FASCIOLOSIS

Edited by J.P. Dalton

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J.P. Dalton

Dublin City University
Republic of Ireland

CABI *Publishing*

CABI *Publishing* – a division of CAB INTERNATIONAL

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A catalogue record for this book is available from the British Library, London, UK

Library of Congress Cataloging-in-Publication Data

Fasciolosis / edited by J.P. Dalton.
p. cm.
Includes bibliographical references and index.
ISBN 0–85199–260–9 (alk. paper)
1. Fascioliasis. I. Dalton, John P.
RC848.F29F37 1998
616.9'63––dc21 98–38015
CIP

ISBN 0 85199 260 9

Typeset in 10/12pt Garamond by Columns Design Ltd, Reading
Printed and bound in the UK at the University Press, Cambridge

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Preface

It is clear that a volume devoted to the causative agents of liver fluke disease, *Fasciola hepatica* and *F. gigantica*, has been wanting for some time. Many advances have been made since the publication of Pantelouris's *The Common Liver Fluke* nearly thirty-five years ago, and it was my goal to fill the hiatus by assembling a book that would comprehensively cover these advances. I am particularly delighted that those scientists that have been responsible for keeping liver fluke research at the forefront of parasitology have contributed chapters in their particular expertise to the book. I have arranged these various chapters in a manner that makes the book read like a single-author volume, rather than a multi-authored one.

While the book's main emphasis is on the liver fluke of temperate climates, *Fasciola hepatica*, many chapters also consider the tropical liver fluke, *F. gigantica*; additionally, an extended chapter has been dedicated to this latter parasite. The book begins with classical chapters on the passage of the liver flukes through their definitive mammalian and intermediate snail hosts, with special emphasis on the fascinating mechanisms by which the parasites rupture from their cysts or hatch from their eggs, how they seek out and penetrate their hosts, and develop and adapt as they move from one environment to another. Topics on the control and epidemiology of the disease, and the novel use of Geographical Information Systems (GIS) to estimate the prevalence of disease in different regions are then treated alongside each other. The chemicals used to control fasciolosis in the past and at present are discussed in the context of their structure and mechanism of action, and the problem of drug resistance is addressed. In addition, the pathology, clinical aspects and pathophysiology of liver fluke infections and the diagnostic methods used to detect disease are covered. More specialized chapters review the metabolism of the parasites, their neurobiology and aspects of their molecular biology. Because fasciolosis is an emerging zoonosis a chapter on human fasciolosis has been included to highlight this

problem. The recent successes towards the understanding of the immunology of liver fluke infections and in the development of novel molecular vaccines are also given detailed attention.

This book on fasciolosis should be of value to undergraduates and graduates of veterinary and medical science, to research students and their teachers/supervisors. I hope that readers will agree that while we have gained a tremendous amount of knowledge on these fascinating parasites, there are many gaps needing to be filled. Above all, I hope that this book will go some way in attracting new researchers into working on liver flukes. There is certainly plenty of food for thought in this volume.

John P. Dalton

Acknowledgements

I would like to express my sincere gratitude to all my scientific colleagues for contributing excellent chapters to this book. Many thanks are also due to Dr Diane Clery, Dores Maguire, Professor P.J. Quinn, Sandra O'Neill and Ian Buckley for providing some of the graphics and slides. I also want to thank all those at CABI *Publishing* (particularly Amanda Horsfall, Rebecca Stubbs, Elaine Boyes and Tim Hardwick) who encouraged me greatly along the way and who have done a superb job in putting this book together.

We are grateful for the contribution of Schering-Plough towards the printing of the colour plate section.

John P. Dalton

1 The Life Cycle of *Fasciola hepatica*

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Introduction

Fasciolosis is an economically important disease of domestic livestock, in particular cattle and sheep, and occasionally man. The disease is caused by digenean trematodes of the genus *Fasciola*, commonly referred to as liver flukes. The two species most commonly implicated as the aetiological agents of fasciolosis are *F. hepatica* and *F. gigantica* (family Fasciolidae). *F. hepatica* has a worldwide distribution but predominates in temperate zones while *F. gigantica* is found on most continents, primarily in tropical regions.

The class Trematoda belongs to the phylum Platyhelminthes, and can be divided into two subclasses, the Digenea and Aspidogastrea. The Digenea are characterized by a complex life cycle in which one or more intermediate hosts are involved. Many variations on the life cycle exist, but each typically includes a molluscan primary or intermediate host in which larval multiplication occurs, and a vertebrate final or definitive host in which sexual reproduction occurs. Members of the family Fasciolidae are hermaphroditic and self-fertilization can occur, although sexual reproduction is normally by cross-fertilization in the final host.

The story of the discovery of the life cycle of *F. hepatica* has been recounted before; therefore only a short account will be given here. A list of the principal discoveries is shown in Table 1.1. Integral to the successful completion of the life cycle, the biological factors involved will be discussed in the second part of the chapter. Additional details on many of the aspects of the life cycle will be covered in more depth in subsequent chapters.

The Discovery of the Life Cycle

It would appear that the first trematode or fluke ever to be recognized was *F. hepatic*a. The earliest description of fasciolosis in the literature is contained in a book entitled *Black Book of Chirk*, published *circa* 1200 (Froyd, 1969), in

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Table 1.1. Milestones in the discovery of the life cycle of *Fasciola hepatica.*

Person/year	Milestone
De Brie, 1379	First to observe the liver fluke *F. hepatica*
Redi, 1688	Disproved theory of spontaneous generation
Swammerdam, 1737	First to see cercariae dissected from a snail (see Swammerdam, 1758)
Müller, 1773	Observed cercariae swimming in water
Zeder, 1803	Described miracidium hatching from fluke egg
Nitzsch, 1807	Observed cercariae encysting
Bojanus, 1818	Described redia and development of cercariae
Steenstrup, 1842	Published theory of alternation of generations (see Steenstrup, 1845)
La Valette St George, 1855	Observed infection of a snail by a miracidium
Wagener, 1857	Observed penetration of snail by miracidium and subsequent development of redia
Weinland, 1875	Suggested that the larval stages of the liver fluke occur in *L. truncatula*
Leuckart, Thomas, 1882	Confirmed *L. truncatula* as intermediate host and worked out life cycle of *F. hepatica*
Lutz, 1892, 1893	Confirmed that herbivorous animals acquire adult fluke by ingestion of metacercariae
Sinitsin, 1914	Confirmed route of migration of *F. hepatica* to the liver

which reference is made to liver fluke in sheep. Froyd (1969) also suggests that a reference which may predate this may be in the Gwentian Code of Wales in the laws enacted by Howel the Good in the 10th century.

Jean de Brie (1379), while he was preparing a treatise on wool production and sheep management for Charles V of France, also made mention of the disease 'liver rot' in sheep, but did not actually describe the appearance of the worm in this treatise (Huber, 1890). Unfortunately the original account of his work has been lost and his observations are only known from various editions published between 1542 and 1594. Like many other of the early writers, de Brie did not associate the liver fluke with the disease 'liver rot', but thought that this was a consequence of the liver being affected by toxic substances produced by certain plants eaten by the sheep.

According to Cole (1944) a further recognizable description of liver fluke appears in a book entitled *A newe Tracte or Treatyse moost profytable for all Husbandemen* which was published in 1523 by Sir Anthony Fitzherbert. Shortly afterwards, in 1549, reference is made in a book entitled *De lumbricis alvum occupantibus* to an observation made by an Italian physician by the name of Fanensi Gabucinus who described worms resembling pumpkin seeds in the blood vessels of the liver of sheep and goats. Additional observations on liver fluke disease were recorded during the second half of the 16th century, namely those of Conrad Gesner (1551) and Cornel Gemma (1575). It was still commonly thought at the time that the feeding of particular plants to sheep was responsible for the disease. It was not until 1688 that this theory was challenged by Francesco Redi (after whom the redia stage in the

digenetic life cycle was named), a physician in Italy, who, by showing that parasites lay eggs, destroyed the false doctrine of spontaneous generation (the hypothetical process by which living organisms arise from inanimate matter). It is uncertain in which parasite this observation was first made (Redi described a number of different types of worms from many kinds of animal) but he was to be the first to publish a picture of the liver fluke – a sketch from a specimen removed from the liver of a castrated ram.

The rejection of the theory of spontaneous generation was a significant breakthrough and was to stimulate a new wave of research. Prominent among these researchers was Govert Bidloo, professor of anatomy at The Hague and physician to William III. Bidloo (1698) observed worms in the bile ducts of sheep, stags and calves and recalled having seen similar worms in the livers of humans. (Apparently, John Faber, 1670, was the first to state that the liver fluke lives in the bile ducts, not in the blood vessels (Reinhard, 1957).) He also observed eggs inside the living worm and thought that sheep probably became infected by swallowing the worms or their eggs and that the worms got to the liver in the blood rather than by passing via the small intestine. His results were reported in the form of a memoir to Antony van Leeuwenhoek, which stimulated Leeuwenhoek to investigate further. Leeuwenhoek thought that the worms lived in water and that sheep became ill by drinking this water, but he was not able to explain why he was unable to find such creatures in water samples taken from ditches in fields near the city of Delft. His observations were recorded in letters sent to the Royal Society which were subsequently published in the Philosophical Transactions (Leeuwenhoek, 1700, 1704).

It was not until the end of the 18th century that reference was made in the scientific literature to the intermediate stages of the life cycle. It was a chance observation by a Dutchman named Johann Swammerdam (1758) who, while dissecting a snail (*Paludina vivipara*) in order to examine its internal structure, saw living things which he thought were not of snail origin. Examination of his illustrations of these 'worms' clearly shows them to be the cercariae of some trematode. Later, in 1755, Frank Nicholls, a physician and prominent anatomist in England, presented a report to the Royal Society in which he remarked that the bile ducts in the livers of bullocks infected with 'liver rot' were blocked by 'a wall of stone' around the flukes – this was the first reference to calcification of the bile ducts and thus the earliest insight into the pathology of the disease.

