ethiopia, Zambia, Zimbabwe and Malawi) are committed to a more decentralized approach to tsetse control, including a limited level of community participation and contracting to private operators. In most of these countries, the strategy is justified in terms of cutting costs, rather than a commitment to community involvement *per se*. In some other countries (Côte d'Ivoire, Ghana, Namibia and Uganda) the policy is still control by central or local government, but with community participation to prevent theft of traps and targets. In Kenya, central government has also maintained formal responsibility for control, but there have been several non-government tsetse control projects, as well as a regional project under the control of OAU/IBAR. These have incorporated varying levels of participation. In Burkina Faso and Togo, government policy is unclear, but donor-funded programmes have promoted the commercial supply of pour-on to farmers. Botswana plans to use private contractors for tsetse control, as well as involving tour companies in target deployment and maintenance. There is unfortunately no information concerning policy in relation to

community involvement in tsetse control for the remaining 22 countries in sub-Saharan Africa. This stems partly from deficiencies in the OAU/IBAR and PAAT databases, especially for West and Central Africa, but also from some countries focusing on trypanotolerance and/or chemotherapy rather than tsetse control (seven countries) and others (nine in all) suffering from civil strife. In general, then, the policy commitment to community participation still seems to be at a rather low level.

In terms of control activities on the ground, most community participation in southern Africa has been at the nominal or instrumental level, with the community providing free labour. Barrett and Okali (1998) described three target control operations against two subspecies of *Glossina morsitans* in Zambia in the mid-1990s. Free community labour was provided for target deployment and maintenance.

In other parts of Africa, there are examples of several different approaches to participation, including a few efforts to promote systems that can function with little or no government input. In West Africa during the 1980s and 1990s, Laveissière involved community members in the control of *Glossina palpalis palpalis* by insecticide-impregnated screens in Vavoua focus in Côte d'Ivoire. Inter-ethnic differences between the heterogeneous communities of Côte d'Ivoire, together with inadequate supervision, were considered the main problems (C. Laveissière, Bouaké, 1993, personal communication). In the early 1990s in Togo, villagers and herdsmen provided free labour to maintain targets, but participation declined over time due in part to the excessive work load and difficulties in delimitation of the zones that each herdsman had to maintain (R. De Deken, Antwerp, 1994, personal communication). Use of pour-on has since proved more sustainable. Elected farmer representatives are coached by field staff and private veterinarians to ensure treatment of all cattle. Efforts are being made to establish distribution and sale of pour-on through private veterinarians (Bastiaensen *et al.*, 1999). In Burkina Faso, 5 years of field experience showed that the use of pyrethroids applied to cattle for simultaneous tsetse and tick control was very effective (Bauer *et al.*, 1995). Moreover, it was thought to be a more readily acceptable method of tsetse control than traps and screens for the livestock owners. One constraint was the cost of pyrethroids (the project was obtaining them tax-free); another constraint was the poor state of the livestock industry because of the import of highly subsidized meat and milk products (B. Bauer, Bobo-Dioulasso, 1994, personal communication).

In Central Africa, traps and targets have been used for control of both human and animal trypanosomiasis. In the Bouenza region of the Republic of the Congo, large-scale trials were carried out in the 1980s using unimpregnated traps for the control of *G. palpalis*. Traps and training were provided free to villagers, who then deployed and maintained the traps to effectively control tsetse. However, the quality of trap maintenance declined as fly density dropped (Gouteux and Sinda, 1990) and control was not sustained. In the Central African Republic, traps were used to control *Glossina fuscipes fuscipes* to protect livestock owned by the pastoralist Mbororo. This is one of the few programmes where the level of participation has gone well beyond instrumental to representative, and possibly transformative. There was extensive community education and mobilization using demonstration teams, posters, extension visits and radio programmes (Blanc *et al.*, 1991). Local stockbreeders' associations and pastoral interest groups were involved, and traps were sold to farmers, rather than provided free. A subsequent cost–benefit analysis (Blanc *et al.*, 1995) showed the value of this method for the farmers and the country, although at present only a small proportion of livestock farmers use the method.

The greatest diversity of approaches to community participation has been in East Africa. In Uganda, large-scale control of *G. f. fuscipes* has been carried out using pyramidal traps deployed by government staff, but maintained by local people employed by government (Lancien and Obayi, 1993). There has also been a very limited deployment of locally made traps and targets

(Okoth, 1999). In Ethiopia, under the supervision of government tsetse control staff, farmers maintained insecticide treated traps and targets for controlling *G. morsitans* and *Glossina tachinoides* (Slingenbergh, 1992). A long-running research trial in the Ghibe area has demonstrated sustained demand by livestock farmers for deltamethrin pour-on, albeit at subsidized prices. Factors affecting demand included season, distance to the nearest supply point, and whether neighbours were also using pour-on (Wangila *et al.*, 1999). The latter finding suggested that some collective action in the use of pour-on was occurring informally.

Several tsetse control programmes featuring varying levels of community participation in Kenya have been reviewed by Brightwell *et al.* (2001). A research project to study participation in control of *Glossina pallidipes* was established in the Nyaboro area adjacent to Ruma National Park in western Kenya. The project sought to use representative participation to foster a community-based approach. However, by 1998 there were very few traps still operational, probably because effective tsetse control in the neighbouring national park meant that tsetse control was not a priority for the community. Another smallholder programme at Kathekani also used a representative approach, but was very dependent on outside funding. There have been a number of tsetse control projects in pastoralist areas, including the Olkiramatian and Shompole Community Development Project. This project sought to integrate tsetse control with other rural development activities, using both representative and transformative participation. Despite considerable technical success in controlling *G. pallidipes*, this project ended in 1993 (see above), but some use of pour-on by farmers has continued.

Constraints

Nominal participation

To improve upon past performance, it is necessary to make the constraints explicit and consider how might they be addressed. From the above it is clear that, although there are many examples of nominal and instrumental participation, more substantive forms of participation have been rare. To be effective, the level of community participation must be adapted to anticipated future levels of government action, whether direct or contracted to private operators. If government intends to initiate or maintain control operations, but requires some community contribution to reduce costs, then instrumental participation is sufficient. But if government regards some areas as 'low potential', or is withdrawing from any active tsetse control, then higher levels of participation are essential to produce a genuine farmer- or community-based approach, which people can maintain without the intervention of government. One constraint to farmer-based tsetse control has been the imposition of tsetse control on communities, with only nominal participation.

Collective action

Another constraint results from the considerable dispersal ability of savannah tsetse. Because of this, control activities for these species must cover a relatively large area, usually several hundred square kilometres, in order to minimize the effects of reinvasion. Hence some level of formal or informal 'collective action' is required by the people living in that area. The problem with services such as tsetse control is that they are 'public goods', in that benefits spill over to other members of the community. In such a situation it is difficult to get someone to take the initiative and pay for the service (e.g. traps or targets), as everyone wants to wait and be a 'free rider'. Bauer and Snow (1999) pointed out that this is less of a problem for insecticide treatment of livestock than for traps and targets, since in the former case there is also a private benefit, namely tick control. However, whatever the method, there must still be agreement between the majority of farmers over a large area to use such methods if control is to be effective. So far, most projects have achieved this unanimity of purpose by offering pyrethroid

pour-ons at well below the rate at which it could be supplied by private veterinarians.

One possible solution to this constraint (at least for animal trypanosomiasis) is for control to be spearheaded by smaller subgroups within the overall community. Hardin (1982) showed that a collective good will be provided once an individual or a cohesive subgroup finds that the benefits to itself from creating that good are greater than the cost of creating the collective good for the whole group; the greater the difference in costs, the greater is the likelihood of this occurring. The smaller the subgroup, the more likely it is to achieve the cohesion necessary for collective action. Unfortunately, where representative bodies have been formed in communities to date, they have usually been set up to represent all the community, rather than those livestock keepers most likely to benefit. Small subgroups comprising the main livestock keepers in each area would probably be able to coordinate control efforts much more effectively, whether maintaining availability of pour-on or supervising effective trap or target maintenance.

Cost

The cost of tsetse control is another important constraint, despite the lower costs if it is applied by farmers rather than by government staff. Farmers have little liquid capital; they are reluctant to commit it to control programmes they know little about and are uncertain whether they can maintain. The easiest way to reduce costs is better implementation of existing knowledge. For example, once a tsetse population is reduced, control can be maintained with far fewer traps, targets or treated livestock than were needed to obtain a rapid reduction. The density needed depends on the rate of reinvasion. Both Brightwell *et al.* (2001) and Bauer and Snow (1999) noted that technical guidelines on use of odour baits and insecticides are often not followed, resulting in higher costs than necessary. As for the use of traps versus targets, traps provide the undoubted advantage of farmers being able to see the caught flies. Also, traps continue to work if there are delays in respraying, but at a lower efficacy. However, traps are much more cost effective if also treated with insecticide, and continue to be effective at killing flies even if they are damaged.

Some costs may be more difficult to reduce. The pour-on formulation of deltamethrin would undoubtedly be used much more widely if the price was lower. The benefits of pyrethroid application to cattle are surprisingly well known, to the extent that a number of ranch owners in eastern and southern Africa treat their cattle using agricultural pyrethroids (sold at a much lower price) with diesel as a carrier. We are not aware of any scientific trials to test the efficacy of such practices, but if prices remain at their present level such practices by farmers are likely to become more widespread. Government regulations on pyrethroid acaricides in countries such as Kenya permit use of an alternative drug or insecticide only after widespread resistance has developed to those in use. Such regulations reduce potential sales and hence increase the price. This practice also promotes, rather than diminishes, the development of resistance (Dolan, 1999).

Information

Lack of reliable information is an important constraint to farmers initiating tsetse control. This includes information on diagnosis, treatment and vector control techniques. This is known as a ‘transaction cost’, described by Williamson (1985) as the economic equivalent of friction in physical systems. Without sufficient information available there is no chance that tsetse control can become ‘demand driven’ rather than supply driven. Brightwell *et al.* (2001) noted that, in Kenya, the most frequent technical constraint was the difficulty of obtaining competent technical advice. Many government extension services in Africa are no longer functional, especially in the livestock sector. Research institutions should not be expected to fill this void – scientists are trained and rewarded for doing research, not for designing and implementing extension work.

The solution here lies instead in the privatized veterinary services that are slowly being created across the continent. Until recently, underfunded state-run services have prevented both wealthy and poor livestock owners being served to the extent they desire and can afford. Government regulations have either prohibited private entry or made provision unprofitable and, with no economies of scale, prices have remained high (Leonard, 1993). Privatization should reduce the costs of veterinary services and products through competition and a better targeting of services to different types of farmers. One of those services needs to be the provision of information to farmers on appropriate trypanocidal usage, and how such chemotherapy can be integrated with tsetse control in high-challenge situations.

Provision of such information not only provides farmers with all the necessary options; it also reduces another transaction cost, that of risk. One of the main reasons that farmers are sometimes reluctant to take up new initiatives is the possible risk of failure, in what is anyway a risk-prone enterprise. If information is available on the effectiveness of tsetse control measures, such measures are more likely to be adopted by other farmers. The most effective means to disseminate this sort of information may be through farmers' associations or interest groups, such as those described by Blanc *et al.* (1991).

The state of the livestock industry

One other highly relevant transaction cost for livestock farmers is tied capital. The only way most livestock farmers can mobilize resources is through sale of livestock or livestock products. Yet the state of the livestock industry in Africa leaves much to be desired. Prices are often kept low by governments to keep the urban population content, and it can be difficult to export with the restrictive regulations imposed by many developed countries. Whilst subsidies for the livestock industry are the rule in developed countries, they are almost non-existent in Africa. The situation is exacerbated in some countries by massive imports of meat and dairy products from countries with heavy subsidies (Bauer and Snow, 1999).

Attitudes

The last constraint to be considered is probably the most intractable of all: the attitudes of the key players in the development business, whether donors, government or the technocrats. Cherrett *et al.* (1995) pointed out that in development nowadays 'we need the professionals on tap, not on top'. This rarely happens in the field of tsetse control – the professionals decide when, where, how and if tsetse are to be controlled. This does not leave much in the way of decision making for the people living in an area.

A somewhat controversial example of this is given by Hancock (1989). He describes how, in one African country,

> I met an anthropologist from Manchester University who had been contracted by Britain's Overseas Development Administration to do a survey amongst settled farmers in a tropical area that was about to be extensively sprayed to eradicate tsetse fly (and in which limited spraying had already begun). What he discovered, after conducting detailed interviews, was that the local people were bitterly opposed to the project. Many of their chickens, which contributed an important part of their diet, had been killed by the initial spraying and they did not want to lose any more. In addition, they were apprehensive that once the tsetse flies were gone nomadic herdsmen would move livestock in and destroy their crops (cattle cannot graze in areas of tsetse infestation because of trypanosomiasis). The anthropologist's findings were ignored by ODA, which went ahead with the spraying anyway (indeed, it is difficult to see why the survey was commissioned in the first place: the decision to bombard the area with insecticide had been made some time before and was, according to the anthropologist, irrevocable).

Conclusions

Over the last 25 years, community participation has become an essential component in the project documents of every substantive programme in tsetse control. It even appears as a component of the International Atomic Energy Agency's plans for large-scale eradication of *G. fuscipes* using the sterile insect

technique. Yet with a few notable exceptions, that is where real participation has remained – on the pages of proposals. The attention of donors and governments alike seems to have moved from solving problems to describing them. Innumerable policies have come and gone, but the flies and their disease are, if anything, more prevalent.

Farmers and communities have not controlled tsetse because, with few exceptions, they were not given the opportunity. Continent-wide, tsetse control has remained as top-down as before. Novel vector control technologies, such as traps, targets and insecticide treatment of livestock, have merely added to the traditional techniques expensively applied by government and development staff, or more frequently have not been used at all. From the available evidence, most community participation in tsetse control to date has been nominal or, at best, at the instrumental level.

This may be adequate if vector control is intended to supplement efforts to control sleeping sickness, and there is a long-term commitment from government to maintain tsetse control under the health programme. But if the intention is to control animal trypanosomiasis, then no country in Africa appears to be committed to sustained control using government resources, with the possible exception of Botswana. Elsewhere the livestock farmer is expected to meet the costs. In this situation, nominal and/or instrumental participation can never result in sustained control. Such 'participation' is a cruel façade, and only delays the introduction of cost-effective packages of chemotherapy and vector control.

It seems likely that economic pressures must eventually force a more realistic approach on donors and governments alike. As privatization of veterinary services proceeds, and as drug resistance increases the cost of chemotherapy, veterinarians will face increasing calls from farmers for more effective solutions to their problems. Fortunately the technologies are available to meet these calls. Whether the money is there will depend on the general state of the livestock industry.

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31 Control of Triatominae

João C.P. Dias and Chris J. Schofield

Introduction

Amongst the parasitic diseases of Latin America, Chagas disease has the highest impact in terms of its social, medical and economic consequences. In the early 1990s, some 16–18 million people were estimated to be infected with the causative agent, *Trypanosoma cruzi*, with a further 100 million considered at risk (WHO, 1991). Ranked in terms of DALYs (disability-adjusted life years lost due to the infection) the impact of Chagas disease in the Americas was considered greater even than that of all other parasitic diseases combined (World Bank, 1993). In spite of this, many governments gave little attention to Chagas disease, since it was primarily an infection of the rural poor rather than the urban rich. This scenario is now changing for three main reasons: (i) increasing recognition of the socio-economic impact of the disease; (ii) changing epidemiological patterns with increasing risk of urban transmission; and (iii) proven feasibility of control (see Chapter 19). To a large extent, human demographic changes over recent years – involving extensive migration from endemic rural areas to cities – have also had an impact on the incidence of Chagas disease, as have the extensive Chagas disease vector control programmes, leading to currently revised estimates of around 11 million people infected (Schmunis, 1999). Chagas disease remains difficult to treat (see Chapter 22) but it can be readily controlled by halting transmission – mainly through measures to reduce the risk of tranfusional transmission from infected blood donors (see Chapter 26) and elimination of domestic populations of the insect vectors, i.e. large bloodsucking bugs of the subfamily Triatominae.

The Triatominae (Hemiptera, Reduviidae) are notorious both as bloodsucking pests and as vectors of Chagas disease thoughout Latin America. Domestic infestations can be highly annoying, leading to psychological stress for the householders and to resources of poor families being wasted in ineffective attempts at control. The bugs also take large bloodmeals (see Chapter 9) and are believed to make a substantial contribution to chronic iron-deficiency anaemia. Even peridomestic infestations are considered to affect animal productivity – for example, by reducing the egg production of hens. All of the 138 currently described species of Triatominae seem capable of transmitting *T. cruzi*, but it is only those species that have adapted to live in close association with humans that are considered as important vectors of the human disease. For epidemiological and operational purposes, therefore, a distinction can be made in accordance with the degree of association with human dwellings (Dujardin *et al*., 2000) as follows.