The next contribution towards an understanding of the life cycle was made almost twenty years later by Otto Müller. In 1773, he wrote of finding microscopic tadpole-like creatures swimming in the water of ponds and called them cercariae, a generic name which he gave to all of these creatures characterized by having a tail. He mistakenly thought these cercariae were Infusoria (a term applied to microscopic organisms, including various Protozoa and Rotifera, found in infusions of organic substances), as did many others who subsequently followed in his footsteps. Several different kinds of cercariae were observed and described, including those depicted by Johann Eichhorn (1781) and by Johann Hermann (1783). However, at this time the

life cycle of flukes was still a mystery and no one even suspected that more than one animal host was required for its completion. The first person apparently to have an inkling that this might be necessary was Peter Abildgaard of Copenhagen (1790). Ahead of his time, the results of his experiments (which provided evidence that the development of the tapeworm *Diphyllobothrium* sp., from larval to adult stage, required the larval stage to pass from a fish host to a bird) were soon forgotten and the idea that an immature form of a parasitic worm can require a different host from that of the adult had to wait another 60 years (Küchenmeister, 1852) before being accepted.

In 1803, Johann Zeder reported observing the hatching of eggs from a number of different species of trematode, although not of *F. hepatica*, and the escape of a ciliated embryo (miracidium) into the water. After Zeder's observation, Christian Nitzsch (1807) followed with the first account of cercariae encysting. He had been watching some cercariae swimming in water and had noticed that after a while they attached themselves to a substrate, then lost their tails prior to becoming covered by a gelatinous substance. Having lost all means of movement he assumed that he had witnessed the cercariae dying. Nitzsch continued working with cercariae and nine years later he had described several new cercariae (in Dawes, 1968). In doing so, he noticed the similarity between the anterior end of a cercaria and a distome fluke (Nitzsch, 1817) although he still thought that cercariae were different from flukes and from all other known forms of pond life.

During this period, despite such advances, the only thing that was definitely known about the life history of the liver fluke, or any other fluke, was that eggs were laid from which a ciliated larval form emerged. Although many observations were recorded on other stages of the cycle, such as cercariae and rediae, a connection between them remained elusive. The next observation of relevance was made by Ludwig Bojanus who, in 1818, unaware of Swammerdam's work published in 1737 (see Swammerdam, 1758), rediscovered the redial stage of a trematode. Unlike Swammerdam, who failed to grasp the significance of his finding, Bojanus noted the resemblance between rediae, cercariae and adult flukes. By observing the birth of cercariae from the rediae, Bojanus put forward the idea that there may well be a connection between cercariae and flukes. However, like Swammerdam, Bojanus did not work on the snail *Lymnaea truncatula* and therefore did not observe the larval stages of *F. hepatica*. Despite these publications the consensus at this time was still that cercariae were independent forms of life.

E. Mehlis, a German medical practitioner, made the next significant contribution in 1831 by describing the hatching of 'ciliated embryos' from the eggs of trematodes (it was Friedrich Creplin (1837) however, six years later, who observed such forms (miracidia) hatching from the eggs of *F. hepatica*). Not only did Mehlis observe the hatching of miracidia but he also noted their energetic swimming in water and suggested that this behaviour might be associated with the need to find something that would enable them to develop to a stage that could eventually infect the final host. Such a theory went against the more popularly accepted theory that the final host became

infected by the ingestion of the egg stage. However, in 1852 the latter theory was finally disproved by Professor James Simonds of the Royal Veterinary College, London. Simonds (1880) reported that he failed to find a single fluke or any signs of liver disease in an experimental sheep infected six months earlier with thousands of fluke eggs.

By the middle of the 19th century most of the individual parts of the life history of many species of trematode had been noted. Johannes Steenstrup was the first person to start to bring together the various pieces of the story. In 1842 he published his work *On the Alternation of Generations* in Danish; the same year saw publication of the German edition, which was translated into English (Steenstrup, 1845). He fitted the theory to various forms of life, including trematodes although only brief mention is made of *F. hepatica*; most of his work on trematodes describes echinostome and stylet cercariae. However, the term 'alternation of generations' had previously been used to describe the life cycle of tunicates (benthic invertebrates) in 1819 by the Franco-German poet and naturalist, Louis Charles Adelaide de Chamisso (in Dawes, 1960). Carl von Siebold (1854) provided more evidence to support the theory. He found rudimentary sex organs in some encysted cercariae and suggested that it was likely that this stage, which occurred in invertebrates, was the infective stage for vertebrates, in which the sexually mature fluke occurred. The first suggestion of a definite connection between a specific cercaria and a specific adult fluke was probably made by Rudolph Leuckart in 1852 (in Taylor, 1937). He observed the similarity between a certain fluke found in the intestine of a predacious fish and of encapsulated cercariae found in the gills of its prey. Adolphus von La Valette St George (1855) demonstrated, by feeding-experiments, that certain encysted cercariae from water snails developed into sexually mature flukes in birds, and that cercariae which had not encysted were not infectious.

Further pieces of the jigsaw were put in place by Guido Wagener (1857) who observed the penetration of miracidia into snails and the subsequent development of rediae, and by the German helminthologist David Weinland (1875), who according to Reinhard (1957) was the first person to suspect that larval stages of liver fluke occurred in *L. truncatula*. In 1875, Weinland found 'cercaria sacs' in the digestive gland of this snail and also noted that cercariae showed a strong inclination to leave water and climb on to foreign objects. He conjectured that cercariae encysted on grass in order to be eaten by sheep and that these cercariae were in fact young liver flukes. Twenty years later *L. truncatula* was confirmed as the usual intermediate host of *F. hepatica* (other species of snail may also be infected, see for example, Boray, 1969). The discovery was made independently by Algeron Thomas (1881, 1882a, b, 1883a, b) in the United Kingdom and by Leuckart (1881, 1882) in Germany. It was Thomas who established the right to be acknowledged as the first person to make the discovery, details of which were first published in *Nature* (Thomas, 1882b). The work of Thomas and Leuckart is summarized by Reinhard (1957).

Despite the work of Thomas and Leuckart, certain parts of the life history were still uncertain and required experimental proof. For example, proof was

still required that herbivores acquired the parasite by swallowing metacercariae. The discovery of the exact migration route by which young flukes reached the liver of the final host was also still to be elucidated. Experimental data confirming the first issue were generated by Adolpho Lutz (1892, 1893) who successfully infected guinea pigs, a rabbit, a goat and a brown rat by adding metacercariae to their food. However, according to Joseph Alicata (1938) the species of liver fluke with which Lutz was working was *F. gigantica*, not *F. hepatica*. The final piece of the jigsaw was added by Dimitry Sinitsin in 1914. Sinitsin, a Russian helminthologist, proved that young flukes in the rabbit, after liberation from their cysts in the small intestine, penetrated the wall of the gut and migrated to the liver via the peritoneal cavity. This observation was supported and further investigated by Shirai, 1927; Susuki, 1931; Shaw, 1932; Schumacher, 1939; and Krull and Jackson, 1943.

Outline of the Life Cycle

The life cycle of *F. hepatica* consists of five phases as shown in Fig. 1.1. They are: (i) passage of eggs from the host to the outside environment and their subsequent development; (ii) hatching of miracidia, their search for and penetration of the intermediate snail host, usually *Lymnaea truncatula*; (iii) development and multiplication of the parasites inside the snail; (iv) emergence of the cercariae from the snails and their encystment; (v) ingestion of infective metacercariae by the final hosts and development to adult worms.

Each of these five phases will be briefly reviewed together with the various favourable and unfavourable factors which influence the successful completion of each stage. It should be noted, however, that variations in the typical life cycle can occur; for example, in certain final hosts prenatal infection occurs while in others a zoonotic potential of infection exists by ingestion of raw liver containing immature flukes. These aspects of the life cycle will be covered in more detail in subsequent chapters. It is worth emphasizing that the presence of sufficient moisture and a suitable temperature are the two most important factors which influence the successful completion of the cycle.

Development and survival of the fluke egg

Liver fluke eggs are passed from the common bile duct into the duodenum and subsequently into the faeces. The eggs consist of a fertilized ovum surrounded by a large number of yolk granules. They are yellowish brown in colour, oval in shape, 130–145 µm long by 70–90 µm wide and have an indistinct operculum (Fig. 1.2 A–C, Plate 1). The eggs which are passed out in the faeces on to pasture are undeveloped and undergo embryonation outside the host. Several physico-chemical factors, especially temperature, humidity and oxygen tension, are known to influence embryonation.

Liberation from faeces

Although partial development of the egg can occur while still inside moist or wet faeces, complete development and hatching will only occur after the egg

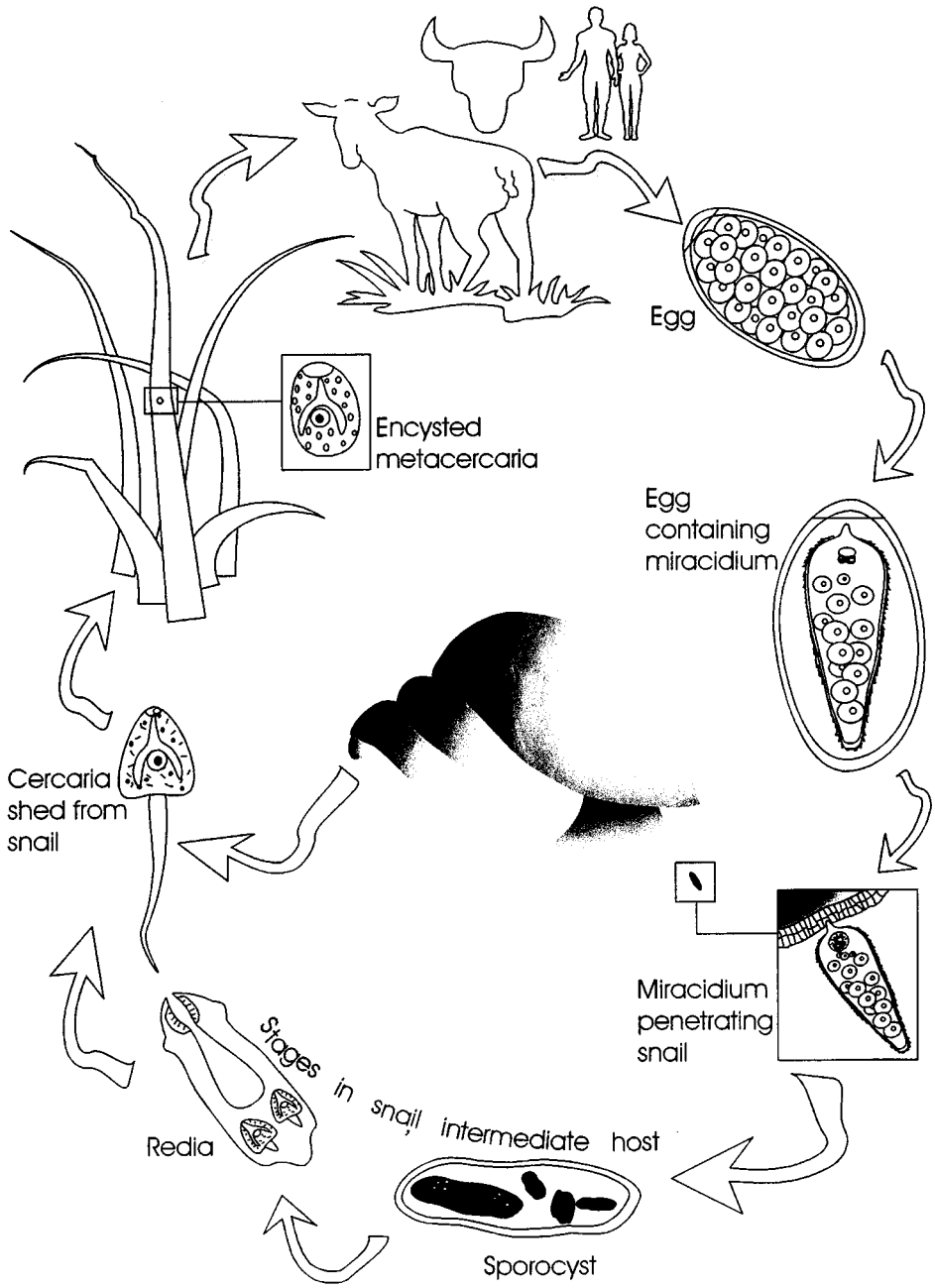

Fig. 1.1. The life cycle of *Fasciola hepatica*. (Drawn by Mr Ian Buckley.)

has been liberated from the faeces, a requirement that is normally facilitated by factors such as the action of heavy rain, the deposition of faeces in water, and the trampling action of animals. The inhibitory effect of faeces is likely to

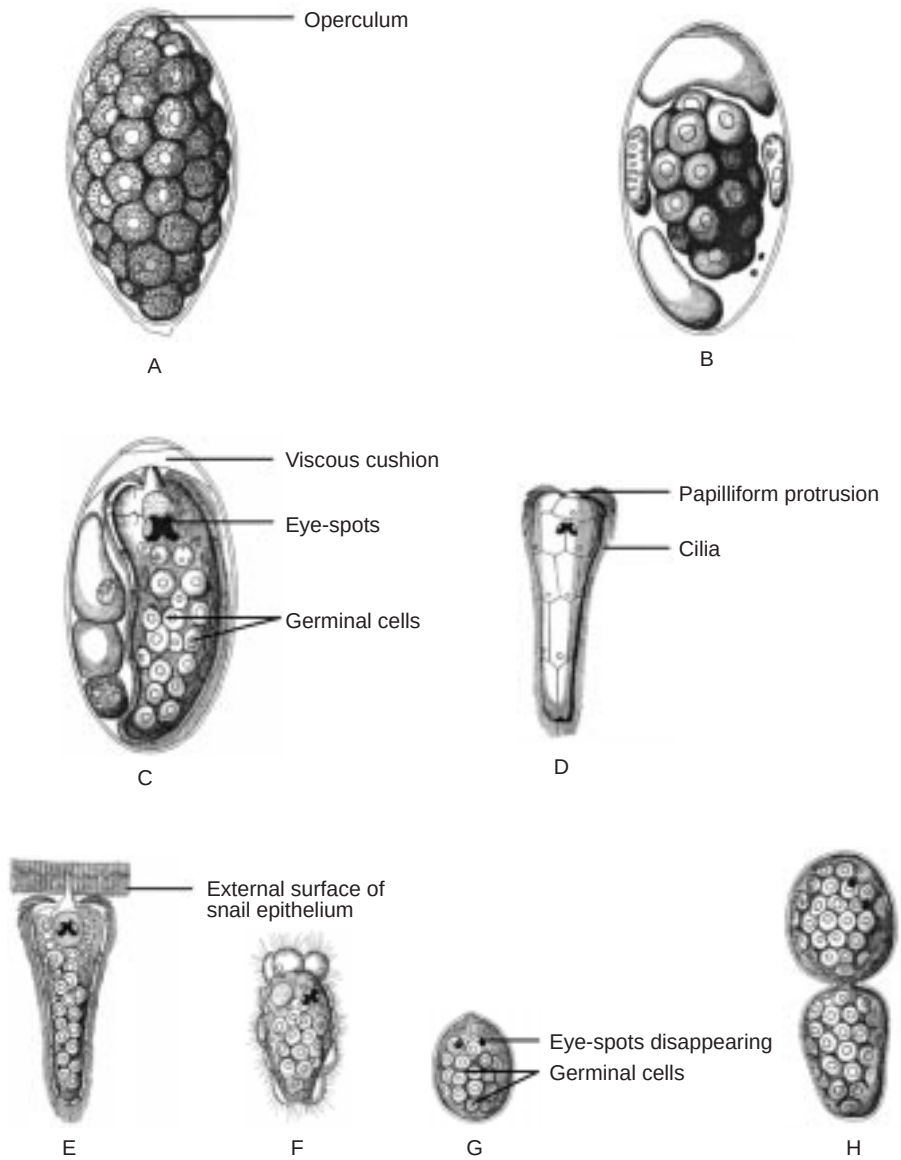

Fig. 1.2 (*and opposite*). Stages in the life cycle of *Fasciola hepatica*: A, undeveloped egg – note operculum (cap) and difference between embryonic cells (small mass in upper central part of egg) and yolk-bearing cells; B, morula – embryonic cells have grown at the expense of the yolk cells; C, fully developed egg ready to hatch – miracidium – note cilia and eyespots; D, a free-swimming miracidium, external view; E, a miracidium penetrating a snail, internal view; F, after penetrating the snail, the miracidium loses its cilia and becomes a sporocyst (G); G, sporocyst; H, the sporocyst dividing; I, the sporocyst forming redia (form with sucker and primitive gut); J, a more mature redia – note other embryonic morulae in the body cavity. The two lateral projections are characteristic of this stage; K, a fully mature redia showing developing redia and cercariae (the forms with the tail); L, the cercaria; the free-swimming form which encysts on vegetation. (Adapted, with permission, from the description of the life cycle of *F. hepatica* published in Whitlock, J.H. (1960) *Diagnosis of Veterinary Parasitisms*. Henry Kimpton, London, 236 pp. The original definitive description was published by A.P. Thomas in *The Journal of Microscopic Science* (1883) 23, 87–99.)

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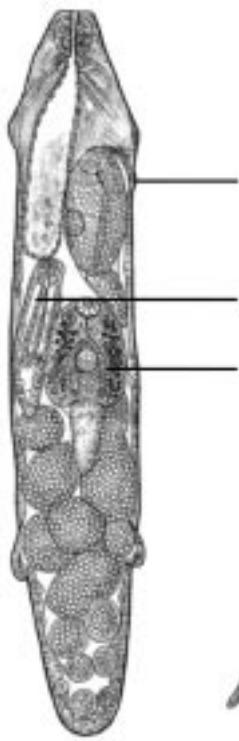
J



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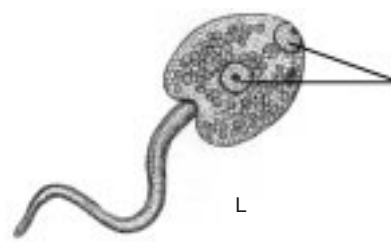

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Fig. 1.2 (*continued*).

be caused by a number of factors, e.g. competition for oxygen by micro-organisms or the presence of toxic substances. Eggs, however, can remain viable in faeces from 3 weeks to several months, according to various conditions and the time of the year. Egg viability persists for a longer period during the winter than in the summer months and tends to decrease with increasing dryness (Rowcliffe and Ollerenshaw, 1960).

Temperature

Thomas (1883a, b) observed that development of the egg was influenced by temperature and moisture. Although he made no specific measurements to determine the relationship, he did note that eggs recovered from freshly passed faeces were undeveloped and that temperatures of about 23 to 26°C were most favourable for embryonation. At these temperatures eggs became fully developed within 2 to 3 weeks. Development was prolonged to 2 to 3 months at the lower average temperature of 16°C.

A temperature of at least 10°C is necessary for embryonation (Ross and McKay, 1929). Under laboratory conditions, the rate of development of the egg increases with temperature within the range 10 to 30°C. Thus, at 10°C development of the egg takes about 6 months but at 30°C it is completed in 8 days. Above 30°C, development is increasingly inhibited and at 37°C does not occur at all. Mortality increases the longer the eggs remain at 37°C, with 100% mortality being reached after about 24 days (Rowcliffe and Ollerenshaw, 1960). At temperatures below 5°C, development of the egg stops but can be resumed if the temperature is increased to 13°C. Eggs refrigerated (2 to 10°C) for 2.5 years remain viable, although undeveloped, and after being kept at room temperature for 18 days following refrigeration, hatch over a period of 14 days (Krull, 1934). The failure of eggs to develop at a similar temperature for a shorter period of time, 6 to 11°C for 5 months, has also been noted (Ross and McKay, 1929). Similar findings have also been recorded by Boray (1969) who kept eggs of *F. hepatica* in the refrigerator at 4°C for at least 2 years. After incubation these eggs hatched and the miracidia were infective to snails (*Lymnaea tomentosa*).

Eggs kept at −5°C are killed within about 17 days but may survive for some time under 30–40 cm of snow (Vasileva, 1960); while eggs kept at below freezing point (−15°C) survive for only 24 h (Boray, 1969).

Moisture

The results of very few controlled experiments on the effects of moisture on fluke eggs have been published. It is known that eggs desiccate readily and that water is required for embryonation. The effect of moisture is likely to become critical when conditions dictate slow development of the egg and thus long exposure to other factors in the environment which may not be favourable. Under such conditions, the maintenance of a surface film of moisture around the egg for at least 3 weeks is essential. Eggs on soil, however, will develop without the presence of free surface water, provided that the soil is saturated (Ollerenshaw, 1959). Eggs in moist faeces can survive for at least 10 weeks in the summer and 6 months in the winter in the

UK. However, if the faecal mass dries out, there is rapid mortality of the eggs (Ollerenshaw, 1971). In Spain, mortality can be reduced during the winter if eggs overwinter in wet faeces, with 55% surviving (Luzón-Peña *et al.*, 1992).