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- **Primary domestic vectors** are highly domesticated species such as *Triatoma infestans* in Southern Cone countries, and *Rhodnius prolixus* in Venezuela, Colombia and parts of Central America.
- **Primary domiciliary vectors** are highly domesticated species that also retain local silvatic ecotopes, such as *Triatoma brasiliensis* in northeast Brazil and *Triatoma dimidiata* in Central America. The distinction between domestic and domiciliary vectors is designed primarily to reflect the mode of arrival of the species into the specified domestic environment. Domestic species such as *T. infestans* arrive mainly by passive transport from other infested houses, so that for most of their distribution they are found only in domestic habitats and not in local silvatic habitats. Domiciliary species arrive at the house by active or passive transport from neighbouring silvatic habitats. Thus domestic populations can be considered as candidates for local eradication, whereas the control of domiciliary species requires long-term vigilance and repeated selective intervention.
- **Secondary vectors** are peridomestic species that generally occupy chicken coops and other animal enclosures associated with rural dwellings, such as *Panstrongylus megistus* in central and eastern Brazil, *Triatoma maculata* in Venezuela, and species of the *Triatoma sordida* group in Southern Cone countries.
- **Silvatic vectors** are species that seem predominantly associated with silvatic habitats. Some, such as species of Alberprosenini, Bolboderini and Cavernicolini, are so highly adapted to particular silvatic ecotopes that they would seem unlikely to adapt to domestic or peridomestic situations, and are considered to have no epidemiological significance. However, there are many silvatic species that have been recorded entering and colonizing peridomestic and domestic habitats, which have varying degrees of epidemiological importance. There are many examples, including *Rhodnius pallescens* in Colombia and Panama, *Rhodnius stali* and *Panstrongylus rufotuberculatus* in Bolivia, *Rhodnius neglectus* in central Brazil, and *Triatoma rubrovaria* in southern Brazil and Uruguay.

Noireau (1999) offered similar distinctions, using the terms 'major vectors' rather than primary domestic vectors, 'secondary vectors' for primary domiciliary vectors, 'candidate vectors' for secondary vectors, and considering the silvatic vectors as either 'basically silvatic' or 'exclusively silvatic'.

These distinctions are not categoric and can change with time and place. During much of the last century, for example, *P. megistus* was the main domiciliary vector in central and eastern Brazil, but was progressively replaced by *T. infestans* spreading from Bolivia (Schofield, 1988). Domestic populations of *P. megistus* were also spread into the reconcavo of Bahia (northeastern Brazil) where they constituted the main domestic vector species before being eliminated in the early 1990s. *T. dimidiata*, although a widespread silvatic species, is the primary domestic vector in Ecuador (where no silvatic populations have been reported) and the primary domiciliary vector in many areas of Central America (where silvatic, peridomestic and domestic populations can occur). Similarly, in northeastern Brazil, the main vector is *T. brasiliensis* – a species that occupies domestic, peridomestic and silvatic habitats. Some silvatic species considered as little more than 'academic curiosities' during the 1970s have since adapted to become locally important domiciliary vectors in some areas. *Rhodnius ecuadoriensis*, a silvatic and peridomestic species in Ecuador, is now the primary domestic vector in parts of northern Peru, where silvatic populations are unknown. Thus, although there are highly effective techniques for eliminating domestic populations of Triatominae, there is a constant need for entomological surveillance to monitor the domestic trend of peridomestic and silvatic vectors.

Techniques and Strategies

Sampling methods

Sampling methods for domestic Triatominae are primarily designed to assess the presence

of bugs in the house and in associated peridomestic habitats. The most widely used is the timed manual collection (*hora-hombre* in Spanish) whereby a field inspector searches the house and peridomestic habitats using a torch to see into cracks and crevices, and long blunt forceps to withdraw the bugs. The resulting counts of bugs collected can give a crude index of relative infestation rates, but as a quantitative approach this method depends on the skill and experience of the inspector, and also on the way in which the 'man-hour' is calculated (it may be, for example, two times the result of one man searching for 30 min, or of two men searching for 15 min each, and so on) (Schofield, 1978). In operational studies, where the objective is just to establish whether or not a house is infested, the manual collection is generally modified so that two men search the house for 15–20 min each or until the first bug is found, whichever is the sooner. Manual searches for bugs can be aided by the use of dislodgant sprays of non-residual irritant pyrethroids, of which the most effective seems to be 0.2% tetramethrin in water (Pinchin *et al.*, 1980).

Passive sampling devices for domestic Triatominae include various designs of artificial refuges, such as the Gómez-Núñez box, the Cohen trap and the TDR Biosensor. These are essentially open cardboard boxes pinned to the house wall, which bugs may enter over a period of a few weeks. The presence of bugs may be revealed on inspecting the boxes and finding the bugs themselves, their exuviae, eggs or eggshells, or streaks of bug faeces deposited after a bloodmeal. The faecal streaks are the most common evidence of a bug infection, and can also be detected simply by pinning a sheet of paper or calendar to the wall and examining this at intervals. A dichotomous key is available to help confirm that the faecal streaks indeed represent a triatomine bug (Schofield *et al.*, 1986). Experience shows, however, that passive sampling devices are not equally effective for all species – especially for those such as *T. dimidiata*, that are generally at very low densities in houses. Attempts to improve sampling efficiency by using baited traps (e.g. with bug faecal extracts, ammonium bicarbonate, CO_2 releasers etc.), though often showing promise in laboratory studies, have yet to prove effective in field use.

Two further approaches are widely used in control and surveillance operations. The first is to show prepared specimens or life-size photographs of the bugs to householders, noting whether or not these are recognized and if the householders report recent findings of the bugs. The second is to give the householders a self-sealing plastic bag into which they can collect any bugs they find.

Operational sampling procedures generally make use of all the above techniques: manual search of houses during the preparatory phase, passive sampling devices installed just after spraying, and householder reports during the ensuing entomological vigilance.

For research purposes, the absolute density of bugs in houses has been estimated by dismantling the house structure and collecting all bugs found, preferably using a Lincoln index to estimate collection efficiency. From such studies, the highest infestations recorded to date are 6034 *T. infestans* in one house in central Brazil (Dias and Zeledón, 1955) and 11,403 *R. prolixus* in one house in Colombia (Sandoval *et al.*, 2000).

Control methods

Development of techniques for the control of Triatominae has focused almost entirely on domestic populations, since these are of greatest medical, social and economic importance. Almost all possibilities have been explored, as summarized below.

Insecticides

Compounds from almost every class of chemical insecticide have been trialled, including organochlorines, organophosphates, carbamates and pyrethroids. Gas fumigation using cyanide or methyl bromide has also been used experimentally but is quite impractical. The compounds most

widely used in large-scale campaigns prior to 1980 were gamma-BHC (also known as lindane or gammexane) and dieldrin, but synthetic pyrethroids are now the compounds of choice – mainly deltamethrin at 25 mg active ingredient (a.i./m^2), lambda-cyhalothrin at 30 mg a.i./m^2, cyfluthrin at 50 mg a.i./m^2, betacyfluthrin at 25 mg a.i./m^2, or cypermethrin at 125 mg a.i./m^2. These pyrethroids are highly effective when applied at the appropriate dose in a good quality residual formulation such as wettable powder (WP) or flowable suspension concentrate (SC). Liquid formulations such as emulsifiable concentrates (EC) are not suitable because they do not provide sufficient residual activity on porous mud walls. A polyvinyl acetate (PVA) matrix formulation of malathion (known as Durathion®) provides extended residual activity but is difficult to apply and not widely used. Fumigant canisters generating insecticidal smoke have been widely promoted but have often proved dangerous and ineffective, and have only been widely used in Argentina.

Insect repellents

Bar Zeev (1980) examined the repellency of over 25 standard and experimental compounds, including well-known repellents such as deet, indalone and dimethyl phthalate. Most were completely ineffective in deterring feeding by *P. megistus* and further studies also indicated only limited repellence against *R. prolixus* (Buescher *et al*., 1985). More recent work suggests that deet may have slightly more effect against *T. infestans* and may prove useful for personal protection in certain situations (Alzogaray *et al*., 2000).

Insect growth regulators (IGRs)

Juvenile hormone mimics act to prevent the final moult to adult (the final moult instead gives rise to a supernumerary nymph). Precocenes act in the converse sense, inhibiting secretion of natural juvenile hormone and so promoting moulting of very young nymphs to precocious adults. Both were tested in laboratory and field trials during the 1970s but proved very slow to act, often with negligible effect on the total bug population.

Insect pathogens

A wide variety of insect pathogens has been proposed for control of Triatominae, including fungi (particularly strains of *Metarrhizum anisopliae* and *Beauveria bassiana*) and entomophagous nematodes such as species of *Neoaplectana*. In field trials such pathogens were invariably limited by climatic conditions, as they generally require very high atmospheric humidity.

Biological control

Control of Triatominae with insect predators has been widely proposed, as has the use of hymenopterous egg parasitoids such as *Telenomus fariai*. Laboratory and field trials have invariably proved disappointing and computer simulations illustrate that such techniques would be ineffective. In the case of egg parasitoids, for example, inundative release would be followed by local extinction of the parasitoid (since no more triatomine eggs would be available) but the bug population would then quickly recover – through development and reproduction of the nymphs and adult bugs unaffected by the egg parasitoids. Non-inundative release would tend to lead to balanced parasitism around the average rate of about 14% found in many natural bug populations. The egg stage of Triatominae typically carries only around 1% of the reproductive value of each generation (Rabinovich, 1972), which means that destruction of all eggs of a specified age would only reduce the resulting adult population by 1%.

Genetic control

Genetic control by the release of sterile or sub-sterile males was proposed in the 1970s. However, it proved difficult to achieve viable sterility or sub-sterility by radiation, due to the holocentric nature of bug chromosomes, and the idea of releasing male bugs into a domestic population would be considered

unethical. Sterilization by chemical methods proved possible in the laboratory but inappropriate for domestic use, because of the teratogenic and carcinogenic risk to householders. More recently, a strain of the symbiotic *Rhodococcus* bacteria that are often found in triatomine guts has been genetically modified to express L-cecropin. This results in bugs in which *T. cruzi* cannot develop, and has been proposed as a means to control transmission (Dursavala *et al*., 1997). Notwithstanding the practical difficulties of spreading genetically modified bacteria amongst domestic bug populations, however, such an approach would be unethical when methods exist to eliminate the bug population altogether.

Traps

A wide variety of traps has been proposed for the control of domestic Triatominae, and several have been patented. These range from various designs of cardboard box that act as artificial refuges (e.g. the Gómez-Núñez box, the Cohen trap, the Maria sensor or TDR biosensor) to similar designs containing non-drying adhesive and/or IGR or insecticide formulations. Similarly, a wide range of possible attractants has been tested, including beta-lights, chicks or small mammals, various volatile fatty acids, and ammonium bicarbonate. Without exception, all have proved ineffective and impractical as a control measure though artificial refuges are sometimes used as a sampling technique, and adhesive traps baited with a small mammal are proving highly effective for sampling silvatic bug populations (Noireau *et al*., 1999; Abad-Franch *et al*., 2000).

Housing modifications

Structural modifications to rural housing have been widely used in the context of domestic triatomine control, sometimes including modification of the peridomestic structures. The aim in all cases is to reduce the availability of dark cracks and crevices where the bugs can hide, though improvement of rural housing is also a recognized development objective irrespective of the presence of domestic Triatominae. The techniques generally involve replacement of thatch roofs with tiles or sheets of corrugated metal or fibre-cement, improvement of the beaten-earth floor using tiles or poured concrete, and various forms of wall-plaster (Schofield and White, 1984; Schofield *et al*., 1990). Many of these improvements can be carried out by the householders themselves, provided that they are appropriately motivated and have access to financial credit enabling the purchase of materials (Briceño-León, 1990). Some projects involve replacement of the dwelling, using low-cost construction techniques. These may involve use of prefabricated concrete sections, fired bricks, or compressed soil blocks. Food aid to poor rural communities, and/or unemployment benefit, has been used to promote house improvement, by providing food or other payment to householders in return for their labour in carrying out supervised house construction.

Housing modifications are more expensive than insecticide interventions, but their main limitation as a means to control domestic Triatominae is the slow rate at which such modifications can be carried out on a large scale and the difficulties of ensuring adequate maintenance. Where house infestation rates are high, it is difficult to carry out modifications that will entirely inhibit bug colonization, and extensive modifications in an entire rural community are very time consuming and often difficult to maintain. For these reasons, it is now generally accepted that housing improvements should form a subsequent phase of Chagas disease vector control, after the original domestic bug populations have been eliminated by insecticides (Guillen *et al*., 1997).

Health education

Health education is an important component of any Chagas disease vector control programme, because the affected communities should be informed and prepared to accept the surveillance and control interventions. To a certain extent, well-informed communities can also create demand for appropriate interventions. During the vigilance or sur-

veillance phases of any intervention programme, active participation by the community becomes a vital element. Householders effectively act as the 'eyes' of the control services, by reporting the presence of any bugs found in their houses or animal enclosures. For this type of surveillance to be effective, the householders must be well informed about the bugs and the risks posed, and there must be a nearby volunteer post (such as a local shop or clinic) where reports of bugs can be registered. An inspector then visits these posts every few weeks to confirm the reports of bugs and to arrange selective respraying where necessary.

Implementation strategies

From the accumulated research and field experience, the various ideas for the control of domestic Triatominae have been refined to include just three primary elements: chemical insecticides, community health education and rural housing improvements. Implementation strategies vary but generally follow three main phases: preparatory, attack and vigilance.

The preparatory phase involves training, administrative and financial organization and geographical reconnaissance to map the affected areas. Each house in the designated localities is visited to record its position on an operational sketch map, inform the householders of the activities and check the house for presence of bugs. The attack phase involves spraying all houses in infested localities, irrespective of whether or not each individual house is known to be infested. The reason for this is that available sampling methods are imprecise and so during the attack phase it is considered more efficient to spray houses that may not be infested, rather than risk not spraying a house that is infested.

Vigilance should be organized during the course of the attack phase. Its objective is long-term monitoring of new or residual house infestations, accompanied by selective respraying where appropriate. Vigilance may be 'active' whereby each house is periodically checked by a team of trained field inspectors, or it may be 'passive' or community-based whereby householders report the finding of bugs to a nearby volunteer post. The field inspector then checks these reports and organizes selective respraying where necessary.

Organization of the interventions can be modified in response to different epidemiological scenarios, with different decision-making thresholds depending on the operational objectives. For highly domestic vectors such as *T. infestans* and *R. prolixus*, the operational objective is complete elimination of all domestic and peridomestic populations. This is feasible in areas where there are no silvatic populations of these species. For this reason, the attack phase targets all houses in each locality where even one house has been found to be infested and any respraying during the vigilance phase will generally include the infested house together with any neighbouring houses within a radius of 200 m. For other vectors, however, complete elimination may not be feasible because of extensive silvatic populations; hence the objective is to maintain houses clear of infestation by a combination of regular vigilance and selective house spraying. For control of *T. brasiliensis* in northeast Brazil, for example, all houses are sprayed in localities with more than 20% house infestation rates, but where house infestation rates are lower then only the infested houses are sprayed. Similarly, for *T. dimidiata* in Central America a series of different intervention strategies has been tested, indicating that the most cost-effective strategy would be active vigilance combined with spraying only those houses with confirmed infestations (Acevedo *et al*., 2000).

History and Progress of Chagas Disease Vector Control

1909–1990

Carlos Chagas first described the disease, which took his name, in 1909. An exceptional clinician, Chagas also became skilled in public health and sanitation and quickly realized the importance of controlling domestic Triatominae. Prior to the Second World War,

however, there were few suitable techniques. Attempts were made to control *P. megistus* in Brazil using kerosene or boiling water thrown over the walls of infested houses and there were some limited trials with military flame-throwers (Fig. 31.1). Isolating the house with a canvas cover and injecting cyanide gas was more effective, although quite impractical on a large scale (Fig. 31.2). But Chagas also recognized the importance of improved living conditions to control domestic Triatominae: he reasoned that, since the bugs lived in cracks and crevices in house walls, then improved structures should provide fewer resting places for the bugs. Under his leadership and that of his colleagues, such as José Pellegrino and Emmanuel Dias, several experiments were made in rural housing improvement, which today remains an important element of Chagas disease control.

The advent of synthetic insecticides during the Second World War was the first major breakthrough in techniques for Chagas disease vector control. DDT was quickly found to be ineffective against *P. megistus* and *T. infestans* and had only a latent effect against *R. prolixus*. Two other organochlorines, dieldrin and gamma-BHC (also known as HCH, lindane or gammexane), were found to be highly effective when sprayed over house walls at relatively high doses (typically 500 mg a.i./m^2) (Dias and Pellegrino, 1948; Romaña and Abalos, 1948). To put this in context, it is worth recalling that triatomine bugs generally have a much higher tolerance to insecticides, compared with other medically important insects such as mosquitoes. For example, World Health Organization guidelines for testing insecticide susceptibility, published during the 1970s, suggested exposure of mosquitoes to papers impregnated with 1.6% dieldrin for 2–4 h. This should kill all susceptible mosquitoes. Comparable guidelines for *T. infestans* recommend exposure to 4% dieldrin for 4 days (WHO, 1975).

For many years BHC remained the mainstay of Chagas disease vector control trials and campaigns though dieldrin was more widely used in trials in Venezuela from 1957 and during the national campaign against *R. prolixus* formally initiated in 1966. The Venezuelan campaign was the first large-scale programme against Chagas disease vectors and achieved impressive results. By

Fig. 31.1. Early attempt to control domestic Triatominae using military flame-throwers in Bambui, Minas Gerais, Brazil (1944).

Fig. 31.2. Early trial of gas fumigation against domestic Triatominae in Minas Gerais, Brazil (1940). The rural house has been sealed in a canvas cover and cyanide gas is being injected.