Oxygen tension

No development of the egg will take place if present in a concentrated faecal suspension, although eggs will survive for more than twice as long in aerobic conditions as in anaerobic conditions. Eggs kept in cultures without faeces show little variation in mortality, but those in aerobic conditions hatch in one-fifth of the time taken by those at a lower oxygen tension (Rowcliffe and Ollerenshaw, 1960). Boray (1969) observed that during the summer months in Australia the development of eggs is delayed and that the hatching rate is lower in stagnant pools with a lot of organic matter compared with habitats with moderate water movement.

pH

Eggs incubated at 27°C will develop and hatch within a pH range of 4.2 to 9.0, but above pH 8.0 development is prolonged (Rowcliffe and Ollerenshaw, 1960). The optimum pH for embryonation appears to be 7.0 (Al-Habbib, 1974).

Hatching of the miracidium from the egg and its penetration of the intermediate snail host

Hatching of the egg

It is clearly of importance to the survival of the fluke that its eggs should be stimulated to hatch by environmental conditions outside its vertebrate host, thus providing the miracidia with a reasonable opportunity for finding and infecting the molluscan intermediate host. At the same time, it is also advantageous that the hatching process should be inhibited by conditions occurring within the definitive host, thus avoiding premature hatching. However, as noted by Thomas (1883a, b), fully embryonated eggs exposed to the same conditions do not always hatch together, a certain number hatching on successive days for some weeks, even months. Such a strategy is obviously of practical importance rendering a suitable habitat dangerous for grazing animals for a long period of time.

It is generally accepted that light and temperature affect the hatching of eggs of *F. hepatica*. It is common laboratory practice to induce mass hatching of miracidia by placing incubated eggs in strong light, although the necessary stimulus can be provided by suddenly placing the eggs in a comparatively large quantity of cold tap-water (Jepps, 1933). Experiments carried out by Roberts (1950) showed that eggs of *F. hepatica* incubated for 14 days in the dark hatched only on exposure to light. Chilling or vigorous stirring had no effect. Roberts (1950) also investigated whether a particular wavelength of light was essential for hatching when eggs had been incubated at 25°C for 14 days. She concluded that violet and blue wavelengths of the spectrum appear to be an essential part of the light stimulus. No hatching occurred when violet

and blue light was excluded by the use of filters, but when these filters were removed, hatching occurred in 1–3 min. Al-Habbib (1974) found that short wavelengths (yellow, green and violet) had a strong stimulatory effect, whereas Geller and Bausov (1977) reported that red light had a poor effect in activating hatching. Gold and Goldberg (1976), however, found no marked differences in hatching at various wavelengths or at different light intensities. Mitterer (1975) demonstrated a discontinuous hatching of eggs during exposure to alternate periods of light and dark in an atmosphere of carbon dioxide. Gold and Goldberg (1976) also concluded that temperature changes, especially lowering of the temperature, were important stimuli for hatching.

Although the hatching process has been fairly extensively studied (Erasmus, 1972) the precise mechanism(s) by which light and temperature facilitate hatching is still uncertain. Four different theories have been proposed to date:

1. Thomas (1883a, b) suggested that increased movement and stretching of the embryo was responsible for rupture of the operculum and that the cilia started to move as soon as contact with water was made, resulting in the miracidium freeing itself from the egg.
2. Mattes (1926) considered that an increase in internal osmotic pressure was responsible for the rupture of the opercular bond.
3. Rowan (1956, 1957) suggested that light stimulates the miracidium to release a proteolytic 'hatching' enzyme that digests a substance binding the operculum to the shell; a change in this bonding substance permits the egg to open. Just before hatching, within a few seconds, the viscous cushion situated at the opercular end of the egg swells to about twice its size. As a result the vitelline membrane ruptures, the cushion material flows out of the shell and usually dissipates. The miracidium promptly follows the cushion material through the narrow opening.
4. Wilson (1968a) rejected Rowan's hatching theory and his experiments. He concluded that it is the miracidium that is stimulated into activity by light and that this leads to an alteration in the permeability of the membrane of the viscous cushion. Prior to expansion, the cushion (a fibrillar mucoprotein complex) is in a dehydrated or semi-hydrated state. Hydration of this material following a change in permeability of the enclosing membrane results in the swelling of the cushion and the internal pressure forces the operculum open, causing expulsion of the miracidium. Many workers favour the 'hatching enzyme' theory, albeit in different species of trematodes (see for example, Onorato and Stunkard, 1931; Durie, 1953).

Survival of miracidia

The miracidium is about 130 µm in length, broad anteriorly and tapering posteriorly to a blunt end. The cuticle is ciliated, and there is an anterior papilliform protrusion and a pair of darkly staining eye spots visible near the anterior end of the body (Fig. 1.2D and E). Once hatched from the egg the miracidium becomes active, immediately starting to swim at great speed (on average, 1 mm s^{-1}; Wilson and Denison, 1970). Characteristic swimming

movements have been used as a means of determining the infectivity of miracidia. Those swimming in circles are not usually infective. Speed of movement of the miracidia after hatching has also been found to be a good indicator of their infectivity; those taking about 30 s to move 1 cm do not infect snails, while those requiring 4–12 s to travel 1 cm are generally infective (Boray, 1969).

The need to find a suitable host to penetrate is an urgent one, for those miracidia failing to do so generally die within 24 h (Thomas, 1883a,b; Hope Cawdery *et al*., 1978), at a rate which is age dependent (Smith and Grenfell, 1984). Such age-dependent mortality is a common feature of short-lived, non-feeding larval stages in the Digenea and is associated with the depletion of finite energy reserves (Anderson *et al*., 1982). It has been shown, using histochemical techniques, that glycogen levels in the miracidia of *F. hepatica* decline with age (Wagner, 1965). Why the duration of this searching phase should be so short is rather puzzling. It has been suggested that since snails tend to have a highly aggregated distribution, and highly discontinuous ('patchy') habitats, strategies involving repeated short searches in many different locations are more effective than larger searches repeated in just a few locations (Smith and Grenfell, 1984). Although miracidia are capable of covering long distances during their short lifetime (over 50 m at temperatures between 10 and 15°C), in practice, they are often confined to small bodies of water just a few centimetres across, most of which contain no snails at all (Smith, 1978).

There appears to be no evidence that miracidial mortality varies with pH of the medium, at least in the range 6–8 (Smith and Grenfell, 1984). On the other hand, these authors showed that mortality does vary both with the temperature of the medium and the age of the miracidia. The mean expected life span of miracidia decreases from about 36 h at 6°C to about 6 h at 25°C. At 10°C the mean life span is about 1 day (Al-Habbib, 1974). However, miracidia can be kept alive for 3 days in a slightly alkaline solution of peptone (Thomas, 1883b).

Location and penetration of the snail host

The behaviour patterns of miracidia have been extensively studied especially with regard to their phototactic, thermotactic, geotactic and chemotactic responses, but many of the experimental results remain equivocal. Three main areas have received special attention: (i) the extent to which a miracidium is 'attracted' towards a snail; (ii) whether such responses (if they occur) are specific for a particular species of snail; and (iii) whether the general responses of a miracidium are merely responsible for bringing a miracidium into an ecological niche similar to that of the snail so that contact is readily established (Smyth and Halton, 1983; Haas and Haberl, 1997).

It is now widely accepted that the sequence by which a miracidium finds its snail host can be divided into three main steps (Wright, 1959; Ulmer, 1971; Saladin, 1979; Christensen, 1980): (i) host habitat selection – the newly emerged miracidium makes its way to the environment of its specific snail host; (ii) a period of random movement in the host habitat; (iii) host finding – the miracidium orientates towards the host using tactic or kinetic mechanisms. The

miracidium of *F. hepatica* is strongly phototropic, which is generally believed to be an adaptation for locating the snail host. The intermediate snail hosts are amphibious. In Europe and some parts of Asia, the intermediate snail host is usually *Lymnaea truncatula* and elsewhere several other species of *Lymnaea* are implicated. Their habitat is typically along the edge of small ponds, ditches and marshy land in areas which are subject to alternate flooding and desiccation. The ability of the miracidium to move towards the light ensures that it will not waste vital energy reserves by exploring the bottom of ponds where *L. truncatula* is not to be found. An interesting comparison can be made with the miracidium of *F. gigantica* which infects different species of snail. Studies on the infection of snails show this species of *Fasciola* to be less host-specific than *F. hepatica* (Kendall, 1954; Boray, 1966). In Africa, where *F. gigantica* primarily infects *L. natalensis* (a species which lives in deeper water), the miracidium is actively repelled by light and therefore dives further down into the water, where the snail is more likely to be found (Taylor, 1964).

Other forms of tropism have been investigated, such as the perpendicular distribution of miracidia in water, and rheotaxis (see for example, Neuhaus, 1941; Yasuraoka, 1953), but most work has been concentrated on chemotaxis.

Thomas (1883a, b) tried to infect several different kinds of freshwater snails and noted that the miracidia appeared to choose instinctively which host to enter. This work was repeated by Kendall (1950). Besides *L. truncatula*, the only other species of snail into which Thomas observed miracidia entering were young specimens of *L. peregra*, although little development occurred inside this species. He hypothesized that some difference in the nature of the secretion of the surface of the body in these two species of snail served as a guide for the miracidia. Mattes (1936) maintained that, though miracidia became attached to any soft body with which they came into contact, specific attachment and subsequent penetration in *L. truncatula* might be due to the texture of the epidermis or to the nature of what he called the 'histolytic ferment'. A positive chemotactic reaction by the miracidia to *L. truncatula* occurs up to a distance of 15 cm (Neuhaus, 1953). Beyond this distance contact between the miracidia and the snail depends on chance. Therefore, it is to be expected that the greater the density of snails, the greater the chance of a miracidium successfully locating an individual snail.

The presence of a wide range of potential stimulant molecules in the mucus of snails is well documented (see for example, Wilson, 1968b; Wilson and Denison, 1970; Wilson *et al.*, 1971). Concentrations of glucose, 16 amino acids and various lipids have been identified from the mucus of *L. truncatula* (Wilson, 1968b). However, the stimulant(s) involved have yet to be fully characterized. Short-chain fatty acids C_7–C_9 (>0.1 mM) will stimulate miracidia of *F. hepatica* to attach to *L. truncatula* but such treatment appears to have a detrimental effect on the miracidia after about 10 min; chain lengths of between C_6 and C_9 (>0.01 mM) stimulate a turning response by the miracidium (Wilson *et al.*, 1971). It therefore appears that a single compound, having features in common with short-chain fatty acids, is capable of stimulating two different behavioural responses, depending on its concentration. Experiments by Nansen *et al.* (1976) and Christensen *et al.* (1976b) confirmed the existence of a special

chemical attraction of miracidia of *F. hepatica* not only towards *L. truncatula*, but also towards other species of *Lymnaea* in which the parasitic development is usually interrupted, such as *L. peregra*, *L. palustris* and *L. stagnalis*. It was also shown that miracidia are not able to penetrate intact egg clusters of *L. truncatula*.

Although light is known to stimulate the hatching process, and the miracidium is positively phototropic, light does not appear to be a stimulus for the infectivity of the miracidium (Christensen, 1975). Infectivity of the miracidium is independent of pH (in the range 5.4–8.4), but dependent on water temperature. At or below 5°C, the miracidium is not infective, regardless of the exposure time. Optimum temperature is in the range of 15–26°C and a clear inverse relationship has been demonstrated between the environmental temperature and the duration of the host-finding capacity of the miracidia. At 8, 16 and 24°C the host-finding capacity ceased after 24–30, 20–24, and 13–20 h, respectively (Christensen *et al.*, 1976a).

Penetration of the miracidium of *F. hepatica* into *L. truncatula*, and its transformation into a sporocyst, has been described by a number of workers including, at the light microscope level (Thomas, 1883a, b; Faust, 1920; Eales, 1930; Mattes, 1949; Roberts, 1950; Dawes, 1959, 1960; Southgate, 1970), and at the transmission and scanning electron microscope level (Wilson *et al.*, 1971; Blankespoor and van der Schalie, 1976; Køie *et al.*, 1976; Coil, 1977). The process is complex and an understanding requires an appreciation of the detailed structure of both the miracidium, in particular the body wall, and the body tissues of the snail, which are both beyond the scope of this chapter. However, the penetration process involves a mechanical boring action by the miracidial anterior papilla and is also likely to be facilitated by the secretion of proteolytic enzymes (Smyth and Halton, 1983). Tissue at the point of penetration, generally near the branchial aperture, is observed to be degraded (Wilson *et al.*, 1971).

Presence and infection of snails

Since the presence of the snail is essential to the completion of the life cycle, an appreciation of the ecology of the snail is required before considering the development phase of the fluke within the snail. The many factors involved in the ecology, particularly those influencing the presence or absence of the snail intermediate host, have been thoroughly reviewed elsewhere (see for example, Ollerenshaw, 1959; Taylor, 1964; Pantelouris, 1965; Boray, 1969), and summarized by Boray (1967b). The most important factors are the snails' ability to survive long periods in dry soil (aestivation) and low temperatures (hibernation), their extremely fast rate of reproduction and their active and passive migration.

Development and multiplication inside the snail

Although aspects of this part of the life cycle were described in the original work by Thomas (1883a) and have subsequently been expanded by numerous

other workers, it was not really until Taylor and Mozley (1948) described methods of maintaining the snail in cultures in the laboratory that it became possible to examine critically the host–parasite relationship. One of the most favourable factors contributing to the successful completion of the life cycle, and thus the propagation of the liver fluke, is parthenogenetic multiplication within the snail. It was first demonstrated by Krull (1941) that a snail infected with a single miracidium can produce about 4000 metacercariae. For details of some of the factors affecting the development of the fluke within the snail, see for example: Faust, 1920; Ross, 1930; Rees, 1931; Schumacher, 1939; Kendall, 1949, 1953; Kendall and McCullough, 1951; Kendall and Parfitt, 1959; Kendall and Ollerenshaw, 1963; Boray, 1963, 1966, 1967a, b; Hodasi, 1972; and Rondelaud and Barthe, 1987. A brief account of the process is given here, and is discussed in more detail by Graczyk and Fried in Chapter 2.

Once inside the snail, the young sporocyst (Fig. 1.2F) migrates via the blood vessels or lymph channels primarily to the digestive gland (often referred to as the liver), which is situated in the upper spirals of the shell. Here the sporocyst (Fig. 1.2G and H) begins to grow. Consisting initially of a minute ball of tightly packed germinal cells in which remnants of the eye-spots can be seen, each germinal cell gives rise to a ball of new germinal cells from which the next larval stages, the rediae, develop. Ultimately, the sporocyst, distended by rediae (Fig. 1.2I), ruptures liberating the rediae into the digestive gland. The rediae move about more actively than the parent sporocysts, and cause considerable damage to this gland.

The redia is roughly cylindrical in shape and possesses a pair of marginal lappets (bulging projections) at the posterior end and a raised collar-like structure just behind the anterior end of the body (Fig. 1.2J and K). There is a mouth which leads into a muscular pharynx and posteriorly to a simple unbranched intestine. The body of the redia contains numerous germinal cells which, like those in the sporocyst, multiply to form germinal balls from which the final larval stage, the cercaria, is produced. Between 16 and 20 of these germinal balls are produced within each redia. Under adverse conditions (not clearly understood) rediae may give rise to a second generation of rediae which then gives rise to the cercariae. It is worth noting that this unusual further multiplication phase was observed by Thomas (1883a, b) (see also Fig. 1.2K). The mature rediae measure 1–3 mm in length and are capable of considerable movement. Their migrations can cause serious damage and, in heavy infections, death of the snail. Normally, however, the snail shows a remarkable power of regeneration. When the cercariae are fully developed they escape from the redia by way of the birth pore which is situated laterally behind the anterior collar. Snails larger than 5 mm are more likely to shed cercariae than smaller snails (Olsen, 1944).

The cercariae are tadpole-like with a discoidal body and a long tail. The body measures 250–350 µm and the tail is twice as long. There is an oral sucker and a ventral sucker in the centre of the body (as in the adult fluke). Leading from the oral sucker there is a pharynx, on either side of which are very conspicuous cystogenous glands, an oesophagus and a forked intestine (Fig. 1.2L). The mobile cercariae generally leave the snail 4–7 weeks after

infection by migrating through the tissues. Different larval stages may, however, coexist in a single snail (Agersborg, 1924). It follows, therefore, that the cercariae do not mature at the same time and, as demonstrated by Faust and Hoffman (1934), leave the snail over a period of time.

Emergence of cercariae from snails and their encystment

For a short time after they have emerged from the snail the cercariae swim freely in the water. They are very active and frequently change direction, both horizontally and vertically, although tending to keep near to the surface rather than going down into deeper water. The process of encystment and the structure of the cyst wall is complex and has been described by Wright, 1927; Dixon and Mercer, 1964; Dixon, 1965; Stirewalt, 1963; Køie *et al*., 1977; and Smyth and Halton, 1983. Alicata (1938) described the structure of the cyst wall of *F. gigantica*.

Encystment

During a few minutes to 2 h after emergence, the cercaria settles on various objects, including blades of grass, and attaches by means of the ventral sucker. Encystment may also take place upon the surface of the water (Wright, 1927). Once attached, the body contracts inwards releasing the outer layer of the cyst which has been preformed. Simultaneously, as the embryonic 'epithelium' is shed and the outer layer is laid down, the tail separates from the body. The tail is sometimes shaken off before the encystation begins but, as a rule, the tail remains in connection with the body during the process (Thomas, 1883a, b). The cyst is white when laid, and is almost immediately infective to the definitive host. After a day or two the cyst gradually becomes yellow in colour due to the presence of quinine, and darkens as it hardens.

Structure of the metacercarial cyst

The structure of the cyst wall is complex. Essentially, it consists of an outer cyst and an inner cyst. The outer cyst is composed of an external layer of tanned protein and an underlying fibrous layer of mucoprotein. The inner cyst has a complex mucopolysaccharide layer subdivided into three, and an additional layer (layer IV) of laminated or keratinized protein. A region of layer IV is specialized to form the ventral plug. The outer cyst wall probably acts as a barrier against bacterial and fungal infections, and is also important for attachment to the substrate, normally grass (Dixon, 1965). Strong adhesion to grass for long periods is important for the survival of metacercariae and the infection of the final host. As the cysts may survive for long periods and remain infective if the outer wall is removed, the inner cyst walls must play a more important part in the survival of the metacercariae (Boray, 1963).

Longevity of metacercariae

The longevity of metacercariae has been investigated by several workers and though records indicate that metacercariae may survive for more than 1 year on pasture it is probable that a dangerous level of infection does not remain

viable for such a long period (Soulsby, 1965). Survival for long periods is mainly dependent on sufficient moisture and moderate temperatures. Although metacercariae are rapidly killed by very low temperatures, they can retain their infectivity after being maintained at −2°C for 8 weeks (Taylor, 1949), and may survive for as long as 11 months at temperatures varying from −3 to 2°C if the average temperature remains above freezing (Shaw, 1932). Approximately 50% of metacercariae encysting on herbage in September can survive winter conditions in the UK (Ollerenshaw, 1967). Metacercariae are resistant to freezing between −2 and −10°C but lose their infectivity at −20°C (Boray and Enigk, 1964). At higher temperatures, metacercariae produced by *L. tomentosa* can survive for 6 months at temperatures of 12 to 14°C, with 25% surviving for 8 months. When stored at between 2 and 5°C, only 10% will survive for 1 year; others will survive for 8 weeks at 20°C, but none will survive for more than 6 weeks at 25°C (Boray, 1963). Under natural conditions in the USA, it has been shown that metacercariae are destroyed by heat and drought during the four summer months (Olsen, 1947). Similarly, metacercariae will not survive a typical Australian summer (Boray and Enigk, 1964).

The survival of metacercariae on forage crops, particularly hay and silage, has been considered by various workers. For example, metacercariae can survive for 8 months on hay if harvested during rainy weather (Marek, 1927). Boray and Enigk (1964) have suggested that high relative humidity (90%) is required for survival in hay, which would have to be stored at a low temperature. Enigk and Hildebrandt (1964), however, found that metacercariae placed in hay at low temperatures can survive for 2–3 months at apparently lower relative humidity. Metacercariae of both *F. hepatica* and *F. gigantica* do not survive in silage (Alicata, 1938; Wikerhauser and Brglez, 1961), which may therefore be fed to animals during the winter without serious risk of infection.