1976, evaluation of 382,071 rural houses in the six most highly affected states (Aragua, Carabobo, Cojedes, Portuguesa, Trujillo, Yaracuy) indicated that the proportion infested (house infestation rate) had been reduced from 31.1% to 5.6%. Similarly, overall seropositivity of the rural population declined from 44.44% in the 1959–1963 survey to 11.69% in 1980–1984. Seropositivity in children 0–9 years old declined from 20.49% to 1.50%, indicating a substantial cut in active transmission (Fig. 31.3).

In spite of its success, the Venezuelan campaign against *R. prolixus* was gradually reduced. It had been born from a sense of confidence in the successful control of malaria and suffered accordingly as malaria resurged after 1969. It had never been a national campaign and so heavily affected states such as Barinas were not controlled. Nevertheless, the Venezuelan campaign was an important demonstration that large-scale control of Chagas disease vectors was practicable and the Venezuelans went on to gain enormous experience in rural housing improvement which has helped to consolidate some of the advances made.

During the 1950s to 1970s, most classes of synthetic insecticide were trialled against Chagas disease vectors as they became available. None showed significant advantages over BHC or dieldrin and most were more expensive. The early 1980s saw the launch of synthetic pyrethroid insecticides, which were to prove a major technical breakthrough. Comparative trials in Brazil and elsewhere soon established that the new compounds were considerably more effective than BHC in eliminating domestic bug infestations, even at very low doses and even when applied just once. The new compounds were considerably more expensive (per kilogram) than BHC, but because of the low doses ease of use and infrequent applications they were more cost-effective. Moreover, they left no unpleasant smell and did not mark the treated house walls and so were more readily accepted both by spraymen and householders.

A crucial year in the history of Chagas disease control was 1979. By now, the scientific community had the first results of large-scale serological surveys indicating the true magnitude of Chagas disease in most countries of Latin America. They had compelling studies on the pathology and social impact, particularly for chronically infected cardiopathic cases. Experience from Venezuela and

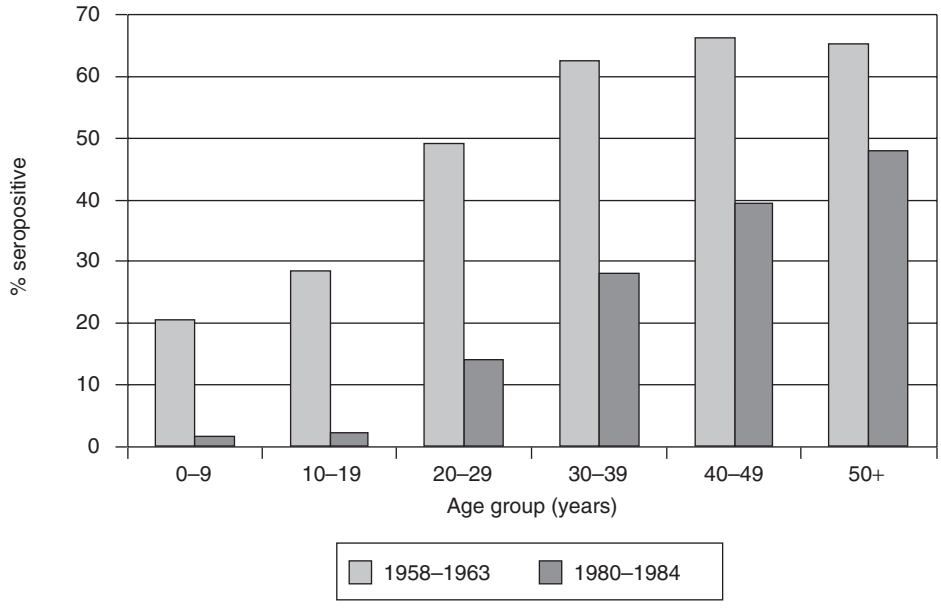

Fig. 31.3. Consolidated results of the Venezuelan programme against Chagas disease, 1968–1980. The histogram shows age-related seroprevalence for *Trypanosoma cruzi* infection before control (surveys during 1959–1963) and after control (surveys during 1980–1984). Data derived from Guevara de Sequeda *et al.* (1986).

from the Brazilian state of São Paulo showed that large-scale vector control was feasible, and the first results of field trials with synthetic pyrethroids were becoming available. The problem was being clearly identified and quantified and the technical solutions could be demonstrated. The International Congress of Chagas disease in Rio de Janeiro was the forum, and the then President of Brazil, João Batista Figueiredo, became one of the convinced (Fig. 31.4). Planning was initiated at the highest levels and in 1983 the first national campaign against Chagas disease was launched in Brazil (Dias, 1987). This was a truly national campaign, including amongst its objectives the complete eradication of the main vector, *T. infestans*, from national territory. It was designed to cover all the endemic states with a comprehensive mapping of infested localities and spraying of infested houses, initially with BHC but progressively replaced with synthetic pyrethroids. Once sprayed, a system of community-based vigilance was set up, whereby householders could report the finding of bugs to a local volunteer post (known as PIT – Posto de Informação sobre Triatomineos). The public health inspectors would periodically visit the PITs, confirm the presence of bugs and organize selective respraying where necessary.

The 1983 Brazilian campaign was ably planned and skilfully executed by staff of SUCAM (at that time, the executive arm of the Ministry of Health). By 1986, almost 75% of the geographical objectives had been attained, in the sense that infested localities had been mapped, sprayed and placed under community-based vigilance. Then in 1986, the epidemiological situation changed drastically with the return of *Aedes aegypti* to Brazilian coastal cities. *Ae. aegypti* is the urban vector of yellow fever, which had been successfully eliminated from most of South America by the 1960s. Its return in the 1980s brought with it not just the threat of renewed yellow fever transmission, but also epidemics of dengue. Uncomplicated dengue is far from life-threatening and even dengue haemorrhagic fever or shock syndrome can usually be successfully treated – but dengue affects urban populations, including journalists. There was public and

Fig. 31.4. João Batista Figueiredo (President of Brazil, 1979–1984) at the First International Congress of Chagas Disease, Hotel Gloria, Rio de Janeiro, 1979. The President (centre of picture, wearing spectacles) is being shown a model of a rural house, complete with *Triatoma infestans*, set up in the hotel lobby. The bugs are being indicated by Dr Carlos Chagas Filho (son of Carlos Chagas) (wearing pinstripe suit with his back to the camera).

political alarm and the rural Chagas disease campaign became subordinate to a new urban *Ae. aegypti* campaign.

1990–2000: the Southern Cone Initiative

By 1990 it was becoming clear that the problem for Chagas disease vector control rested less with technical questions and more with political decision. Only five countries of the Americas had official programmes against Chagas disease and none of these had fully prioritized objectives. In Venezuela, the Chagas disease programme was subsumed into the national programme against malaria, with additional activities carried out by the rural housing authorities. In Brazil, the national Chagas programme was being starved of resources by the demands of urban *Ae. aegypti* control, malaria control in the Amazon region and the long-running reorganization and decentralization of the executive health services. In Argentina, the national Chagas programme had been decentralized in 1983, but although there was active Chagas disease vector control in some provinces, such as Cordoba, Salta and Jujuy, others were much less active. In Chile, a partially decentralized Chagas programme carried out periodic house spraying in response to local demand, but without concerted effort except in the most heavily affected IV region. Only in Uruguay, where a national programme had been formalized in 1988, were consistent control activities attempted, but this national programme was grossly underfunded (the 1990 operational budget was US$15,000 for the whole country) and depended on voluntary contributions from each municipality for the spray programme.

In parallel with the basic political question, there was also an obvious biological problem. As with other insect pests, Triatominae are no respecters of national borders and can be readily transported from untreated to treated areas – especially

amongst the belongings of visitors (see Chapter 9). This had already been recognized in the Brazilian campaign of 1983–1986, when Brazil made frontier agreements with neighbouring Paraguay and Uruguay for control of *T. infestans* on the other side of their border.

The enlightened response to both problems – political and biological – came in the form of the Southern Cone Initiative. This was a joint agreement, signed in 1991, between the governments of Argentina, Bolivia, Brazil, Chile, Paraguay, Uruguay and later Peru, to control Chagas disease by the elimination of the main vector, *T. infestans*. These seven countries encompass the entire geographical distribution of *T. infestans*, which was recognized to have originated in central Bolivia but to have been transported in association with human migrations to other countries (Schofield, 1988). Throughout its distribution, it appeared to be exclusively domestic or peridomestic, with no silvatic populations except in parts of central Bolivia. However, genetic studies had indicated incipient speciation and a lack of gene flow between the silvatic populations and neighbouring domestic populations in central Bolivia (Dujardin *et al*., 1996, 1997a,b, 1998a) (see Chapter 9), so that the idea of complete elimination of all domestic and peridomestic populations of *T. infestans* seemed biologically feasible as well as politically acceptable.

The underlying rationale behind the Southern Cone Initiative was the idea that international agreement would favour the continuity of national policy for Chagas disease control, while the common objectives would reduce the risk of accidental cross-border carriage of the vectors, or of infected blood products. The primary objective of eliminating *T. infestans* included the idea of suppressing or controlling populations of other species that might be of local importance. The second objective was to reduce the risk of Chagas disease transmission by blood transfusion (see Chapter 26). In general, each country within the initiative finances its own national activities and retains complete autonomy for programme implementation. Operational aims, methods and achievements are discussed annually at the meeting of the Southern Cone Commission coordinated by the Pan-American Health Organization (PAHO).

A preliminary analysis of likely costs and benefits of the Southern Cone programme predicted that total costs over a 10-year period (1991–2000) would be between US$190 million and US$350 million but, against this, direct benefits from savings in medical costs alone would amount to around US$53 million/year, with an estimated annual rate of return on investment of just over 14% (Schofield and Dias, 1991). To date (1991–1999), the combined investment of the seven governments is estimated at just over US$302 million (OPS, 1999). Point studies that include benefits from reduced morbidity as well as savings in medical costs indicate actual rates of financial return of around 30% in Brazil (Akhavan, 2000) and over 64% in Argentina (Basombrio *et al*., 1998).

The Southern Cone Initiative has been highly successful, as shown by the current rarity of *T. infestans* over much of its previous distribution and by the sharp decline in the infection rates of children born since the programme began (Fig. 31.5) (Schofield and Dias, 1998; Schmunis, 1999). Uruguay and Chile were formally certified free of human Chagas disease transmission in 1997 and 1999, respectively, and six of the previously endemic states of Brazil were similarly certified in March, 2000 (Goiás, Mato Grosso, Mato Grosso do Sul, Paraíba, Rio de Janeiro, São Paulo). Two further states, Minas Gerais and Rio Grande do Sul, were considered probably free of transmission but requiring further data for certification. (Minas Gerais was formally certified free of transmission in 2001–2002, along with four provinces of Argentina – Jujuy, La Pampa, Neuquen and Rio Negro – and the Amambay Department of Paraguay.) In practical terms, this means that even the poorest rural families in these regions, living in even the poorest conditions, can live in houses free of domestic Triatominae, free of the risk of *T. cruzi* infection. In addition, because of stringent efforts to improve the screening of blood donors, the supply of blood and blood products has

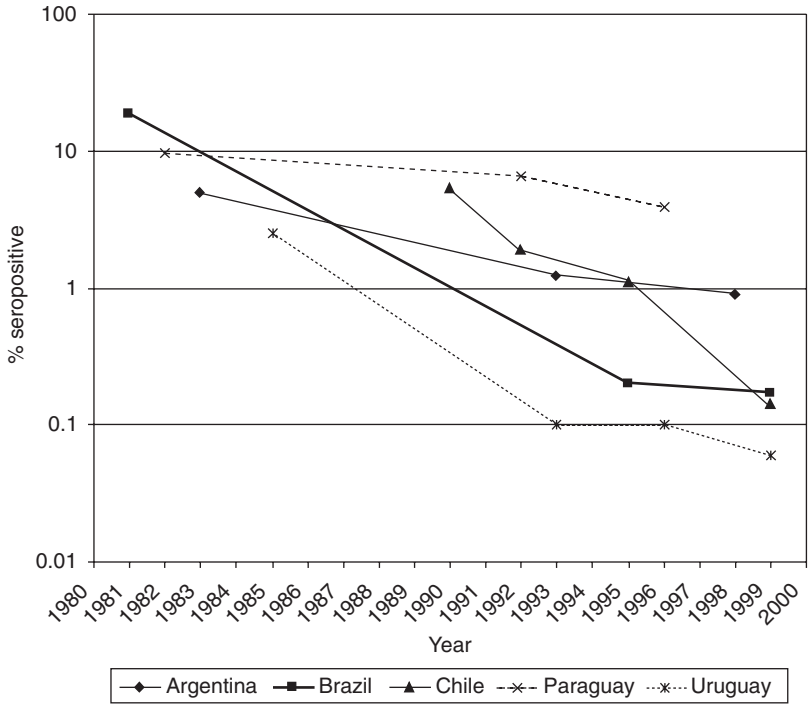

Fig. 31.5. Recent changes in the seroprevalence of *T. cruzi* infection in Southern Cone countries. In all cases, the incidence of new infections has been drastically reduced (data from OPS Southern Cone reports, courtesy Dr G.A. Schmunis and Dr R. Salvatella).

become much safer throughout the Southern Cone region. Moreover, epidemiological data are indicating some unexpected clinical benefits as improvements in disease progression amongst those people already infected before the control programme, together with a reduced likelihood that infected mothers will pass the infection to their offspring by transplacental transmission. The reasons for this are not clear but may relate to the absence of reinfection as a result of the vector control interventions (Dias *et al*., 2002a).

Control of *Triatoma brasiliensis*

As the central and southern states of Brazil are being cleared of the main domestic vector, *T. infestans*, the northeastern states face the problem posed by *T. brasiliensis* and associated secondary vector species such as *Triatoma pseudomaculata* and *Rhodnius nasutus*. These states include Ceará, Rio Grande do Norte, Alagoas, Sergipe, Paraíba, Pernambuco and parts of Maranhão, Piauí and Bahia, which now account for most of the remaining vector-borne transmission of *T. cruzi* in Brazil. Unfortunately, these states also include the poorest regions of Brazil and Chagas disease control operations have not always been implemented on a regular basis.

T. brasiliensis resembles *T. infestans* and both appear to have originated from silvatic rockpile habitats. Whereas the rockpile habitats of *T. infestans* are confined to small areas of central Bolivia, those of *T. brasiliensis* are widespread in the arid catinga of northeast Brazil and this species seems readily able to recolonize peridomestic and domestic habitats. Moreover, the peridomestic environment of houses in northeastern Brazil tends to be very extensive, often with large goat corrals built of stone or thorn branches, which represent difficult structures for conventional insecticide treatment. Control

trials and campaigns throughout the north-eastern states have demonstrated the feasibility of eliminating domestic populations of *T. brasiliensis* but peridomestic populations often remain. In some cases this is because not all of the peridomestic structures were sprayed. Often, however, it seems that the insecticide formulations are less effective in peridomestic habitats, due to accelerated breakdown on exposure to sunlight and also because the sprayed deposits may become quickly covered by dust and animal wastes.

Strategies for the control of *T. brasiliensis* have recently been extensively revised and are now based on an extended system of community-based vigilance accompanied by periodic spraying of infested localities (Cadernos de Saúde Pública Vol. 16, supplement 2, 2000).

The Central American and Andean Pact Initiatives

Stimulated by the Southern Cone success against *T. infestans*, two further regional initiatives were launched in 1997. The Central American Initiative mainly involves Guatemala, El Salvador, Honduras and Nicaragua, while the Andean Pact Initiative involves Venezuela, Colombia, Ecuador and northern Peru. In both cases, the main targets are *R. prolixus* and *T. dimidiata* (and *R. ecuadoriensis* in northern Peru). *R. prolixus* has a discontinuous geographical distribution, in Venezuela and Colombia and in the central part of Central America. It has never been recorded from northwestern Colombia, nor from Panama or southern Costa Rica and it was quickly eliminated from northern Costa Rica by insecticide spraying in the early 1950s (Ruiz, 1953). Historical reconstruction backed by genetic and morphometric comparisons indicates that *R. prolixus* was accidentally imported into Central America just prior to 1915 and, like *T. infestans* in the Southern Cone, it appears to have been spread as a domestic species in association with human migrations (Schofield and Dujardin, 1997; Dujardin *et al*., 1998b). There is also some evidence that *R. prolixus* was spread by humans from Venezuela into the central Magdalena valley of Colombia (Schofield and Dujardin, 1999). As far as is known, no silvatic populations of *R. prolixus* occur either in Central America or in central Colombia, and so in both regions this domestic species is considered a feasible candidate for complete elimination.

In contrast to *R. prolixus*, *T. dimidiata* has many silvatic ecotopes in southern Mexico and Central America, though silvatic specimens have been only rarely reported from Colombia and not at all from Ecuador. Current genetic studies suggest that *T. dimidiata* may have originated in the Yucatan region, followed by a natural spread northwards and southwards, so that there is some evidence of clinal variation as far south as Panama (Solis-Mena *et al*., 2000; Schofield, 2002). In contrast, Ecuadorian populations of *T. dimidiata* show close genetic similarities with those of southern Mexico (Solis-Mena *et al*., 2000; Abad-Franch *et al*., 2001), suggesting that they may have been transported to Ecuador along pre-Columbian maritime routes that are well established by archaeological evidence (Meggers and Evans, 1963).