The infectivity of metacercariae depends not only on various climatic conditions, as mentioned above, but also on the temperature during their development through the larval stages in the snail (Davtyan, 1956; Boray, 1963).

Ingestion of infective metacercariae

Within an hour of infection, metacercariae begin to excyst in the small intestine. Within two hours following infection these have bored through the wall of the intestine and can be found in the abdominal cavity *en route* to the liver. However, before such migration can start, the liver fluke has to free itself from its cyst.

Excystation

Extensive literature is available on metacercarial excystation, including Dixon, 1966; Erasmus, 1972; Lackie, 1975; Smyth and Halton, 1983; Sommerville and Rogers, 1987; and Sukhdeo and Mettrick, 1986, 1987. An account of the more recent literature (Fried, 1994) will be given here. The process is complex and

is likely to involve extrinsic factors (e.g. elevated temperatures, reducing conditions, pH, pCO_2 and the presence of bile salts) and intrinsic factors such as secretions by the fluke. Many aspects of the process have been studied *in vitro* and relatively few studies have been carried out on excystation *in vivo*. The mechanisms of how extrinsic factors work during excystation are still poorly understood and probably the most significant recent advances to be made have been concerned with intrinsic factors.

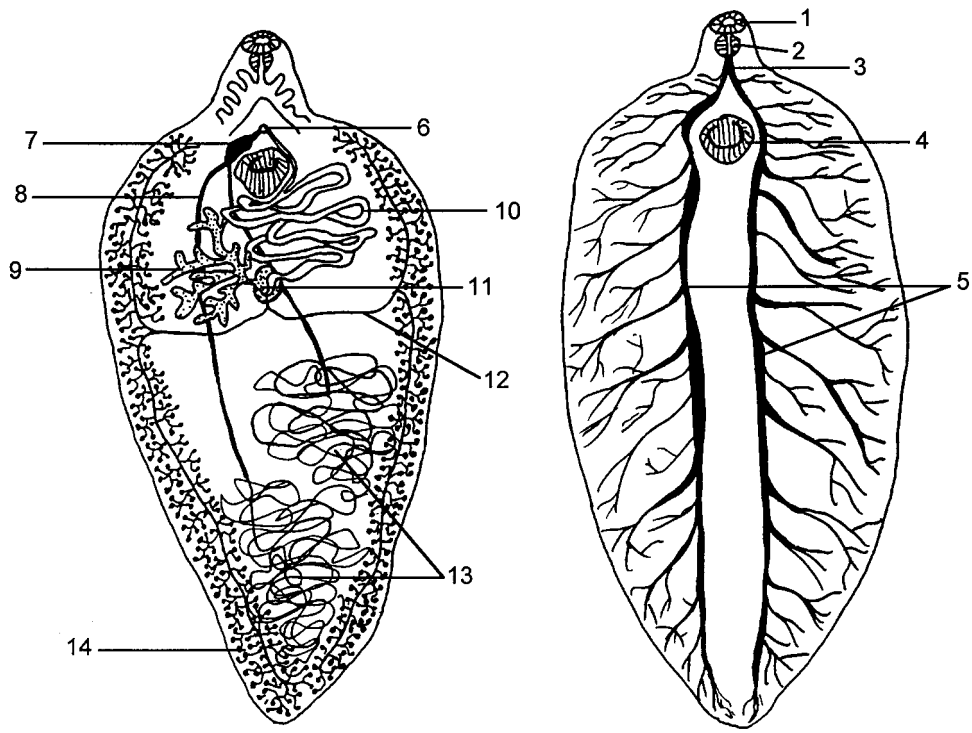
There are basically two phases of excystation: a passive activation phase followed by an active emergence phase. These have been described by Dixon (1966) and Smith and Clegg (1981) and reviewed by Sukhdeo and Mettrick (1987). Activation is believed to occur in the stomach or rumen and is a prerequisite to emergence. Conditions which stimulate activation *in vitro* are high pCO^2, temperature about 39°C and reducing conditions. During activation the metacercariae rotate vigorously for a while before the quiescent phase (Dixon, 1966), during which time the predominant activity appears to be the emptying of their caecal contents (Sukhdeo and Mettrick, 1986). It would appear that these caecal contents contain secretions which affect the inner cyst wall, thus aiding emergence.

Excystation occurs in the small intestine of the host below the opening of the *ductus coledochus*. However, Hughes (1959) and Dawes (1961) have shown that young flukes can emerge from cysts injected into the peritoneal cavity of hosts, suggesting that, apart from a temperature of about 39°C and possibly the presence of a low concentration of carbon dioxide, excystation may require very little additional stimulus. That host digestive enzymes are required would appear doubtful. The emergence phase is probably triggered by bile and its presence may activate an enzyme secreted by the parasite, inducing muscular movements of the young fluke (Dixon, 1966). Various hypotheses have been proposed for the mechanisms by which bile initiates such muscle activity (Dixon, 1966; Lackie, 1975). Sukhdeo and Mettrick (1986) examined the effect of bile salts on pre-activated metacercariae. They found that bile salts which did not possess an aromatic structure identical to cholic acid were ineffective in stimulating emergence. Glycine-conjugated bile salts were more effective at stimulating emergence behaviour than taurine-conjugated bile salts, and optimal emergence occurred after treatment with cholic acid conjugated to glycine. The emergence response to glycocholic acid is dose dependent and the dynamics of the dose–effect relationship is characteristic of specific ligand–receptor binding, where a significant fraction of the receptors must be occupied before a noticeable response to the ligand occurs (Ross and Gilman, 1985; Sukhdeo and Mettrick, 1987). It should be noted, however, that Fried and Butler (1979) found glycocholate ineffective in stimulating such excystment. The implications of this work are discussed by Sukhdeo and Mettrick (1987) who suggest that the fluke must be able specifically to recognize the ventral plug region and coordinate its activity to effect an escape.

Migration to the liver

Following on from his classical study referred to earlier, Schumacher (1956) provided the first real insight, in the form of photomicrographs, of early

migration of the liver fluke through the liver. Details of the process in different animal hosts, including the penetration of the intestinal wall, movement through the abdominal cavity and penetration of the liver, have been described in great detail (and also reviewed) by Dawes (1961, 1962, 1963), Dawes and Hughes (1964) and Boray (1969), and will be covered in more depth in Chapter 3. A synopsis will be given here to illustrate the general features in a typical host, the sheep. It should be appreciated that, depending on which animal host is affected, many of the features, including the specific timing of key events and the severity of the pathology, can vary quite markedly. After



Internal structures of *Fasciola hepatica*.

1. Oral sucker
2. Pharynx
3. Oesophagus
4. Ventral sucker
5. Caeca
6. Genital pore
7. Cirrus sac
8. Vas deferens
9. Ovary
10. Uterus
11. Ootype
12. Vitelline duct
13. Testes
14. Vitelline gland

Fig. 1.3. Diagram of an adult *Fasciola hepatica* showing major structures. (Drawn by Dr Diane Clery.)

the metacercariae have excysted in the small intestine, the newly excysted juvenile flukes rapidly penetrate the intestinal mucosa, in doing so breaking down epithelial cells, connective tissue and unstriped muscle fibres, and move into the peritoneal cavity. Once in the peritoneal cavity, the flukes apparently browse on whatever tissue is available, occasionally penetrating organs including the local lymph nodes. The liver appears to be reached by random wanderings, although recent evidence suggests that newly excysted juveniles may migrate towards the liver in response to some stimulus, an orthokinesis (Sukhdeo and Mettrick, 1987). Once through the liver capsule (a process which typically takes between 4 and 6 days after infection), the young flukes burrow through the liver for between 5 and 6 weeks causing extensive haemorrhage and fibrosis. Significant growth of the fluke occurs during this period (see Plate 1). There is some evidence that young flukes prefer to feed on hepatic cells rather than blood, although some blood is inevitably ingested. The flukes eventually reach the bile ducts, about 7 weeks after infection, in which they grow to adults and become permanently established. Occasionally, immature flukes may be found in unusual sites, e.g. lungs, pancreas, lymph nodes and thymus and some may infect the fetus in pregnant animals. From 8 weeks after infection, eggs are found in the bile, and later in the faeces, thus completing the life cycle. Some adult flukes may live for a considerable time in the liver (Fig 1.3 and Plate 1, respectively, illustrate the adult fluke and aspects of its life cycle). For instance, flukes 11 years of age have been recorded in sheep by Durbin (1952), each producing up to 20,000 eggs per day. It is noteworthy that fertile eggs can be obtained from an animal infected with a solitary fluke (Hughes, 1959).

An overview of the historical discovery of the life cycle of *F. hepatica* has been given here, together with the main biological factors involved in the successful completion of the cycle. Further information can be found in an extensive literature including: Taylor, 1937, 1964; Reinhard, 1957; Dawes, 1960, 1968; Pantelouris, 1965; and Smyth and Halton, 1983.

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2 Development of *Fasciola hepatica* in the Intermediate Host

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Introduction

For this chapter we have reviewed the primary literature published between 1985 and 1997. Most of the earlier literature on physiological aspects of larval *Fasciola hepatica*–snail relationships was covered in the classic work by Smyth and Halton (1983). Moreover, the recent book edited by Fried and Graczyk (1997) provided a nice capstone to the literature, as many of the chapters in that book provided citations useful for our coverage.

Our primary literature citations are selective rather than exhaustive. In preparing for this review we have examined a number of useful books, monographs and reviews that have helped us with this topic. We consider the following works most useful for coverage on structure, function, ecology, behaviour, biochemistry, immunology of all aspects of the host–parasite relationships of larval *F. hepatica* and their snail hosts. These works are: Kendall (1965, 1970), Pantelouris (1965), Smyth (1966), Dawes (1968), Boray (1969), and Erasmus (1972).

The Intermediate Snail Host

The principal intermediate snail host for *F. hepatica* is *Lymnaea truncatula* (Plate 1). In regions of the New World where the disease was introduced other varieties of snail, which may not be distinct species from *L. truncatula*, may be important. These include the snails *L. cubensis* and *L. viatrix* in South America, and *L. tomentosa* in New Zealand and Australia (Boray, 1985). *Lymnaea truncatula* snails have been shown to be capable of adapting to many different environments such as the high altitude (>4000 m) of the Bolivian Altiplano (see Chapter 12 of this volume). Interestingly, the intermediate snail host of *F. gigantica*, *L. auricularia sensu lato*, is resistant to *F. hepatica*, while *F. gigantica* does not appear to be capable of developing in *L. truncatula* (Boray, 1985; see also Chapter 15 of this volume).

Fasciolosis (ed. J.P. Dalton)

Miracidium: Biology, Behaviour and Snail Infection

Hatching from the egg is achieved by muscular contraction of the miracidium which, when stimulated by light, secretes proteolytic enzymes from the apical glands that digest the plug of the egg (Buzzell, 1983). The difference in osmotic pressure between the inside and outside of the egg then expels the miracidium, even if it is dead. The miracidium is the first free-living larval stage of *F. hepatica* and is a non-feeding organism. Its life span is determined by the amount of energy reserve, e.g. glycogen, and the length of miracidial survival is influenced by environmental factors. Under optimal conditions, miracidia of *F. hepatica* have up to 25 h to find a suitable intermediate host or die.

Adaptations to snail penetration and to the external environment

The surface of the *F. hepatica* miracidium consists of 21 large, flat (1.5 µm thick) epithelial plates arranged according to the formula 6 + 6 + 3 + 4 + 2 = 21 (Ginetsinskaya, 1988). The epithelial cells contain cilia arranged in regular longitudinal rows (Køie *et al.*, 1976). The anterior tip of the miracidium has a mobile, reversible, muscular proboscis (terebratorium) which plays an important role in penetration of snail tissue. The anterior end of the miracidium contains a large flask-shaped apical gland and four unilateral glands all of which have ducts which pass to the tip of the apical papilla (Buzzell, 1983).

Miracidia begin to swim immediately after hatching at the rate of 1.3–2.5 mm s^{-1}; the rate increases with a rise of temperature and decreases when the temperature drops (Ginetsinskaya, 1988; Chipev, 1993). At 6°C, *F. hepatica* miracidia lose their ability to move. The swimming rate decreases when the glycogen level is low (Chipev, 1993). *Fasciola hepatica* miracidia are aerobic and are extremely sensitive to low oxygen concentrations and die at oxygen concentrations lower than 0.7 mg l^{-1} (Ginetsinskaya, 1988). As miracidia do not feed during their short life span, tissue glycogen is their only source of energy. Glycogen concentrated in the epithelial plates is used for: (i) hatching; (ii) swimming; and (iii) snail penetration (Ginetsinskaya, 1988).

The most important taxes displayed by *F. hepatica* miracidia include: (i) movement to the source of light (positive phototaxis); (ii) movement against the force of gravity (negative geotaxis); and (iii) chemical stimuli (chemotaxis) (Haas and Haberl, 1997). The complex array of taxes displayed by miracidia is related to the ecology of their intermediate hosts, pulmonate snails, which inhabit shallow water and remain at the water surface in order to access atmospheric air. This array of complex miracidial taxes assures that this larval stage will enter immediately the active zone of its intermediate hosts (Atema, 1988).

The ability to penetrate snail tissue develops during the life of the *F. hepatica* miracidium. A miracidium is incapable of snail invasion during its first few minutes of life (Ginetsinskaya, 1988). However, optimal invasion capability of *F. hepatica* miracidia is achieved at 1.5–2.0 h after hatching and then this capacity slowly declines (Ginetsinskaya, 1988).

Recognition of and penetration into the snail

The invasion process of a snail by *F. hepatica* miracidia includes: (i) attachment to the host surface; (ii) penetration of the snail body surface; and (iii) migration to the site of localization (Ginetsinskaya, 1988; Chipev, 1993). Penetration of the snail surface has been divided into three phases: (i) less than 1 min after attachment the ciliated cells of the anterior tier of the miracidium are shed and swim away; (ii) the cilia of the remaining cells beat violently and after 5 min cilia are detached near the cell surface; and (iii) the miracidia remain embedded at the snail surface for about 15 min before initiation of migration through the snail tissue (Køie *et al*., 1976).

Fasciola hepatica miracidia change their normal straight-on swimming mode when they enter the active space of a host (Chipev, 1993). The miracidia then demonstrate an increased rate of change of direction known as the first behavioural pattern (Haas and Haberl, 1997). *Fasciola hepatica* miracidia can already discriminate between different snail species during the process of snail approachment (Chipev, 1993; Haas and Haberl, 1997). This behavioural response has been classified as chemoklinokinesis with adaptation (Haas and Haberl, 1997). Once inside the host's active zone, the *F. hepatica* miracidium shows the second behavioural pattern, i.e. a sharp 180° turn that prevents it from leaving the host's active zone (Haas and Haberl, 1997). These two behavioural patterns result in an accumulation of miracidia around the snail (Chipev, 1993). Attachment is stimulated by short-chain fatty acids present in snail mucus (Haas and Haberl, 1997). Miracidia begin to secrete the contents of their apical and unilateral glands before penetration of the snail tissue (Buzzell, 1983). Mechanical interactions of the miracidial apical papillae and snail microvilli are necessary for stable attachment (Ginetsinskaya, 1988). Macromolecular glycoconjugates in snail mucus have been reported as substances capable of stimulating *F. hepatica* miracidia to penetrate (Haas and Haberl, 1997). Penetration movement is synchronized with release of secretions from the miracidial apical glands (Køie *et al*., 1976).

Although *F. hepatica* miracidia attempt to penetrate snail surfaces almost anywhere (sometimes successfully), the penetration is most successful in the area of the pulmonary cavity (Ginetsinskaya, 1988). This is probably because the *F. hepatica* miracidium has a long proboscis that firmly wedges between the columnar epithelium of the snail's pulmonary cavity (Ginetsinskaya, 1988). After finding a snail, the *F. hepatica* miracidium fastens itself by its apical papillae and its entire anterior end becomes flattened and performs regular rhythmic movements involving alternate body contractions and elongations (Køie *et al*., 1976). The presence in the aquatic environment of 'non-host snails' (NHS) that interfere with the capacity of *F. hepatica* miracidia to infect their intermediate hosts is known as 'decoy effect' (Chipev, 1993).

Asexual Development Within the Snail

Tissue migration and morphogenesis

Secretion of proteolytic enzymes from the apical gland and the four unilateral glands continues during miracidial migration through snail tissue (Køie *et al*., 1976). The host tissue is lysed by the gland secretions only in direct proximity of the apical region of the migrating miracidium; there is no visible tissue damage behind the miracidium. The miracidium sheds its epithelial plates during migration. Following penetration of the snail, the miracidium undergoes metamorphosis and transforms into the mother sporocyst which has the shape of a filiform sac (Køie *et al*., 1976). At the optimal temperature of 18°C, metamorphosis takes about 12 h (Ginetsinskaya, 1988). The migration and localization of the mother sporocyst in snail tissue was studied in four species of pulmonate snails monomiracidially infected with *F. hepatica* (Preveraud-Sindou *et al*., 1994). When miracidial penetration occurred through the mantle, the mother sporocyst preferentially localized in the zone surrounding the kidney and the heart (Preveraud-Sindou *et al*., 1994). When miracidial penetration occurred through the foot, the sporocyst localized in the foot, mantle, or the zone surrounding the kidney and heart (Preveraud-Sindou *et al*., 1994).

The mother sporocyst contains a fixed number of germ balls (Ginetsinskaya, 1988). The miracidial apical and lateral glands are visible in the early developing mother sporocyst (Buzzell, 1983). Mother sporocysts reproduce by asexual mitotic divisions eventually giving rise to the first generation of daughter rediae (Buzzell, 1973). The daughter rediae of the first generation (independent rediae) free themselves by breaking through the body wall of the mother sporocyst which subsequently dies (Ginetsinskaya, 1988). The productivity of the mother sporocyst, measured by the number of independent rediae and the number of cercariae in an individual redia, is related to the size of infected snails (Rondelaud and Barthe, 1987). In sexually mature *L. truncatula* snails, mother sporocysts produce more rediae which subsequently produce more cercariae, than do mother sporocysts in juvenile snails (Rondelaud and Barthe, 1987). In daughter rediae, the germ cells continuously give rise to subsequent generations of rediae (dependent rediae); each redia generation is composed of the cohorts, e.g. first, second, third, etc. (Ginetsinkaya, 1988).

Regulation of redia populations

Interspecific competition

Interspecific competition involves interactions between larval trematodes of various species that infect the same snail host. The interactions result in: (i) elimination of the larva of one species (predation), or (ii) delay or enhancement of larval development of one trematode species (Abrous *et al*., 1996; Augot *et al*., 1996). The effect of interspecific competition among trematode larvae in snails infected with *F. hepatica* has been measured by: (i) the prevalence of snail infection (= infection rate); (ii) the intensity of snail

infection (= redial burden); and (iii) cercarial shedding (Abrous *et al*., 1996; Augot *et al*., 1996).

Interspecific competition was investigated in sexually mature and juvenile snail hosts of *F. hepatica* (Abrous *et al*., 1996). When juvenile (4 to 6 mm height) *L. glabra* were exposed to a single *Paramphistomum daubneyi* miracidium after being exposed to a single *F. hepatica* miracidium and vice versa, the prevalences of snail infections with each trematode species were similar (13%) (Abrous *et al*., 1996). However, no infection was observed in snails exposed to only one trematode species, indicating that in juvenile snails cross-exposure enhances development of the trematode larvae (Abrous *et al*., 1996). The development of redial burdens and the prevalence of infection were studied in sexually mature *L. truncatula* subjected to the successive cross-exposure of *F. hepatica* and *P. daubneyi* miracidia (Augot *et al*., 1996). In another experiment, sexually mature *L. truncatula* were exposed simultaneously to miracidia of these two trematode species (Augot *et al*., 1996). Overall, the snails harbouring larval forms of both trematode species were fewer in number, and the prevalence of snail infection with *P. daubneyi* (61%) was higher than with *F. hepatica* (37%) (Augot *et al*., 1996). Interspecific competition in adult snails may result in delayed maturity of free (independent) rediae of *F. hepatica* (Abrous *et al*., 1996; Augot *et al*., 1996).