Based on these various studies, the current operational appraisal is that *R. prolixus* is a feasible candidate for eradication from Central America and possibly also from central Colombia, while *T. dimidiata* may be a feasible candidate for eradication from Ecuador. At the time of writing, survey work and extensive control interventions have been carried out in Guatemala, Honduras, El Salvador, Nicaragua and parts of Colombia, following the successful methods used in the Southern Cone Initiative. The results suggest that *R. prolixus* may have already disappeared from El Salvador and its distribution is being steadily reduced elsewhere in Central America as a result of the control interventions.

The 'Acapulco Syndrome'

The current feeling amongst those involved with Chagas disease control is one of quiet confidence, albeit tinged with perennial

uncertainty about political continuity for the control interventions. There is little doubt that domestic populations of Chagas disease vectors can be eliminated and that, together with improved screening of blood donors, this will progressively reduce transmission of *T. cruzi*. For some of the primary vectors, particularly *T. infestans* in the Southern Cone and *R. prolixus* in Central America, it seems likely that once the domestic populations are eliminated they will not be reconstituted – at least not by the same species. For other important vectors such as *T. brasiliensis* in northeastern Brazil and *T. dimidiata* in Central America and the Andean Pact, there is a need to improve methods to control peridomestic populations but there is some confidence that, once the domestic populations have been eliminated, then regular community-based vigilance will allow any resurgent domestic populations to be dealt with on a local level. This will involve some strategic and operational modifications within the current concept of decentralized health intervention services, but such modifications have been foreseen and discussed and are in the process of being implemented.

The greatest risk to the current successful trend in Chagas disease control comes, in a sense, from that same success. For as Chagas disease becomes less of a public health problem, then the need for continued surveillance and selective intervention becomes less appreciated at the political level. The greatest difficulty, therefore, is to ensure continuity of surveillance and intervention at the political level and also at the community level, where it is already apparent that many communities have lost awareness of the triatomine bugs that they have not seen for some decades. Amongst medical clinicians also, there is a need to ensure adequate continuity of training, so that future infections will not go undetected.

Today, there are three enormous areas endemic for Chagas disease vectors, where Chagas disease surveillance and control is in its infancy: Amazonia, Mexico and the USA. The Amazon region, comprising much of Brazil, Bolivia, Peru, Colombia, Ecuador, Venezuela and the Guianas, represents a major challenge. Here, human colonization is associated with major changes in land use, which already present a risk for domestication of otherwise silvatic species of Triatominae. In the Brazilian Amazonia alone, over 200 cases of Chagas disease have been diagnosed in the last 10 years (Silveira, 1999) together with evidence of progressive domestication of vector species such as *Panstrongylus geniculatus, T. maculata* and *Rhodnius brethesi*. In other Amazonian countries, there are increasing reports of silvatic species of Triatominae flying into dwellings, with a similar increase in reports of Chagas disease transmission (e.g. Aguilar and Yépez, 1996; Raccurt, 1999). As these countries of South America gain experience in surveillance and control of Chagas disease vectors, it can be hoped that this experience will be applied to the Amazon region, leading to an adequate level of entomological surveillance, selective intervention and research to identify the key features of human colonization and land-use change that can trigger the domestication process in otherwise silvatic species of Triatominae (Dias *et al*., 2002b).

Elswhere, the situation is different. In the USA, Chagas disease vectors are widespread but well known to the scientific community. Also, they are species that rarely enter homes except under extreme conditions – for example, when drought triggers high mortality of their silvatic reservoir hosts. Therefore, vector-borne Chagas disease is rare in the USA. In contrast, blood-borne transmission may be increasing and there is as yet insufficient screening for *T. cruzi* in US blood banks. In Mexico also, screening for *T. cruzi* infection in blood donors does not have high coverage and vector-borne transmission is widespread. Data from large-scale serological surveys suggest well over 1 million cases of *T. cruzi* infection in Mexico (Guzman Bracho *et al*., 1998), while entomological studies reveal a wide variety of domestic vector populations representing several species of the *Triatoma phyllosoma* and *Triatoma protracta* complexes, as well as *T. dimidiata* in the southern states. It is to be hoped that Mexico will soon implement an adequate system of vector surveillance and control throughout the country, together with improved screening of blood donors.

Mexico and the USA also show what may be the idealized end point for Chagas disease vector control – for which the term 'Acapulco syndrome' was coined. Acapulco, a large and prosperous city on the Pacific coast of central Mexico, shows the full range of communities from the very poor to the very rich and privileged. Domestic populations of Triatominae are no longer apparent – although they were reported up to 20–25 years ago, at least on the periurban fringes of the city. Nevertheless, vector-borne transmission still occurs, albeit rarely. We deduce that some 'event' may trigger the flight of an adult silvatic bug which may fly through an apartment window. That bug would be hungry and probably infected with *T. cruzi*. In the apartment, the bug feeds and may transmit the parasite but fails to survive to establish a domestic colony. The result can be an acute case of Chagas disease, perhaps in someone living high up in a luxurious apartment block, but there is nothing for the vector control authorities to control. This may represent the end point of Chagas disease control, relying on clinical surveillance backed by recognition of the bugs if they can be found, with prompt remedial treatment of the rare acute infections that may occur. If we are right and the Acapulco syndrome can be reached throughout Latin America, then the need for adequate clinical training and community awareness deserves to be stressed even in those areas where current Chagas disease control activities are meeting well-earned success.

Acknowledgements

Along with all Latin Americans, we extend a heartfelt acknowledgment to all those who have contributed to the research basis and political development that has led to current progress in Chagas disease vector control, with a special thanks to the dedicated brigades of field workers who implement the control initiatives in the endemic areas. Much remains to be done, but their success is showing that the dream of liberating Latin America from the burden of Chagas disease is at least feasible.

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32 The Sterile Insect Technique as a Component of Area-wide Integrated Pest Management of Tsetse

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The sterile insect technique (SIT) is usually applied as part of an area-wide integrated pest management (AW-IPM) approach. The understanding of the principles of the AW-IPM concept is therefore important for an appreciation of the SIT.

The Concept of Area-wide Integrated Pest Management

Pest management applies practices for suppression, eradication or prevention of unwanted biological organisms that are causing agricultural or environmental problems. Integrated pest management (IPM) aims at intervention against such pest organisms to optimize benefits while minimizing undesired environmental impact and other risks. It involves the integration of different approaches and techniques, including biological, cultural, physical, mechanical, educational and chemical methods in site-specific combinations into a sustainable systems approach for pest management. A key element of most IPM approaches is access to a professional decision-making structure, whereby skilled field monitoring provides the relevant biotic and abiotic information about the habitat and the pest and guides the end-users in their independent IPM efforts.

Most conventional IPM approaches are based on the concept of problem measurement and, as soon as a predetermined (economic) threshold is exceeded, reacting with an integrated systems response, with preference given to non-chemical techniques. In addition to such repeated and *reactive pest suppression* efforts, there are other IPM approaches that also aim at minimizing adverse environmental impact and other risks and at maximizing benefits. This second option is *pest eradication*, which is preferred over continuous or repeated suppression in situations where the (economically) acceptable threshold is very low and/or when the overall benefits (economic and ecological) from a pest-free status, sustained over a certain time, substantially outnumber the benefits from investments in continued or repeated suppression. (Although the term 'eradication' is sometimes used for the extinction of a species from the earth, in this chapter it stands for localized complete removal of a population of a pest species, i.e. creation of a sustainable pest-free zone that – except for quarantine measures – does not require further pest control. The pest species concerned may still exist at other places, in laboratory colonies or distant habitats.) A third more *proactive* IPM approach involves a set of measures aiming at *pest prevention*, i.e. the application of techniques either before a pest is introduced (to prevent its introduction and/or

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establishment) or, for seasonally occurring pests, sufficiently early in the (growth) season to prevent losses.

IPM approaches are usually designed for farmers' application at the 'field-by-field' level (Lindquist, 2000). A particular challenge for efficient IPM is the management of pests in areas where several end-users independently decide whether or not to participate in the intervention campaign. Reichelderfer *et al.* (1984) modelled the percentage of smallholders participating in IPM measures aimed at the conservation of natural enemies of the brown plant hopper (*Nilaparvata lugens*), against the benefits that result to both the campaign participants and to 'free-riders', who opted not to join the intervention (Fig. 32.1). Attractive benefits, i.e. at least one-third of the potential benefits, only occur to the participants if at least half of the smallholders participate. With steadily growing rate of participants the benefits to both groups – participating smallholders and free-riders – increase exponentially. When 90% of the smallholders participate they will gain 83% of the potential benefits and the 10% free-riders gain two-thirds of the potential benefits.

Knipling (1972) (Fig. 32.2) illustrated the importance of highest possible rates of campaign participants or percentage of area covered for the efficiency of pest management operations. In a hypothetical model, which would apply for pest insects such as boll weevil (*Anthonomous grandis grandis*), codling moth (*Cydia pomonella*) or pink bollworm (*Pectinophora gossypiella*), he compared two scenarios over four pest-insect generations: scenario one, whereby control measures are taken that exert 90% control of the *total* population; and scenario two, whereby more intensive control measures are taken that achieve 99% control but only cover 90% of the affected area or pest population. In his model, insects not destroyed are assumed to increase at a fivefold rate each generation. The second scenario, with highly intensive (99%) control but only 90% of the population covered, results in ten times and 100 times more pest insects after two and three generations, respectively, if compared with the first scenario (less intensive control but entire-population coverage). Knipling's calculations also show that 100% control on 99% of the host hectarage falls far short of the suppression that is achieved when 90%

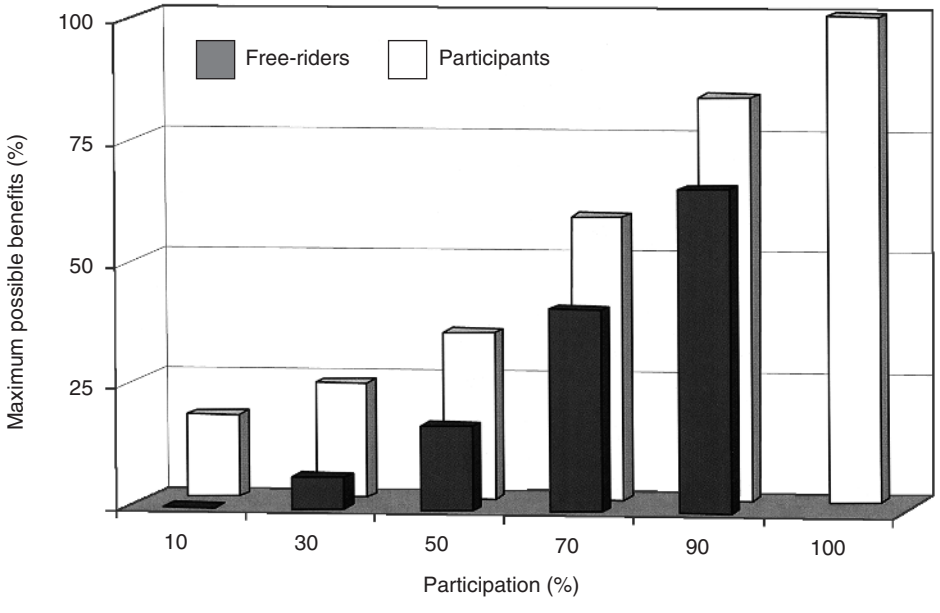

Fig. 32.1. Benefits depending on participation in a biocontrol programme (Reichelderfer *et al.* (1984) and Klassen (2000)).

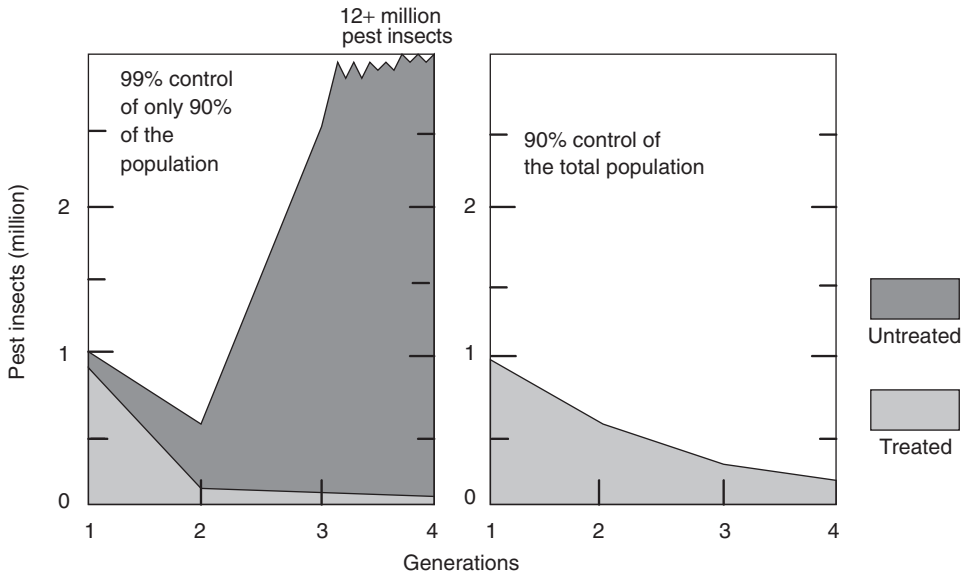

Fig. 32.2. Importance of total pest population suppression (after Knipling, 1972).

control is obtained on 100% of the host hectarage. Knipling (1972) described this area-wide insect population intervention concept as follows:

> Uniform suppressive pressure applied against the total population of the pest over a period of generations will achieve greater suppression than a higher level of control on most, but not all, of the population, each generation.

Thus moderate but consistent pressure applied to each generation of the total pest population results in more effective pest management than intensive pressure applied against small segments of the total population (see http://apmru.usda.gov/Default.htm). Calkins *et al.* (1996) further elaborated on the potential of area-wide IPM.

When the concept of area-wide pest management was developed it was first regarded as an approach for managing a single pest or a small group of pests over a large region, whereas IPM was considered to incorporate all pests within an agroecosystem into a management programme that is primarily conducted on a farm-to-farm basis. A firm basis for merging both approaches has since emerged: according to Chandler and Faust (1998), area-wide programmes now not only monitor and attempt to manage a key pest or pests but also address secondary pests and non-targets, including beneficial arthropods. Therefore, the term 'area-wide IPM' provides a more accurate description for this pest and agro-ecosystems management approach. Among the advantages that result from an area-wide IPM approach, Chandler and Faust (1998) listed: (i) more effective and more efficient pest management than pest control on an individual farm-by-farm basis; (ii) long-term solutions to key pest problems in larger agro-ecosystems as opposed to quick-fix solutions on small hectarages; (iii) integration of the best and most environmentally benign management techniques; (iv) biorational management strategies for secondary and other key pests; and (v) prevention of major pest outbreaks and provision of more sustainable pest management procedures.

Efforts against tsetse and trypanosomiasis (T&T) should take full advantage of the benefits associated with the accepted principles of AW-IPM, or integrated disease and pest management (IDPM). The African trypanosomiases are transboundary disease problems that can effectively be sustained even by low-density tsetse populations. The T&T problem constitutes a key bottleneck for

enhanced sustainable agriculture and rural development (SARD). FAO (2002) advocated that the principles of AW-IPM should guide the planning and implementation of T&T intervention measures, and thus avoid some of the typical shortcomings of some conventional field-by-field IPM approaches.

Principles of the SIT

The SIT involves the production and systematic releases of reproductively sterile insects among the indigenous target population, sustained over several generations of the pest population. When sterile male insects mate and inseminate female insects, these females become effectively infertile for the remainder of their lifespans.

The insects to be sterilized and released are propagated at special large rearing facilities. Males are sterilized by radiation at the appropriate development stage and then taken to the identified target area and released. Aerial release facilitates a homogeneous and area-wide dispersal of the released sterile insects. For increased effectiveness, the released sterile males should significantly outnumber the fertile, native male flies (Knipling, 1955). In contrast to insecticide applications, which cost the same regardless of the insect population density and are, therefore, most cost-effective when the target population density is high, the SIT is most cost-effective when the target population is low. This suggests that a phased and complementary use of both 'conventional' methods and SIT would result in maximum efficiency throughout the phases of intervention.

For some species of insect pest that have strong seasonal fluctuations, SIT releases may be initiated without prior conventional suppression and still cause sufficient 'overflooding' ratios. Contrary to the conventional IPM concept, which suggests interventions should only be made after a pest population exceeds a predetermined economic threshold level, SIT is initiated when the target pest population reaches its seasonal minimum – for example, at the end of the winter, before it starts increasing again.

By repeatedly releasing sterile males in quantities that are sufficient to sustain an 'outnumbering' of the target population over several generations, its reproductive capacity is progressively reduced. Eventually, so few fertile males and females remain that fertile matings do not occur and the population is eliminated.

SIT can be used for suppression, localized eradication or prevention of insect pests. Models by Enkerlin and Mumford (1997) underlined that SIT used for either suppression or eradication of the Mediterranean fruit fly is economically competitive with conventional intervention methods that are based on monitoring and the application of insecticides. The technical feasibility of eradication or suppression using SIT has been documented (Lindquist *et al*., 1990; Barnes and Eyles, 2000; Bloem and Bloem, 2000; Hendrichs, 2000). The successful eradications of the New World screwworm fly from North and Central America, the Mediterranean fruit fly from certain areas in North, Central and South America, the melon fly from Okinawa Islands and the Queensland fruit fly from Western Australia are all well known. Preventive SIT measures against insect pests have already resulted in tangible benefits in the cases of medfly, pink bollworm, screwworm and codling moth. For example, preventive aerial release of sterile medflies, conducted over the Los Angeles basin since 1994, has reduced the average number of annual medfly infestations from 7.5 to 0.3 (USDA/APHIS, 1992; CDFA, 2002). The change from the insecticide-based reactive intervention operations after detection of outbreaks to the new proactive, preventive SIT-based approach resulted in a reduction of malathion use from more than 450 tons to less than two kg/annum, and the yearly intervention costs decreased from US$33 million to US$15 million.

History of SIT against Tsetse Flies

In 1966, at Lake Kariba, Zimbabwe, Dame and Schmidt (1970) field-collected *Glossina morsitans morsitans* adults, treated them with

chemosterilants and released them. The target tsetse population was reduced below detectable level within 26 months. In a subsequent experiment, sterile flies were released as pupae and within 10 months an induced sterility of 95% was recorded. The experiment had to be discontinued because of civil unrest.

In 1976 at Tanga, United Republic of Tanzania, Williamson *et al.* (1983a,b) applied the SIT component following two aerial applications of endosulfan. The project involved the mass rearing of *G. m. morsitans* based on a goat host-animal colony. Late-stage pupae from this colony were radiation-sterilized (^{137}Cs γ-rays) and released into a 195 km^2 control area. The target population was reduced by 81%. A bush-free barrier zone 1 km wide proved insufficient to prevent fly movement into the area.

In 1981 a programme covering 3500 km^2 (with 500–600 km of linear riverine forest) was initiated in Burkina Faso (Cuisance *et al.*, 1986). For the first time: (i) insecticide-impregnated targets for pre-SIT tsetse population suppression were used; (ii) simultaneous eradication of three species, namely *Glossina palpalis gambiensis*, *G. tachinoides* and *G. morsitans submorsitans*, was attempted; and (iii) γ-rays were used to decontaminate the blood diet used in the membrane feeding of mass-reared tsetse colonies. The insecticide-impregnated targets reduced the fly populations by 94%. SIT, thereafter, achieved complete eradication within 4 months. Due to failure to expand the fly-free area to reach the natural barriers of the populations, the area became reinfested in subsequent years.

Between 1979 and 1987 a pilot tsetse SIT project (Biolocal Control of Tsetse, or BICOT) was implemented in Plateau State, Nigeria (Oladunmade *et al.*, 1990). Using guinea pigs as host animals for *in vivo* feeding and a mixture of irradiated fresh-frozen bovine and reconstituted lyophilized porcine blood for *in vitro* feeding of tsetse colonies, 180,000 tsetse females were maintained. After a phase of field research the fly population was suppressed by conventional means (initially, 7–10 weeks continuous placement of biconical traps; later on, use of insecticide-impregnated blue fabric targets) to 4–12% of the initial fly population density. During the operational phase of the project (early 1986 to mid-1987), on average close to 10,700 sterile males were released per week (*c.* 40 sterile males/km linear riverine forest; total some 250 km of linear riverine forest). This was sufficient to establish and maintain in the project area of 1500 km^2 a ratio of released sterile males to wild fertile males of at least 10:1. Within a few weeks an increasing rate of induced sterility was recorded among tsetse females of the wild target population as a result of being mated and inseminated by sterile males. Eradication of the target species was achieved by mid-1987, after 8–12 months of sustained sterile male releases. For the first time, sterile virgin females were released and recaptured to confirm eradication (sterile virgin females recaptured several weeks after release were not inseminated). Owing to a lack of funding and in the absence of a pre-agreed strategy for expanding the fly-free zone to larger agricultural development areas and eventually to natural distribution limits of the species, artificial barriers were not maintained and reinvasion occurred.

An area-wide integrated tsetse SIT project was conducted on Zanzibar, United Republic of Tanzania, between 1994 and 1997 (Vreysen *et al.*, 2000). Following a pre-SIT reduction of the *Glossina austeni* target population mainly by means of pour-on formulations of insecticides on livestock by the Zanzibar Government and FAO/United Nations Development Programme (UNDP), the International Atomic Energy Agency (IAEA) supported a project aimed at tsetse eradication on Unguja island (1600 km^2). Most sterilized males for the SIT campaign originated from colonies at the Tsetse and Trypanosomiasis Research Institute (TTRI) at Tanga, Tanzania. The FAO/IAEA Agriculture and Biotechnology Laboratory at Seibersdorf, Austria, served as a back-up and provided some additional males, particularly during the test releases and the initial weeks of operational releases. More than 8.5 million sterile males were released, in the main operational phase (mid-1995 to late 1997) at a rate of between 45,000 and 100,000/week.

For the first time routine releases were conducted from a fixed-wing aircraft, ensuring an even dispersal of sterile males, including otherwise inaccessible areas. On average 55 sterile males/km^2 were released (ranging between 25 and 550/km^2). This is a low number in comparison with screwworm or medfly SIT requirement, which involves the weekly release of 1200–1500 and 100,000–400,000 sterile flies/km^2, respectively. In early September 1996 the last wild tsetse fly was captured on Unguja. The last case of nagana in cattle was detected in August 1997, and eradication operations were completed in December 1997. In the 5 years thereafter no tsetse suppression or any trypanocidal treatment was organized, but systematic entomological and veterinary surveys were conducted, involving the placement of 150–200 traps during more than 300 trapping days, and random sampling of more than 3000 cattle. Not a single tsetse fly was captured between 1998 and 2002 and no cattle born on Unguja were found infected with trypanosomes; only one animal, which had been imported from the Tanzanian mainland and was examined at Kisakasaka quarantine station, had trypanosomes. In spite of the presence of a dense population of other species of biting flies, the epidemiological relevance of mechanical transmission appears to be negligible in the absence of the cyclical vector. Meanwhile, substantial increases in livestock and agricultural productivity have been observed on Unguja. A socio-economic assessment of the agriculture and livestock situation on Unguja (Tambi *et al.*, 1999) revealed substantial benefits resulting from rendering Zanzibar free of tsetse and the trypanosomiasis problem.

In 1999 the Ethiopian government approved a multimillion US$ project on tsetse eradication in 25,000 km^2 of the Ethiopian southern Rift Valley. Based on a phased, conditional planning approach, the government, IAEA and other collaborators initiated a comprehensive baseline data assessment and provided preparatory support in anticipation of sterile male production and releases. In March 2002 the construction of the first two modules of a tsetse factory was initiated. The factory is scheduled to hold eventually 10 million tsetse females and the projected sterile male output is anticipated to be sufficient to cover 10,000–15,000 km^2 with SIT simultaneously.

Feasibility Considerations Regarding the Tsetse-free Zone Concept and Tsetse SIT

Technical feasibility

There are two different approaches towards the creation of insect pest-free zones. The first is a 'rolling back the carpet' strategy, whereby the intervention area is progressively shifted by expanding the eradication area. Ideally this approach is initiated starting from the edge of natural barriers or distribution limits of the pest concerned and is pursued until the pest-free area reaches the opposite distribution limit or another natural barrier. The second approach relies on the identification of pockets with isolated target populations of the pest that can be removed one by one. Preferably the strategy behind the second approach will pay due attention to the best sequence by which these isolated or at least confined foci should be removed in a subregion, in order to minimize risks of reinfestation from remaining pest foci in adjacent areas.

The area in the western hemisphere that was freed from the New World screwworm (NWS) over the past 40 years using SIT is of similar size to the tsetse-infested area in sub-Saharan Africa. The campaign was based on the first approach and started from the northern distribution limit of NWS in the USA. After more than four decades of continuous expansion of the fly-free zone southwards (Fig. 32.3), the SIT operations reached the isthmus of Panama, where a permanent SIT barrier has been established since 2001 to prevent NWS from reinvading the freed area.

In contrast to the assumedly rather continuous distribution of the single species NWS, the trypanosomiasis problem is sustained by several tsetse species, which are often found in fragmented populations (see

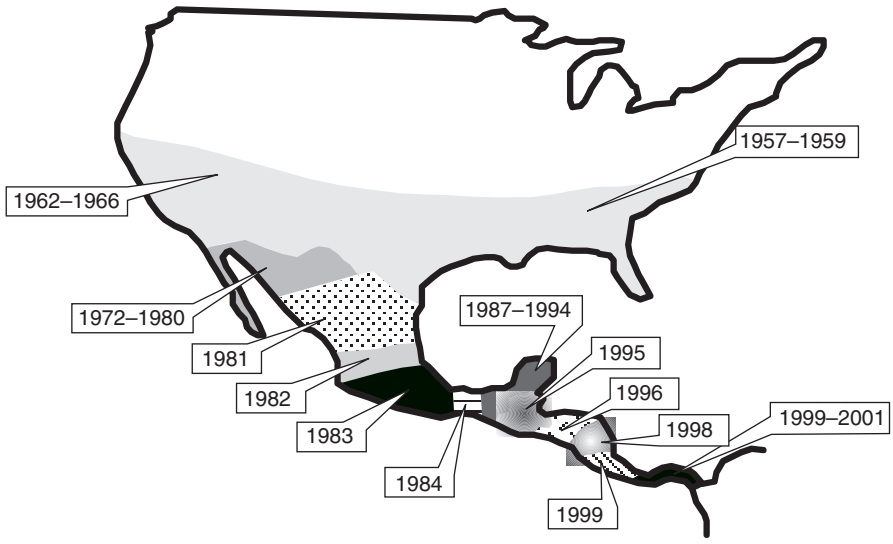

Fig. 32.3. Progressive eradication of the New World screwworm fly from North and Central America.

Plate 14). In addition, the availability of new tools such as Geographical Information System (GIS) allows concerns over a multi-species SIT approach for tsetse flies to be put in perspective: almost one-third (29.8%) of the tsetse-infested area harbours only one species, and between one and three species are found in more than 86% of the 'tsetse belt'. In less than 5% of the tsetse-infested area do five tsetse species coexist and in only 0.07% of the area are there seven or more tsetse species (see Plate 15). Furthermore, only some six to eight tsetse species are considered of economic importance and thus justify intervention measures. Cuisance *et al.* (1986) demonstrated in Burkina Faso that it is possible to eradicate three coexisting species, namely *Glossina p. gambiensis*, *G. tachinoides* and *G. m. submorsitans*, using a combination of conventional techniques and SIT. Tsetse presence/absence risk-prediction maps (Wint, 2001) illustrate that target tsetse species often occur in fragmented populations, an advantage on which the strategy and implementation of intervention operations should capitalize.

In comparison with NWS or medfly, the fundamental biology of the tsetse is such that the flies appear to be better suited for the establishment of sustainable fly-free zones and for the integration of the SIT than is the case with screwworm fly or medfly, the targets for the most successful SIT applications to date (Feldmann and Hendrichs, 2001). The reasons are as follows.

LOWER RISK OF ACTIVE REINFESTATION In comparison with medfly or NWS, tsetse do not move long distances. Although tsetse dispersal is complex (Hargrove, 1981; Rogers, 1990; Williams *et al.*, 1992) and influenced by various parameters such as seasonally fluctuating vegetation types, physiological conditions and other factors involved in the regulation of fly population density, it can generally be assumed that the annual reinvasion fronts of tsetse ranges between 3 and 20 km (Hargrove, 2000). The maximum recorded distance travelled by a tsetse fly over several days is 25 km (Cuisance *et al.*, 1985). In comparison with this, screwworm flies have been observed to travel up to 290 km in less than 2 weeks (Hightower *et al.*, 1965).

LOWER RISK OF PASSIVE REINFESTATION The movement of screwworm-infected cattle into a fly-free area automatically results in fly reinfestation, and any person carrying a worm-ridden fruit can reintroduce a fruit fly into a fly-free area. In the case of tsetse, the larvae develop within the female fly and not on or within animals or fruit. Therefore, ani-

mals or humans carrying fruit or other agricultural products cannot transport the immature stages of tsetse from one location to another. Only the passive transfer of adult flies by vehicles or, occasionally, by animals (e.g. transhumant cattle) needs to be appropriately addressed.

LOWER RISK OF OUTBREAKS An important biological factor of tsetse is their low reproductive rate, which represents an advantage during field eradication programmes: once a wild tsetse population is suppressed by conventional methods and is subjected to overflooding with sterile flies, it has less chance of recovery than is the case for screwworm or fruit fly populations. In the case of fruit flies, each fertile female can lay several hundred eggs in a few days. Even if only one or two wild fertilized fruit fly females survive an intervention campaign, localized outbreaks are likely to occur. With tsetse, on the other hand, any fertile female that survives can only produce one offspring every 9–10 days. Even though undetectable relic populations can be the basis for tsetse populations to recover fully within 3–5 years (Hargrove, 2000), the long tsetse generation period of about 2 months suggests that temporary upsets in sterile fly quality, quantity or distribution have less of a negative impact on the outcome of an eradication campaign, and can therefore be more rapidly overcome with corrective measures in tsetse than is the case for fruit flies or screwworm flies.

LESS COMPLEX TO MASS-REAR Although it requires more time to build up large colonies, the mass-rearing of tsetse has some relevant advantages over that of screwworm flies or fruit flies. Mass-reared screwworm flies and fruit flies require different special rearing conditions at all stages of their development (i.e. egg, larval, pupal and adult). In the case of tsetse, only the pupal and adult stages have to be considered, because the egg and larval stages remain within the pregnant female fly.

LOWER NUMBERS OF STERILE MALES NEEDED The substantially lower number of sterile tsetse males required in the field, i.e. 55–100 sterile males/km^2 vs. 1200–1500/km^2 and 100,000–400,000/km^2 for screwworm and medfly SIT, respectively, is an additional advantage.

The technical feasibility of integrating tsetse SIT with conventional methods has been accepted. The Programme Against African Trypanosomiasis (PAAT, 2000) recognized that 'currently … SIT (Sterile Insect Technique) is the most appropriate method to be used in the final phase of eradication'. Nevertheless, some international criticism concerning the potential role of SIT in the creation of tsetse-free zones persists, which is not surprising if one recalls the controversial history of SIT in screwworm or fruit fly interventions, summarized by Krafsur (1998), and the unsustained results of the pilot SIT schemes against tsetse on mainland Africa.

Environmental feasibility of the creation of tsetse-free zones and of tsetse SIT

The following main environmental concerns are repeatedly raised in relation to the creation of tsetse-free zones and the elimination of the trypanosomiasis problem.

1. Tsetse are considered an important component of the African ecosystem, and their removal would upset the ecological balance of some areas and lead to a reduction in biodiversity.
2. The presence of tsetse protects national parks and the creation of tsetse-free zones would result in uncontrolled agricultural expansion, overgrazing and erosion.

Arguments about the possibility of tsetse playing a role in protecting natural habitats and wildlife from human impacts have been refuted (e.g. Jordan, 1986; Nagel, 1991). Because of their low reproductive capacity, the natural population tsetse density is extremely low, particularly compared with that of other bloodsucking insects. In this context, Nagel (1988) underlined that no predator feeds exclusively, or even predominantly, on tsetse in such a way that the presence of tsetse is indispensable for the existence of any other species. There is also no evidence of *Glossina* spp. being 'key species' in their

ecosystems, nor of the disappearance of tsetse populations causing the destruction of those ecosystems as a result of the loss of the ecosystems' original identity and functionality. The European Union's Scientific Environmental Monitoring Group (SEMG, 1995) also concluded that there is no definite evidence that tsetse are key elements in the ecosystem.

Glossina pallidipes was eradicated between 1946 and 1952 from large areas of South Africa (Dutoit, 1954). In addition, the Kruger National Park has been free of tsetse since the rinderpest pandemic at the beginning of the 20th century. In both cases there is no indication that the absence of tsetse has resulted in a decrease in biodiversity, or that it has affected the ecological balance.

As early as 1948, Hornby (see Ford, 1971) adopted the view that *Glossina* was the principal agent of erosion, causing people to crowd into the fly-free areas. The elimination of *G. morsitans* from most of northern Nigeria not only resulted in agricultural advantages but also was accompanied by environmental benefits and effected a more even distribution of livestock numbers in the area (Bourn *et al.*, 1986). As a result of this and better rains in the subregion as of 1994 (compared with the previous 20 years), the burden of overgrazing in environmentally fragile areas of the Sudano-Sahelian vegetation zone was reduced. This possibly contributed to a slight reversal of the desertification trend in northern Nigeria.

Other ecological reasons supporting the creation of tsetse-free zones

Tsetse, particularly riverine species, frequently expand their distribution after agricultural activities have generated appropriate environmental conditions and provided suitable hosts. In the periurban agricultural system of Bamako in Mali, for example, *Glossina p. gambiensis*, a riverine species of tsetse that previously lived mainly on reptiles, is moving into irrigation schemes, establishing peridomestic behaviour and changing host preference to humans and livestock. The expansion of tsetse into and its threatening of established agricultural systems and other ecosystems have also been reported from other areas (Leak and Mulatu, 1993). The globally predicted higher precipitation and warmer winters that are expected to result in a general spread of disease vectors, including tsetse, may bring about new epidemiological risks that require area-wide corrective and preventive tsetse and trypanosomiasis intervention measures.

In addition to the direct benefits related to tsetse-free zones, a discontinuation of conventional control measures based on chemicals will bring along a reduction of adverse effects on the environment and improved food safety.

Unless sustainable tsetse-free zones can be established in the farmland–game reserve interface areas, tsetse will (despite continuing control measures) survive in small pockets by feeding on wildlife as reservoir hosts and will threaten agricultural production in the vicinity of protected habitats. Farmers, who are well aware of trypanosomiasis and its mode of transmission through the tsetse fly, object to the existence of wildlife and forest reservoirs and are likely to take (illegal) steps to eliminate wildlife reservoir hosts and forest habitats for tsetse. Some park administrations have reacted to this development by introducing tsetse control measures in wildlife parks so as to protect their wildlife indirectly. The financial viability of parks and related tourist industries also depends on protecting tourists from tsetse, which has been an issue, for example, in the Okavango delta of Botswana. Therefore, coexisting game reserves and farmland and rangeland are expected to take mutual advantage of the establishment of tsetse-free zones.

In view of the demographic trends[1] in Africa and the related drastically increasing

[1] Despite the AIDS epidemic, the growth of the human population in Africa is averaging more than 3%, in extreme cases 5% per year (Harrison, 1996). According to the UN ECOSOC's Population Division (ECOSOC, 2002) the World Urbanisation Revision 2001 predicts that by 2030, 52.9% of Africa's vastly growing human population will live in urban areas.

demand for appropriate food, efforts need to focus on enhanced agricultural productivity, to avoid having to open new areas for farming (Borlaug, 2002). Whenever agricultural productivity growth does not increase in step with human demand for food and other farm products, smallholders are generally pushed to expand cultivation and grazing into environmentally fragile margins where yields are low, poverty remains acute, and environmental degradation can be severe (Lee *et al.*, 2000). Strategies for agricultural intensification are therefore required and they need to aim at sustainable food security and environmentally appropriate utilization of available natural resources. In the long term, the creation of tsetse-free zones and the development and implementation of appropriate land-use plans that enable increased agricultural productivity are the only alternatives to the encroachment of low productive agriculture into wildlife areas and may permit a peaceful and profitable coexistence of farmland and protected habitat, including game reserves.

The environmental appropriateness of different tsetse intervention tools is addressed in other chapters. With regard to the environmental feasibility of the SIT, there is no doubt that released sterile tsetse males have an impact only on the tsetse population, without any interference with non-target organisms. SIT is the only absolutely specific technique available. Instead of applying vector controls permanently, the SIT component, where technically feasible and justifiable, can be integrated with appropriate conventional means in an effort to create sustainable tsetse-free zones. This would limit (minor) adverse environmental effects of other components to short periods of the integrated campaign and would involve the environmentally safest tool in a final 'mopping-up' phase.

Economic feasibility

The most persistent argument made in relation to the use of SIT to create tsetse-free zones, starting in identified priority intervention areas in Africa, is the claim that it is not cost-effective. Under situations that do not involve an area-wide approach, this claim may be justified.

Any major investment made in larger-scale T&T intervention will have to be preceded by a benefit–cost assessment. In the case of the areas freed from New World screwworm in North and Central America, the annual direct producer benefits amounted in 1996 to US$1.165 billion, and benefit-to-cost ratios ranged from an average of 12.2 for Central America to 18 for Mexico and the USA, not including benefits from improved human and wildlife health (Wyss, 2000).

Comparing the recurrent investments needed for permanent tsetse control with the additional investments needed for a complementary tsetse SIT campaign that aims at the creation of a tsetse-free zone, it is obvious that the eradication effort requires only that control budgets for a few years are available at once. Based on the current sterile male cost (without economies of scale) and assumed average release rates of 55–100 sterile males/km^2, the present cost for the additional SIT package (sterile males plus 18 months of weekly aerial releases) is estimated at US$800/km^2. Barrett (1997) listed the annually recurrent cost of tsetse control using insecticide-impregnated targets in Zimbabwe during the period 1987–1991 at approximately US$100/km^2 (i.e. Z$600 at that time). Budd (1999) estimated for 1999 the annual cost of placing insecticide-impregnated targets in Zimbabwe at between US$220 and $385/km^2/year, depending on the accessibility of the terrain. According to Allsopp and Phillemon-Motsu (2000), maintaining the status quo of T&T control in the Okavango delta of Botswana requires a recurrent expenditure in excess of US$1.5 million/year, although no cases of nagana were recorded between 1985 and 1998, and the last case of sleeping sickness in Botswana occurred in 1983. The treated area comprised up to 6000 km^2, so the annual cost for target placements as implemented in Botswana therefore amounted to at least US$250/km^2. Based on the two examples above, the implementation of an SIT compo-

nent for tsetse eradication needs an investment over 18 months that equals 2–8 years of recurrent expenditures for tsetse control based on other techniques. In riverine habitats of West Africa, where substantially fewer sterile males are required (10 or fewer sterile males per km^2), the cost for the SIT package is estimated at US$250–300/$km^2$.

Other costs of conventional control that are usually not accounted for include the environmental side effects and related environmental and economic costs of continuous tsetse control, as well as the interference with enzootic stability to various tick-borne diseases. For example, regarding the environmental and economic costs of pesticide use in the USA, Pimentel *et al.* (1992) estimated that an annual investment of approximately US$4 billion saved about $16 billion of US crops; however, the unaccounted environmental and social costs of pesticide use amounted to at least US$8 billion each year. The authors emphasized that a complete long-term accounting of all the (direct and indirect) costs of pesticide use would probably reduce the perceived profitability of applying pesticides by approximately half. Based on such expanded benefit–cost assessments, the option of creating sustainable fly-free zones through integrating the (temporary) use of conventional methods with SIT becomes more attractive in economic terms.

With regard to the benefits accruing from the establishment of tsetse-free zones, this does not only include the saved expenditures for continuous intervention measures. Additional benefits accruing from tsetse-free zones would include significant increases in livestock productivity, both of low-productivity existing cattle breeds but, particularly, resulting from the introduction of high-productivity breeds or cross-breeds. Such productivity increases, and the concomitant large-scale investments to improve the livestock industry, are unlikely to occur as long as tsetse are present – even under a T&T suppression situation. Over time the cost of the SIT package will be further reduced by economies of scale, by the commercialization of various aspects of fly production and by continued development and refinement of various methods that are already partially in use for screwworm and medfly SIT.

As far as cost is concerned, the real challenge may not be cost-effectiveness in the short term. Given the scale of the existing losses and related human suffering, if tsetse-free areas can be established and sustained, it will be worth it (i.e. cost-effective) over time. Benefits will continue to accumulate year after year. The real issue is simply whether or not the funds required to create strategic fly-free areas can be raised. Some critics claim that the economic benefits of eradicating an insect pest population stand or fall with sustaining the pest-free status or experiencing reinfestation, respectively. With regard to the possibility of pest insects reinfesting a freed area, even temporarily rendering an area pest free may be economically attractive, provided that the pest-free phase lasts long enough to allow the investments made to be recovered.

Screwworm eradication in North and Central America solved a problem of relatively advanced livestock systems, whereas tsetse and trypanosomiasis are a problem far closer to the root of (agricultural/livestock) development in its affected countries. In Africa, the impetus for poverty reduction, the positive impact on livelihoods and the related indirect benefits that are expected to accrue from the creation of tsetse-free zones will far exceed the benefits that are accruing in screwworm-free zones in the Americas.

Applying the Tsetse SIT Component

Although SIT can be used for control, eradication or prevention of major insect pests of agricultural, veterinary or medical importance, against tsetse it is currently only applied in identified priority intervention areas as a final component of an area-wide IPM effort that aims at the creation of sustainable tsetse-free areas. The tsetse 'SIT package' consists of: (i) the availability of sufficient and good quality sterile males; and (ii) weekly aerial release capacity over a period of 18 months. Feldmann (1994) summarized general aspects relevant to the mass-rearing of insects, the transport and

release of reproductively sterile insect material and insect pest management.

Sterile males for use in SIT operations are produced at insect factories. The work at the Tsetse and Trypanosomiasis Research Institute (TTRI) at Tanga, United Republic of Tanzania, in support of the Zanzibar tsetse eradication project showed that 1 million tsetse females can supply about 100,000 sterile males for release each week, which is sufficient to cover at least 1000–1500 km^2. For such a production capacity the conventional rearing system, involving trolleys for fly holding, requires a facility of up to 1500 m^2 (fly holding, 510 m^2; membrane feeding, 150 m^2; pupae incubation and 'self stocking' of adults into holding cages 60 m^2; fly handling, quality control and washing, 320 m^2; blood processing and storage at –20°C, 180 m^2; irradiation room, 50 m^2; the rest – at least 200 m^2 – general store). The decision on the location of the factory is mainly influenced by logistics (availability of electricity, water, personnel, supply and repair services, vicinity to international airport, distance to customers, etc.). Twenty-five to 30 trained and dedicated staff are required, willing to work in shifts, if necessary also on weekends. Back-ups for various components are essential, particularly for water and electricity. Also, a back-up 'seed' colony of about 100,000 flies of the colonized strain should be maintained at a different place. Fly proofing is highly advisable and possibly obligatory (depending on the location of the factory and relevant quarantine regulations), and protective measures against chemical or other contaminations (e.g. by air, water used for cleaning or via staff) and against other risks (e.g. measures against ant raids) must be taken. Depending on the species, 1 million tsetse females require, per week, between 170 and 250 l of radiation-decontaminated (1–1.5 kGy) and quality-tested blood. At least a half-year supply of quality-tested fresh-frozen blood should be on store. A fly factory should have separate operational production modules (with different staff; equipment, including spare parts; materials; blood-batch), in order to minimize risks and to facilitate management.

In cases where the sterile males can be purchased from an existing fly factory, only mating compatibility studies need to be conducted in field cages (Mutika *et al.*, 2001) and a contract arranged early enough to permit the colony to reach the required size when needed. Based on the availability of a 'seed' colony (*c.* 100,000 breeding females) of a target tsetse species and assuming acceptable colony performance (i.e. < 1.2% daily female mortality; > 0.6 pupae per female per 10 days; > 85% emergence rate from pupae), a population of 1 million tsetse colony females can be reached within 1 year.

Unless relevant information is already available, test releases with marked sterile males and recapture studies need to be conducted in various habitats and different seasons, in order to obtain information on sterile male survival and dispersal and thus determine the likely release densities required in different areas.

Highly effective suppression tools should be selected that reduce the target fly population to the lowest possible level, because this permits a more efficient utilization of available sterile males. It is important that the pre-release population suppression activities are also based on the area-wide concept, i.e. that the entire tsetse population is suppressed over the complete distribution range of the species in the project area, including game reserves and marginal agricultural land. Different habitats in the project area may require the application of different pre-release fly suppression tools.

The target population densities after tsetse population suppression will determine the numbers of sterile males required for weekly release per square kilometre. Initially the release rates in certain areas will reflect the density of relic wild fly populations, and aim at a resultant ratio of sterile to wild males of > 10:1. As the Zanzibar experience has shown, where no efficient pre-release tsetse suppression tool was available (and where 18 months of continuous placement of about 80 insecticide impregnated targets/km^2 only reduced the *G. austeni* population in the Jozani forest to about 20% of its original density), this may locally require more than 500 sterile males/km^2, and on average

involve 55–100 sterile males/km^2. With decreasing density of the target population, the number of sterile males released over the target area can also be reduced and as soon as the target population reaches or falls below detectable level, a pre-determined 'thin-filming' level of sterile males (e.g. 25–35 sterile males/km^2) can be introduced. Depending on the efficiency of the available monitoring devices, the weekly sterile male releases should be continued for at least three to four generation periods (6–8 months) after the last wild fly is captured. In cases where there is no continuation of SIT operations in the vicinity of the target area and the fly factory will, therefore, eventually discontinue the mass production of the target species, as was the case with the Zanzibar project, it is advised to pursue the fly production and the sterile male releases for a longer period. (In the case of the Zanzibar operation it was decided to pursue the releases for 15 months after the last wild fly was captured, which also took account of the relatively inefficient monitoring devices available and the need to reduce the tsetse production colony substantially upon completion of the project.) If the respective SIT operation is part of a longer-term 'rolling-back' campaign in a larger fly belt and the fly factory continues to produce the target species in large numbers, the release operations can be discontinued earlier, because releases can be reinitiated in case of a local outbreak at any time. Some entomological monitoring will need to be pursued after the discontinuation of the SIT operations.

Large-scale area-wide campaigns will have to cover at least 10,000 km^2 at any time, and it will not be possible routinely to gather comprehensive entomological data at frequent interval from all areas. Instead, a representative monitoring system needs to be introduced. To do so, a 10 × 10 km Universal Transverse Mercator (UTM) cartography grid system is superimposed on land-use/land-cover maps of the area, and in each of these 100 km^2 grids a representative 1 km^2 area of the most suitable fly habitat is identified, which will serve as a fixed monitoring site (FMS). For monitoring at an FMS, five to 25 suitable odour-baited traps (depending on the species) will be positioned for exactly 72 h. Trapped flies are identified and checked for marks, and females are dissected. Particular attention is paid to: (i) the ratio of marked released and unmarked wild males; and (ii) the rate and type of increased reproductive abnormalities (degenerating immature stages – egg, 1st, 2nd or 3rd instar larvae – *in utero*; oviduct blockage; abortion) as a result of the female being inseminated by sterile sperm. As it will not be possible to visit all FMS each week, 20% of the FMS that before intervention showed the highest apparent density of the target population will be covered, together with 10% of sites that are selected randomly from the remaining FMS. The monitoring will be done in a rotational manner, thus obtaining new data every week and visiting each of the selected 20% FMS every 4–6 weeks.

Vreysen *et al.* (2000) summarized the handling of tsetse males earmarked for release. The aerial releases, either dropping biodegradable release cartons with 50–200 sterile males each or using a system of chilled adult releases, take place at an altitude of 200–300 m at a ground speed of 160–250 km/h. The effective swath width between neighbouring parallel flight lines used each week is 1–2 km. Different release flight patterns may be applied along riverine habitats or in valley systems. Ground releases are possible but less preferred, because they are more expensive, take relatively long (which may affect the quality of released males) and do not guarantee that all areas receive sterile males. A climatic data logger accompanies the sterile males from the handling, irradiation and packaging until actual release. Random samples of sterile males are retained at the insect factory and in a field insectary after release, for standardized quality assurance procedures.

There are several situations where the creation of tsetse-free zones and the possibility of an SIT component as part of an integrated area-wide campaign appears feasible and justifiable, and the SIT may be considered under one or several of the following circumstances.

- Demographic development demands the introduction of more productive livestock systems/intensified and diversified agricultural systems, for which the complete elimination of the T&T problem through the creation of a tsetse-free zone is advised.
- There is environmentally detrimental, uneven distribution of cattle and signs of overgrazing adjacent to tsetse-infested areas with very low livestock density.
- Tsetse-infested wildlife reserves and agricultural areas are in close proximity and constitute a threat to each other.
- The tsetse target population is located at the periphery of the tsetse belt, permitting a phased expansion of the fly-free area.
- The target population is isolated in ecological 'islands' or 'peninsulas', as confirmed by population genetic studies.
- There is evidence for tsetse and trypanosomiasis advancing into new, previously uninfested agricultural areas.
- Recurrent expenditures for continuous tsetse/trypanosomiasis control are not acceptable.

Future Prospects for Tsetse SIT

It is likely that the SIT for use against tsetse will – in the foreseeable future – continue to be a subject of controversy. The same was the case with SIT against medfly and NWS (Krafsur, 1998), and in the views of many collaborators the concept of proactive and area-wide insect pest management and the investments required for fly factories seem to conflict with preferences towards solely community-reliant, threshold-responding intervention methods in selected areas. Such 'picking the cherries from the cake' approaches may undoubtedly result in remarkable short-term benefits but obviously risk unsustainability within a short time after the institutional support is discontinued (Jordan, 1995). Therefore, a combination of both approaches, substantial tsetse population suppression, involving local communities wherever feasible, and 'mopping-up' with a final SIT component, should be considered in situations where: (i) the creation of a fly-free zone appears to be sustainable and results in maximized benefits; and (ii) the SIT component is feasible and respective investments are justifiable. The inclusion of the strongest driving force for the survival of a species – namely, sexual interaction and reproduction – as the key to fight insect pests in an integrated and area-wide approach remains a highly attractive intervention approach. Nevertheless, before tsetse SIT will become as successful as SIT against fruit flies or NWS, several issues need to be addressed.

Increased ownership and appropriate commitment of the authorities and other stakeholders in T&T affected countries and a harmonization and concertation of international support are a prerequisite for scaled-up action against the T&T problem. The launching of the Organization of African Unity's Pan-African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC, which reflects ownership and determination of affected African countries to address the T&T problem as a priority development and health issue) at the occasion of the 26th meeting of the International Scientific Council for Trypanosomiasis Research and Control (ISCTRC) in Ouagadougou, Burkina Faso, on 4 October, 2001, and the subsequently initiated harmonization process between PAAT, PATTEC and the ISCTRC enhanced the initiation of transboundary planning of area-wide integrated intervention efforts, a very complex, multidisciplinary exercise that requires input from various sides. One component is the gathering of information on subregional tsetse distribution and the identification of isolated or confined tsetse populations, a prerequisite for the initial creation of tsetse-free zones. This exercise makes use of two relatively new tools: (i) the GIS-aided preparation of tsetse presence/absence risk prediction maps for the economically most important tsetse fly species (Wint, 2001); and (ii) the systematic sampling of information on tsetse population genetics, indicating gene flow and the degree of isolation between neighbouring populations of target tsetse fly species.

For most areas in eastern and southern

Africa, the subregional risk prediction maps show a sufficiently structured probability pattern for tsetse presence/absence, based on which transects with sampling sites can be identified relatively easily. The situation in most of western and central Africa is different, as for some riverine species the mapping resulted in no gradients or patterns regarding the tsetse presence/absence risk but, rather, huge continuous areas of highest probability of tsetse presence. The population genetic sampling in these areas will need a different approach, specifically: (i) the superimposition of a 50 × 50 km UTM grid system on to land-use/land-cover maps (including river basins); (ii) in each 2500 km^2 grid, the identification of one or several sampling sites in a suitable habitat along a river; (iii) the collection of some 50 flies per species per site (if possible); (iv) standardized processing of the flies (head and thorax for DNA examinations; gonadal systems – except for the spermathecae and the uterine content – for examinations of gonadal symbionts; the remaining part of the abdomen for bloodmeal analysis and/or investigations on cuticular hydrocarbons); (v) conversion of the molecular genetic results into a gene-flow database, indicating the number of individuals per species and generation crossing between neighbouring populations; and (vi) (if possible) the preparation of a phylogenetic map that hopefully corresponds to the different river basins. The results are anticipated to facilitate the development of a phased subregional strategy for area-wide integrated intervention campaigns against the tsetse and trypanosomiasis problem.

The initiation of tsetse mass production for SIT will normally be based on the availability of a mass-rearing adapted 'seed' colony of about 100,000 female flies. Standardized mating-compatibility field-cage tests will be applied to ascertain the behavioural and mating compatibility of the mass-reared strain with the different tsetse target populations. In this context additional work will need to be conducted for better understanding the phases of mating behaviour of the different tsetse species, which appears to be not simply a rape type of mating but to involve, for example, elements of conspecific phonotaxis. Further work is also required on the development of internationally accepted fly-production process and product quality assurance procedures, which will find particular application for fly quantity- and quality-related payments by customers (T&T intervention projects applying the SIT) to the supplying fly factories. Depending on the need for tsetse SIT, the tsetse production capacity will necessitate in the foreseeable future the construction of subregional fly factories in Africa for more than 50 million mass-reared colony females and, in addition, several back-up/'seed' colonies at different locations, each with at least 100,000 tsetse colony females. The listing of criteria for an optimal location of fly factories and the preparation of generic plans describing the relative space needed for holding, feeding and processing of identified colony sizes will greatly facilitate the planning work and provide a better basis for respective decision making and budgeting.

Improved procedures for efficient long-distance transport of sterile males from subregional fly factories to release projects need to be elaborated. Systems for automated release of unboxed adults and, if necessary, simultaneous release of more than one tsetse species exactly at the required release rate need to be developed.

In addition to the application of the 'conventional' SIT, investigations could explore the potential role of hybrid sterility and/or its use in combination with SIT in integrated area-wide T&T intervention campaigns (e.g. sterile/substerile *G. m. morsitans* against *G. m. centralis* or sterile/substerile *G. palpalis palpalis* against *G. f. fuscipes*). Efforts also need to be invested in the development of models for better understanding and improved application of the area-wide concept against T&T and the development of predictive tools that facilitate decision making on the required duration of intervention activities and at what stage local 'eradication' of the target population can be assumed and intervention measures be discontinued.

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33 Biting Flies: Their Role in the Mechanical Transmission of Trypanosomes to Livestock and Methods for Their Control

Martin J.R. Hall and Richard Wall

Mechanical Transmission of Trypanosomes

Mechanical transmission of trypanosomes occurs when they are transferred directly from the blood of an infected host to another host without undergoing development in the vector. At its most straightforward, mechanical transmission can occur in the absence of a living vector by the syringe passage of infected blood between animals. This can occur by accident (for example, when the same needle is used to inject more than one animal during an inoculation campaign) but mechanical transmission is more usually associated with the transfer of blood from infected to uninfected hosts by biting flies.

There are two distinct aspects to the discussion of this phenomenon: mechanical transmission in areas free of tsetse and mechanical transmission in areas where tsetse are present. When infected cattle have been moved into new, tsetse-free countries and continents, selective pressures have resulted in the evolution of trypanosomes that are unambiguously capable of transmission by mechanical means alone. These species, or populations, do not need to undergo cyclical development in a vector. Where trypanosomes occur in tsetse-infested areas, considerable debate exists about whether mechanical transmission occurs at all.

Mechanical transmission in tsetse-free areas

In areas of the world known to be outside the range of tsetse flies, it is clear that the transmission of trypanosomes between hosts must be by vectors other than tsetse. In Asia and in Central and South America, *Trypanosoma evansi*, which may have originated from *Trypanosoma brucei*, is transmitted mechanically by biting flies (Luckins, 1988). In Latin America, *Trypanosoma cruzi* is transmitted cyclically by Triatominae. The transmission of *Trypanosoma theileri* by tabanids is somewhat unusual in that it involves ingestion of infected tabanids by the hosts and so is not mechanical transmission in the sense used elsewhere in this chapter. These three trypanosome species are not normally transmitted by tsetse. However, even species of trypanosome for which tsetse are the normally acknowledged vector in sub-Saharan Africa can be transmitted outside the range of tsetse. Thus, *Trypanosoma vivax*, an important agent of bovine trypanosomiasis, occurs in the absence of tsetse flies in Mauritius and Latin America; the most likely vectors of *T. vivax* in these areas are biting flies. In South America, *T. vivax* has become well established in the northern countries of the continent in the absence of its original vector (Dwinger and Hall, 2000) and has even extended its range to the central region of Brazil and

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Bolivia in the last decade (Silva *et al*., 1998). The characteristics of some strains of *T. vivax* from tsetse-free areas have changed to such an extent that they are now incapable of development in tsetse. The fact that transmission of *T. vivax* is maintained in South America in the absence of tsetse suggests that the trypanosomes have undergone adaptive changes that enhance their mechanical transmission compared with the African strains.

Mechanical transmission in sub-Saharan Africa

The mechanical transmission of trypanosomes in sub-Saharan Africa has been the subject of speculation since tsetse were first identified as the biological vectors (Krinsky, 1976). This debate includes both animal and human forms of the disease. During the 20th century, a large number of studies proposed that trypanosome transmission could occur in the apparent absence of tsetse (Wells, 1972 and references therein). However, tsetse often occur at very low population densities and as a result may be very difficult to detect. For example, bovine trypanosomiasis was first diagnosed on Zanzibar in 1908, but it was not until the 1940s that *Glossina austeni* was first detected there. Until that elusive species was captured, it was thought that transmission of trypanosomes was most probably by mechanical means, species of Tabanidae being the main candidates.

In other areas of Africa where trypanosome transmission occurs in the apparent absence of tsetse, it is often argued that, like the situation on Zanzibar, tsetse are responsible but have just not been found. Jordan (1986) and Leak (1999) both considered the evidence for and against mechanical transmission of animal trypanosomes in Africa. They came to the same conclusion that much of the evidence for its occurrence was circumstantial and did not stand up to scrutiny. The evidence was in two categories: either (i) field instances of transmission without apparent tsetse; or (ii) experimental infections in the laboratory. Successful examples of the latter are the mechanical transmission by African species of *Stomoxys* of *T. brucei, T. evansi*, *T. vivax* (Mihok *et al*., 1995) and *T. congolense* (Sumba *et al*., 1998) from infected to uninfected mice. Jordan (1986) summarized that mechanical transmission might play a role in the spread of disease, especially in the seasonal peaks of biting-fly abundance, but that it was not capable of maintaining normally tsetse-transmitted trypanosomiasis in the absence of tsetse.

It is also argued that, since *T. vivax* show local adaptation to mechanical transmission outside the range of tsetse, this might also occur within the tsetse range and, if this is the case for *T. vivax,* similar variation could be present in other trypanosome populations. The increasing proportion of *T. vivax* infections in cattle herds at increasing distance from the main tsetse foci in Africa, or with time following a tsetse eradication campaign, has been presented as evidence of mechanical transmission. However, it can be explained in terms of tsetse transmission without the need to implicate other vectors (Leeflang, 1975). More recently, in Kenya, Baylis and Stevenson (1998) showed that, after dipping cattle in insecticide, the observed reduction in trypanosomiasis by both *T. congolense* and *T. vivax* was greater than that expected from the impact of the insecticide on the tsetse population. They suggested that mechanical transmission by other biting flies such as tabanids and *Stomoxys* may have been playing a much larger role in the spread of infection than had been assumed previously. Nevertheless, while of immense interest and potential epidemiological importance, it remains the case that there are no unequivocal demonstrations of mechanical transmission of trypanosomes by biting flies in the tsetse-infested areas of sub-Saharan Africa. The remainder of this chapter will, therefore, focus on the known cases of mechanical transmission.

Biting Flies as Mechanical Vectors of Trypanosomes

Mechanical transmission is facilitated by the following conditions:

- The parasitaemia of the peripheral blood supply of the infected host is high.
- The population of biting flies is high.
- There is a high proportion of interrupted feeds of flies, due to their disturbance during feeding (e.g. disturbance by host defensive behaviours or by competing flies) (Davies, 1990).
- There is a minimal delay between feeding on an infected host and resumption of feeding on a second, uninfected host – this is most likely when hosts are closely associated, as in herd animals.
- Biting flies are highly mobile.
- A large amount of infected host blood remains on the mouthparts of biting flies between interrupted feeds.

There will be interaction between all of the above factors. For example, a host with an acute infection and high parasitaemia might be considered a good source of infectious agents, but if that host is listless due to the disease and unable to respond to fly attack with strong defensive behaviours, then interrupted feeds and subsequent mechanical transmission are unlikely to occur. In addition to the possibility of a disease moderating host defensive behaviours, these behaviours vary greatly between individual animals and even within one animal depending on a range of factors including the actual biting-fly density, giving rise to host-mediated competition between flies.

When a biting fly feeds on an infected animal it is perhaps not surprising that some trypanosomes are ingested with the bloodmeal. These trypanosomes may subsequently be detected on the mouthparts or in the gut of the biting fly. Through such observations, numerous species of biting fly have been proposed as potential vectors of trypanosomes (e.g. in the families Culicidae, Ceratopogonidae, Simuliidae and Muscidae as vectors of *T. vivax*). However, there are a number of factors other than the simple ingestion of trypanosomes that need to be considered in incriminating vectors and most workers in the field consider that the most likely mechanical vectors among the biting flies are tabanid flies and muscid flies in the genus *Stomoxys* (e.g. *S. calcitrans*, *S. sitiens*, *S. nigra*), on which this chapter will concentrate. The major difference between these two groups in terms of their biting behaviour is that only female tabanids bite (Fig. 33.1), whereas both sexes of *Stomoxys* take bloodmeals. Despite being a cosmopolitan and very obvious group of biting flies, the horn flies, *Haematobia irritans* and *H. exigua*, are unlikely mechanical vectors of trypanosomiasis because of their close association with a single host individual. Even after disturbance they usually alight back on the same host; hence, the opportunity to transfer infectious agents between hosts is minimal.

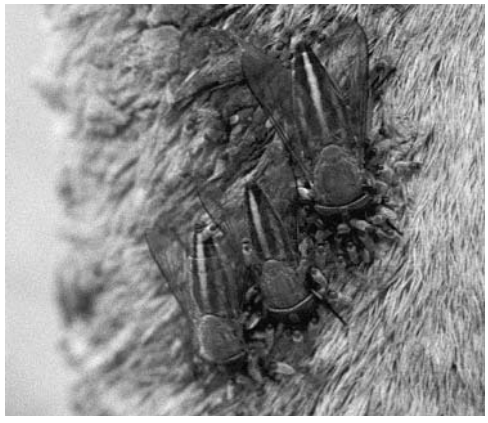

Fig. 33.1. Adult females of *Tabanus occidentalis* feeding on cattle leg, with numerous non-biting Diptera competing for blood at the sites of wounding. (Martin Hall.)

Luckins (1988) reviewed the factors that make tabanid flies, especially in the genus *Tabanus*, efficient mechanical vectors of *T. evansi*. These included: (i) the painful biting nuisance of the flies that provokes host defensive responses and, therefore, brief interrupted feeds; (ii) the persistence of the flies in feeding despite host defensive activity; (iii) their ability to acquire and pass on infections in brief feeding intervals (5 s); and (iv) the protection of blood against desiccation in the enclosure of the mouthparts created by the labellae. These same factors make tabanids 'good' potential vectors of a wide variety of other pathogens (Krinsky, 1976; Foil, 1989). With regard to the actual amount of blood ingested, tabanids consume relatively large volumes compared with other biting flies. Thus, whereas the blood-

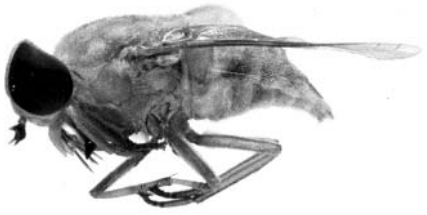

Fig. 33.2. Adult female of *Cryptotylus unicolor*, a proven mechanical vector of *Trypanosoma vivax* (Ferenc *et al.*, 1988). (The Natural History Museum Photographic Unit.)

meals of mosquitoes are in the order of 0.005 ml and those of *Glossina* species are around 0.05 ml, Hollander and Wright (1980) measured the intake of eight species of tabanid in North America as between 0.02 and 0.65 ml per bloodmeal. However, only a small fraction of a biting fly's bloodmeal actually remains on the mouthparts; for example, Foil *et al.* (1987) demonstrated that *Tabanus fuscicostatus* carries a residue of 10 ± 5 nl blood on the mouthparts (fascicle, palpi and labium) immediately after feeding.

In South America, mechanical transmission of *T. vivax* from an infected to an uninfected bovine has been demonstrated with several tabanid species in simple yet effective experiments: with *Cryptotylus unicolor* (Ferenc *et al.*, 1988) (Fig. 33.2) and *Tabanus importunus* (Raymond, 1990) in French Guyana and with *Tabanus nebulosus* in Colombia (Otte and Abuabara, 1991). The numbers of interrupted feeds per host was very similar in the experiments with *C. unicolor* (about 40) and those with *T. importunus* (44). The latter study involved interrupted feeding of flies over a 3-day period, from 8 to 10 days after the experimental inoculation of the donor host with *T. vivax*. Each fly was caged in a 5 × 3.5 cm polystyrene tube and this was placed on the back of the donor host. Flies were allowed to feed for 30–60 s and then were immediately moved in their tubes to the recipient host and allowed to recommence feeding, again for 30–60 s. If they did not complete feeding in that interval the process was repeated (Raymond, 1990). Using a similar experimental set-up, Otte and Abuabara (1991) demonstrated transmission with even fewer flies, just 17 and 19 *T. nebulosus* being involved in each of two successful trials. It is highly likely that similar studies with other species of tabanid would lead to further examples of direct incrimination of tabanids as mechanical vectors of trypanosomes. Interestingly, in the study of Otte and Abuabara (1991), whereas *T. nebulosus* readily resumed feeding after interruption, neither *Tabanus pungens* nor *Tabanus claripennis* would resume an interrupted bloodmeal, suggesting that they would be less effective as mechanical vectors. However, they might be more likely to resume feeding in a less artificial environment.

As well as the direct evidence given above, there is indirect evidence of the role of biting flies in mechanical transmission, through spatial and temporal correlation of disease with the presence of tabanids and with their feeding activity. Thus, in Colombia, *T. vivax* infections were associated with low-lying swampy areas where tabanids breed (Otte *et al.*, 1994). In addition, there was a highly significant positive correlation between the incidence of *T. vivax* infections in cattle and the population of *T. pungens/claripennis* observed feeding on cattle. In the absence of tsetse and other biological vectors, these correlations of disease and tabanids assume greater importance than would be the case in sub-Saharan Africa.

Control of Mechanical Vectors of Trypanosomes

The control of biting flies that are implicated as mechanical vectors of trypanosomiasis closely parallels the methods of tsetse control described elsewhere in this volume. Thus the use of land clearance, insecticides, traps and treatments of hosts have all been advocated at one time or another. The range of options available for the control of tabanids and *Stomoxys* are outlined below.

Sterile insect technique

Although the sterile insect technique (SIT) for control has not been tried with tabanids, it has been used in limited trials against

Stomoxys. It is relatively simple to culture *Stomoxys* in the laboratory, feeding the bloodsucking adults on blood-soaked cotton pads or blood-filled membranes, as for *Glossina*, and rearing the larvae in a medium of decomposing plant material. In one control trial, sterilization of *S. calcitrans* was brought about by exposure of pupae to 2 krad from a ^{60}Co source. Adults were allowed to emerge in the laboratory and then released in areas of high cattle density. Each day sterile flies were released initially at equal and, eventually, at double the estimated initial wild population. Despite daily losses of about 35% of the released males through mortality and emigration, and the fact that the released males were only about half as competitive as wild males, after one generation over 85% sterility was found in the native population. With continued releases, sterility rapidly increased to 100%, while the field population fell in numbers by 97%. Although sterility remained high in the release area for a number of weeks after the last release of sterile males, subsequent reinfestation was rapid (LaBrecque *et al*., 1975). In another trial, sterile flies were released on St Croix, US Virgin Islands, as part of a 3-year feasibility study, running the SIT programme alongside insecticidal and physical techniques for *Stomoxys* control. The population fell by more than 99.9% during the trial, but fertile flies were caught throughout the study and at its termination the wild population rose at an average rate of 8–9 times per generation (Patterson *et al*., 1981).

Use of the SIT has never been seriously proposed for tabanid control. One reason for this is difficulties with the production of sufficient numbers of pupae for sterilization. Tabanid flies have not been successfully reared in the laboratory. Even if these problems were overcome, the great diversity of tabanids (more than 4000 species in at least 137 genera), with many species on a single pasture, makes use of such a species-specific control method highly unlikely. In addition, there are significant problems with the impact on livestock of the release of large numbers of biting flies, unless releases of only the non-biting male tabanids could be made.

Habitat alteration

In the early days of tsetse control, vegetation clearance was carried out to reduce tsetse habitat, using heavy chains pulled by one or more tractors or bulldozers (Jordan, 1986). This had significant adverse effects on the environment and on wildlife areas and, therefore, habitat clearance has been largely discontinued as a deliberate policy. Chaining treatments have been evaluated recently for control of *Tabanus abactor* on ranches in Texas, USA, where the environmental impact is not so great. These demonstrated a significant reduction in the numbers of tabanids caught in traps on chained plots of land compared with control plots (Holmes *et al*., 1998). Other significant habitat alterations, such as the draining of the swampy breeding sites favoured by tabanids, can be of benefit in tabanid control. This may be more acceptable from an environmental perspective if the land is scheduled for development after draining, rather than being drained solely for fly control. However, under specific conditions, some drainage schemes have been implicated in actually creating habitat for tabanid larvae.

Reducing the quantities of manure and decomposing plant material (hay, straw) present in the environment greatly reduces the potential breeding sites for *Stomoxys* and so has a very significant effect on populations of this biting fly. Good sanitation, which has the effect of altering the larval habitat, is probably among the most effective methods of *Stomoxys* control around livestock premises (Foil and Hogsette, 1994).

Application of insecticide to the environment

For control of tabanids and *Stomoxys*, insecticides can be applied to the environment against both the larval and the adult stages. For control of *Stomoxys* larvae around animal units this can be a relatively straightforward procedure, because the breeding sites of wet straw, stable bedding and manure are discrete and applications of larvicides (including insect growth regulators, IGRs) can be well targeted. In addition, the walls where

adults rest can easily be treated with a residual spray, taking measures to avoid induction of resistance. However, tabanids breed in a great variety of aquatic, semi-aquatic and terrestrial habitats and the adults live away from livestock housing facilities. Therefore, the focused application of insecticides against tabanids without affecting non-target organisms is extremely difficult.

Space sprays to target adults rather than the immature stages have been used for tabanid control. Howell *et al.* (1949) used aerial sprays of methoxychlor, toxaphene, chlordane or DDT (in fuel oil and cyclohexanone) on wooded areas between pasture, where tabanids were assumed to rest. Their results were somewhat equivocal, due partly to coincident natural changes in the tabanid populations. Hansens (1981) achieved somewhat more short-term success in aerial ultra-low volume and small volume mist blower sprays against *Chrysops atlanticus*, a nuisance biter in recreational areas. Application rates of resmethrin ranged from 0.0078 to 0.112 kg/ha and gave > 95% reduction of deer fly annoyance 1 h after treatment, 27–97% reduction after 8 h and 0–96% reduction after 24 h. Populations returned to pre-treatment levels within 1–3 days, due to reinvasion. These trials have not been repeated on a wider scale, mainly due to concerns over reinvasion, cost (especially for use in livestock situations) and the environmental impact of such indiscriminate treatment of the environment.

Application of insecticide to host animals

To control biting flies on animals in a herd, insecticides (usually organophosphates or pyrethroids) may be applied topically to all the animals in the form of pour-on, spot-on or dip formulations, to give residual-topical or systemic effects. The macrocyclic lactones (ivermectin, doramectin, eprinomectin, selemectin and moxidectin) have a relatively limited systemic efficacy against biting flies or triatomine bugs at normal theraputic doses (Wilson, 1993).

Maintaining the residual effect of conventional topical insecticides applied as dips or sprays can be difficult, especially in bright sunlight, rainy weather or when animals are grazing in wet grass due to rain or dew. Unfortunately, these difficult rainy-season conditions usually coincide with the tabanid season in the tropics. Other than frequent manual reapplication, a possible answer to the problem of insecticide loss might be the use of automated applicator devices, as used with good effect against tabanids in the 1950s, where the animals effectively treat themselves as they make daily visits to a salt lick, water or food source.

Leak *et al*. (1995) carried out an experiment in tsetse control in which they applied cypermetherin to cattle in the Ghibe Valley of southwest Ethiopia. Applications were made each month by pour-on along the backline, at an approximate dose of 1 ml per 10 kg body weight. In addition to tsetse numbers, the numbers of tabanids and *Stomoxys* caught in monitoring traps were recorded. They showed a significant reduction of tabanid and *Stomoxys* catches in a 2-year control period compared with the 5-year period before control. This appears to be the first demonstration of the long-term effects on the numbers of biting flies, other than the target species, of such a control strategy. As with all uses of insecticide, care needs to be taken to ensure that insecticide resistance is not selected for, not just in the target groups but also in non-target groups of ectoparasites, such as ticks.

An alternative to topical treatments for the application of insecticide is the use of ear-tags, collars or necklaces impregnated with insecticide – usually pyrethroids such as permethrin, cypermethrin or flumethrin. Parashar *et al.* (1989) found that 10% permethrin impregnated ear-tags remained effective for 1–2 months in control of *Stomoxys calcitrans* and the tabanid *Haematopota dissimilis*. A significant factor for consideration with the use of ear-tags is the rate of transfer of insecticide from the impregnated devices to the preferred landing sites of the flies. Thomson (1987) demonstrated that 4% w/w deltamethrin ear-tags in cattle failed to control tsetse because of the poor translocation of insecticide. Most insecticide was located on the head and neck, whereas the tsetse

preferred to land and feed on the bottom of the legs and lower torso. A 0.0046% deltamethrin spray (1.4 mg active ingredient/kg) did cause significant mortality of alighting flies, although neither spray nor ear-tags prevented tsetse from landing and feeding. Tabanids have very distinctive alighting sites on the host, and Leprince *et al*. (1991) found a similar result with tabanids to that of Thomson with tsetse, i.e. fenvalerate sprays were much more effective than fenvalerate ear-tags in causing mortality of feeding tabanids. An evaluation of ear-tags impregnated with lambda-cyhalothrin represents the first report of ear-tags effective in killing tabanids and suggests that these devices might have some merit in tabanid control (Leprince *et al*., 1992).

The median lethal doses (LD_{50}s) of pyrethroids to tabanids are some 10–150 times lower than those of organophosphates. A useful comparison of organophosphates and pyrethroids, both as pour-ons and ear-tags, is that of Presley and Wright (1986). In a test of ten different insecticide formulations, they found that only 1% permethrin 'pour-on' and 10% permethrin ear-tags produced greater than 55% mortality of field populations of *Tabanus abactor*. Three water-based sprays (0.06% coumaphos, 0.05% permethrin and 0.11% dioxathion + 0.005% dichlorvos) and five polyvinyl chloride ear-tags (8% fenvalerate, 7.5% flucythrinate, 16% chlorfenvinfos, 4% fenvalerate + 16% crotoxyphos and 7% cypermethrin + 5% chlorpyrifos) caused no greater than 36% mortality. None of the formulations prevented *T. abactor* from biting and taking a bloodmeal; therefore, they did not reduce the immediate irritation caused by tabanid bites. It is also unlikely that they would reduce the likelihood of mechanical transmission. In this study, as noted above, tabanids preferred to bite on the legs and lower body, the regions where insecticide is poorly distributed from both ear-tags and pour-ons.

The general conclusions from studies of insecticidal applications against tabanids are that pyrethroids are the insecticides of preference and that they should be applied as sprays or pour-ons rather than as ear-tags or other impregnated devices. When using any insecticidal treatments on livestock, efforts should be made to distribute the sprays or attach the impregnated devices as near as possible to the preferred landing and feeding sites of the target species.

Application of repellents to host animals

To reduce the incidence of bites, repellents are sometimes applied to host animals. Formulations of repellents include sprays, wipes, waxy applicator sticks and impregnated tags. The active ingredients include hydroxyethyl butyl piperidine carboxylate, citronella oil and an increasing number of other plant extracts, the most well-known of which is *N*, *N*-diethyl-meta-toluamide ('deet'). As with the application of topical insecticides, their effectiveness can be greatly reduced by rainfall after application, or by the host animal moving through long vegetation that brushes off the chemical. Their use on herd animals needs to be considered carefully, because it is likely that fly biting activity will be diverted to the least protected animal.

Selective application to those animals that are most attractive to biting flies might be a possible way of reducing the use of insecticides or repellents if the most attractive hosts could be readily identified. For this purpose some insecticides may be applied along with a repellent while others, such as the commonly used pyrethroid deltamethrin, also have pronounced intrinsic repellent effects. However, in the use of topical repellents and insecticides and combinations of the two, there is a delicate balance – misuse of these chemicals could actually exacerbate the problem of mechanical transmission. If used only on selected animals, these compounds may serve only to deflect flies on to other potential hosts. In addition, repelling flies from an insecticide-treated animal renders the insecticide useless as a control measure, unless the fly is able to pick up a lethal dose during a brief landing. Furthermore, to minimize the potential for mechanical transmission from an infected animal, the preferred options for biting activity of flies on that animal would be for either no feeding or feeding to repletion. Both would negate

the possibility of an interrupted feed and mechanical transmission. Therefore, a repellent or insecticide that is only partially effective and causes irritation to a fly part-way through a feed, so that the fly leaves to complete its feed on an uninfected host, has the potential to increase the risk of mechanical transmission over the ideal situation of zero or full feeding. The possibility of this risk was demonstrated by Foil *et al.* (1990), who recorded feeding times of tabanids on cattle treated with sublethal doses of fenvalerate some 27–39% less than those on control cattle, with a consequent reduction in engorgement weight of 31%. The likelihood of such flies resuming and completing their feed on another host before knockdown takes effect should be determined. Almost 100% knockdown was recorded at 1 h after feeding. The chances of survival of trypanosomes on tabanid mouthparts, and, therefore, the chances of successful mechanical transmission, will decrease the longer the period of knockdown before fly recovery.

Trapping

Tabanid traps have been developed over many years, originally concentrating on visual stimuli and latterly also including olfactory stimuli (Hall *et al.*, 1998). The most commonly used traps developed specifically for tabanids with a strong visual element are the Manitoba trap (Thorsteinson *et al.*, 1965) and a variant, the canopy trap (Catts, 1970) (Fig. 33.3). The Malaise trap (Malaise, 1937) (Fig. 33.4) has a lesser visual element, depending on the colour of netting used, and is primarily a flight intercept trap for all flying insects, including tabanids. As for tsetse, the use of carbon dioxide and 1-octen-3-ol as odour baits can significantly improve the catch of tabanid flies at baited traps compared with the catch at unbaited traps. Ammonia has also been shown to attract tabanids. Roberts (1975) demonstrated that increasing the release rate of carbon dioxide at a trap resulted in increased catches of tabanids up to a dose of 4000 ml/min – catches were reduced at release rates above that level. Unfortunately, CO_2, whether from dry ice or gas cylinders, is not a particularly convenient odour bait for large-scale use, but continued research should result in identification of more practical odour baits.

Despite the seemingly large numbers of tabanids that can be caught in traps, they are usually considered more for use in monitor-

Fig. 33.3. Version of the canopy trap (Catts, 1970), with spherical black 'target' suspended under the canopy, being deployed in Bolivia. (Martin Hall.)

Fig. 33.4. Version of the Malaise trap (Malaise, 1937) with white roof and black walls, deployed at pasture edge. (Martin Hall.)

ing than in control programmes. Because of the high fecundity of female tabanids, the proportion of the population that would need to be caught each day to have an effect on the population would be much greater than for tsetse. There is also the problem of the reproductive stage at which tabanids are attracted to traps. In a major trial of box traps to control *Tabanus* species (95% *Tabanus nigrovittatus*), although there was a decrease in the nuisance levels of these flies, most of the females entering the traps had already laid fertile eggs, because they were an autogenous species (Wall and Doane, 1980). Therefore, attempts to reduce the tabanid populations in the long term were likely to be unsuccessful, because those flies caught would be replaced by individuals that developed from the initial egg batches laid by females before they became blood-seeking and attracted to the traps. However, traps may have merit in reducing the biting activity of tabanids on livestock if used in a concentrated fashion at locations where livestock gather, e.g. at water sources and stock yards. The possibilities of this were shown by Wilson (1968), who recorded a reduction in tabanid numbers on cattle that were confined to a 5.7 ha pasture, surrounded by 19 evenly spaced sticky traps, baited with CO_2 released from dry ice. A Russian study on the use of traps over several years in cattle pastures gave a similar result, with a 77–97% reduction of tabanids, sufficient to eliminate their disturbance of cattle (Pavlova, 1985).

Most modern traps for *Stomoxys* are based on variants of the sticky traps developed by Williams in 1973. The Williams trap used fibreglass panels (Alsynite) coated with a non-setting adhesive. It was found that traps made with translucent white panels were more effective than those with red or black panels. These traps have been developed more for monitoring than for control purposes. An improved form of this trap is the cylinder trap (Broce, 1988) and this was subsequently developed as a commercial product, combined with either adhesives, insecticides or IGRs. Electrocuting traps have also been developed commercially for use in control of *Stomoxys* around calf pens, dog kennels or large-animal shelters (Pickens, 1991). These traps were found to be more effective in the long term than sticky traps (which needed adhesive sheets to be replaced daily to maintain the catch rate) and without the hazards associated with insecticides.

Biological control

Numerous reports exist of the wide range of infectious diseases, parasitoids, parasites and predators that affect tabanids, but the biological control of tabanids has not received much serious attention, with only one successful inundative release of an egg parasitoid recorded (Legner, 1995). It has been estimated that only 2% survival from egg to adult is required to maintain annual populations of tabanids, which indicates that an enormous depletion by other mortality factors, including biological control agents, can be sustained. The most dramatic examples of adult predators of tabanids are the Bembicine wasps. If a careful watch is made during the season of adult wasp activity, wasps in the genera *Stictia, Rubrica* and *Bembix* can frequently be seen catching tabanids from livestock for provisioning their nests. However, they are unlikely to have a sustained and significant impact on tabanid populations because their habitat zones and seasons of activity are usually restricted compared with those of their prey.

More success has been had with the biological control of *Stomoxys* (Legner, 1995). However, despite the availability of commercially reared Hymenoptera for fly control, in most instances biological control of *Stomoxys* has not yet been demonstrated with sufficient repeatability to be considered as other than an experimental tool. In one trial, inundative releases of Pteromalid parasitoids had no significant effect on the numbers of stable flies trapped, yet the cost of releases exceeded the cattle production losses estimated to occur due to stable flies in the absence of control measures (Andress and Campbell, 1994).

Cultural control

Secure, fly-proof housing of livestock can provide effective relief from the effects of biting flies. For some tabanid species that avoid entry into enclosed areas, it might only be necessary to have a roofed shelter, without walls, to achieve a significant reduction in fly numbers. As the name suggests, stable flies are found in large numbers around livestock housing, but proper disposal of contaminated waste hay and manure around barns and stables facilitates control of these flies, by reducing the larval development sites. Tabanids tend to prefer thick vegetation such as wooded areas or forest edges to open pasture, therefore livestock that graze in the latter areas will be less troubled by tabanids.

Livestock owners worldwide practise movement of animals to avoid tabanid attacks on either a daily or seasonal basis. Animals may also themselves select habitats that are associated with low rates of tabanid attack. Although these movements may be directly related to minimizing the biting nuisance of tabanids, they must certainly also contribute to reducing the risk of mechanical transmission by lowering the biting rate. In the Sudan, Hall *et al.* (1984) concluded that objective evidence for the implication of tabanids in the mechanical transmission of bovine trypanosomiasis was lacking, but that tabanids were a major factor in causing seasonal migrations of cattle.

Where it is not possible to avoid tabanid attacks, then the risk of mechanical transmission can be reduced if a wide enough separation is maintained between infected and uninfected hosts. On the basis of mark–recapture studies, Foil (1983) predicted that if two horses were separated by some 44 m, < 1% of the *Tabanus lineola* disturbed during feeding on one horse would transfer to the other. At greater separations of the order of 100–200 m, if a fly is interrupted while feeding on the infected host, the chances of it resuming a feed on the uninfected host are so low as to be insignificant.

The future – integrated fly management

Despite the variety of control options outlined above, Foil and Hogsette (1994) considered that the only effective chemical strategy to reduce the incidence of tabanids on livestock was the use of repellents or partial repellents. Clearly the decision as to whether or not to initiate these controls would depend on a balance of epidemiological and financial considerations, in part dependent

on the likely involvement of biting flies in transmission. Although the different strategies are considered in isolation above, probably the greatest benefits for future control against biting flies will come from the combined use of a number of techniques in an integrated pest management programme. An example of this could be the 'push–pull' use of repellents on livestock (the 'push') together with attractant traps (the 'pull').

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Index