Cercariogenesis

Fasciola hepatica cercariae are leptocerous (simple-tailed) and gymnocephalous (Nasir, 1988; Fried, 1997). Although cercariae encyst externally on aquatic vegetation, they may sporadically encyst internally in pulmonate snail tissue, e.g. *L. truncatula* (Vareille-Morel *et al*., 1993). Cercarial shedding can occur as early as 27 days post-infection (Lee *et al*., 1995). Cercariae do not feed during their free life, but use reserve materials, e.g. glycogen and fat, accumulated from the snail tissue by rediae. Most of the cercarial glycogen is accumulated in the tail and this reserve is used first. Fat is accumulated in the cercarial body. Cercarial movement is intense but becomes less intense with depletion of glycogen and fat levels (related to the age of cercariae) (Ginetsinkaya, 1988). Cercarial life span is determined by the amount of glycogen and fat, temperature, physiological conditions of a snail host, and oxygen concentration in the water (Ginetsinkaya, 1988). As cercariae are aerobic, low oxygen concentrations tend to be lethal. An increase in temperature causes high-intensity movement and consequently decreases the glycogen reserve.

Adaptations of cercariae to emergence from the host and to the external environment

Fasciola hepatica cercariae display positive phototaxis and negative geotaxis (Ginetsinkaya, 1988). Consequently, cercariae emerge from snails during bright sunny days and immediately reach the water surface. Bright sunlight accelerates encystment of cercariae whereas diffuse light retards this process. In darkness cercariae will not encyst but will continue to swim until there is total glycogen and fat depletion (approximately 25 h) after which they die (Ginetsinkaya, 1988).

Dynamics of cercarial production, and patterns and intensity of cercarial shedding

Fasciola hepatica cercariae which leave the redia and enter the snail haemocoel are not yet fully developed (Ginetsinkaya, 1988). Once in the snail haemocoel, cercariae accumulate glycogen from that site. Cercariae emerge from a snail after the process of glycogen accumulation has been completed (up to 11 days) (Ginetsinkaya, 1988). Intensity of cercarial shedding depends on the amount of glycogen in the snail tissue. Anaerobic conditions disrupt the glycogen cycle and, if infected snails experience such conditions, the production and emergence of cercariae may cease (Ginetsinkaya, 1988; Roberts and Suhardono, 1996).

Cercarial shedding is influenced by environmental factors. Optimal temperature for cercarial shedding coincides with the temperature needed for optimal snail activity; adverse environmental conditions inhibit the development of the parasite and also lower snail activity. The number of cercariae shed daily depends on the age and number of rediae (= redial burden, = intensity of infection), and on the number of miracidia that infected the snail (Lee *et al*., 1995). *Lymnaea viridis* experimentally infected with three or five miracidia produced considerably more cercariae that those snails that were infected monomiracidially (Lee *et al*., 1995). The number of cercariae shed daily is not uniformly distributed during the day (daily rhythm). Periodicity of cercarial shedding is controlled by succession of day and night temperatures and daylight (Ginetsinkaya, 1988). Maintenance of infected snails in total darkness or in continuous light resulted in disruption of daily rhythm and periodicity of cercarial shedding. Mass emergence of cercariae, 1–2 h prior to the snail death, is frequently observed during the summer (Ginetsinkaya, 1988).

Adaptation of cercariae to passive entry into the definitive host

Cercarial adaptation involves encystment on aquatic vegetation which is consumed by the definitive host. The definitive host becomes infected by the ingestion of encysted cercariae (metacercariae). Cercariae shed by a snail rapidly reach the surface of the water and settle on the vegetation near the water surface (Nasir, 1988). A cercaria sheds its tail and forms a transparent glassy cystoid membrane using secretions from the cystogenous glands (Fried, 1997). The resulting metacercaria remains viable for several months (Ginetsinkaya, 1988) and has the capacity to overwinter (Luzon-Pena *et al*., 1994).

Pathogenic Effects of the Intramolluscan Larvae on Snails

Disruption of snail metabolism

Intramolluscan larvae of *F. hepatica* inflict histopathological changes that disrupt the normal course of snail metabolism: (i) infected molluscs release up to three times more heat than uninfected ones; (ii) oxygen consumption increases; (iii) metabolism is intensified; and (iv) snail size may increase (gigantism) (Wilson and Denison, 1980; Ginetsinkaya, 1988). Increased metabolism causes glycogen reserves in the digestive gland and musculature of

the foot of the snail to be reduced. The increases in size and biomass of snails infected with *F. hepatica* coincide with the migration of mother sporocysts through snail tissue (Thompson, 1997), and continues through the period of cercarial shedding (Thompson, 1997). Increased snail size results from increases in wet body mass, volume and shell (Thompson, 1997). At 56 days post-infection the dry snail mass (including tissue, shell and parasites) was approximately twice that of uninfected *L. truncatula* (Wilson and Denison, 1980). When the biomass of control (uninfected) snails (*L. truncatula*) and their eggs was compared with the biomass of infected snails that had not laid eggs because of parasitic castration, the differences were not statistically significant (Wilson and Denison, 1980). This indicates that gigantism is a result of a switch in nutrient supply from reproduction to somatic tissue growth and parasite growth (Wilson and Denison, 1980). Gigantism of snails infected with *F. hepatica* is of nutritional rather than endocrine origin (Wilson and Denison, 1980; Thompson, 1997).

Destruction of the digestive gland

Fasciola hepatica larvae are in the connective tissue of the digestive gland and in severe infections the larvae penetrate the gland (Preveraud-Sindou *et al.*, 1994). The pressure caused by larvae on the tissue causes disappearance of the lumen of the tubule (Ginetsinkaya, 1988). Consequently, oxygen-rich haemolymph cannot perfuse the digestive gland resulting in anoxia and subsequent autolysis of the digestive gland. This, together with an accumulation of larval metabolites, induces disintegration of the digestive gland and substantially decreases the level of glycogen (Thompson, 1997).

Parasitic castration and fecundity

Parasitic castration can be histological due to total destruction of the reproductive system, or physiological, i.e. associated with temporal atrophy of the reproductive glands. Parasitic castration of infected snails will cause the energy for reproduction to be directed towards growth (Wilson and Denison, 1980; Thompson, 1997). In *L. truncatula*, parasitic castration was noted on day 21 post-infection as a result of direct consumption of the ovotestis by *F. hepatica* rediae (Wilson and Denison, 1980).

A loss of reproductive potential due to parasitic castration is a common feature of *F. hepatica*–snail interactions (Thompson, 1997). The decreased number of eggs laid by the snail is directly related to destruction of the gonads. Interestingly, the biomass represented by egg loss is balanced by the increase of the snail soft tissue biomass (Thompson, 1997). Cessation of egg production of infected *L. truncatula* occurs at 21 days post-infection (Wilson and Denison, 1980).

Protective snail reactions

Immunoresponses of molluscs differ from those of mammals. Pulmonate snails do not produce antibodies; they do not have T or B cells, and they do

not display immunologic memory (Adema and Loker, 1997). Antigens that cross the epithelial surface of snails are recognized by lectins (self–non-self recognition) (Renwrantz, 1986). The major defences against invasion by trematode larvae are cellular reactions mediated by haemocytes (phagocytic cells) (Adema and Loker, 1997). Phagocytes (haemocytes showing phagocytosis) can mechanically damage trematode larvae to the extent that the larvae will die (Adema and Loker, 1987).

Although miracidia of *F. hepatica* can penetrate a variety of lymnaeid snail species, the production of cercariae (patent infection) is limited to several species (McReath *et al*., 1982).

Haemolymph of snails infected with trematode larvae contains soluble substances that immobilize miracidia. Approximately 60% of *F. hepatica* miracidia were immobilized by haemolymph drawn from another *F. hepatica*-infected snail (Ginetsinkaya, 1988). Extensive studies on *F. hepatica* in snails indicate that a snail with a patent infection cannot free itself from invasion, and death of the parasite is associated with the death of the snail (Ginetsinkaya, 1988).

The course of *F. hepatica* infection in an inappropriate snail host is rapidly halted by a cellular encapsulation response against the mother sporocyst (McReath *et al*., 1982). The response is divided into two phases: (i) development of a cellular capsule; and (ii) the concomitant degeneration of the sporocyst, removal of the remains and dispersal of the capsule (McReath *et al*., 1982). The efficiency of the cellular encapsulation response increases with the snail age (McReath *et al*., 1982). The intensity of amoebocytic reaction (cellular type reaction) in snails against migrating miracidia is responsible for abortive *F. hepatica* infections (Rondelaud and Barthe, 1981a). The amoebocyte-producing organ, located in *L. truncatula* between the pericardium and the kidney, is activated after initial infection with *F. hepatica* (Rondelaud and Barthe, 1981b). The amoebocyte-producing organ begins to produce two types of amoebocytes (Type I, and Type II amoebocytes) (Rondelaud and Barthe, 1981b).

Environmental Factors Affecting Transmission of *Fasciola hepatica* by Intermediate Hosts

Prevalence of infection (= infection rate), intensity of infection (= redial burden), and cercarial shedding represent the parameters commonly used to epidemiologically and epizootiologically measure transmission of *F. hepatica* by intermediate hosts (Roberts and Suhardono, 1996).

Transmission of *F. hepatica* by snails shows a strong seasonal pattern and occurs mainly in the spring (Roberts and Suhardono, 1996). However, there are exceptions. For instance, in Montana (USA), transmission occurs only in the late autumn (Rognlie *et al*., 1996). Infection of definitive hosts with the pathogen can occur in early spring because intramolluscan stages of *F. hepatica* can overwinter and consequently produce high numbers of metacercarial cysts in early spring (Luzon-Pena *et al*., 1994). The overwintering rate is similar for snails harbouring prepatent or patent *F. hepatica* infections in the autumn

(Luzon-Pena *et al*., 1994). Embryonated eggs deposited in the water in the autumn retain their viability during the winter (Luzon-Pena *et al*., 1994) giving rise to miracidia that infect snails in the spring. The stage of egg embryonation is a factor determining egg survival in the environment; unembryonated eggs do not overwinter. Also, *F. hepatica* metacercariae can retain their viability during the winter (Luzon-Pena *et al*., 1994).

Development of larvae proceeds undisturbed in snail tissues if the temperature is above 12°C. Development of rediae is particularly accelerated in the summer by evening temperatures of 12 to 15°C and day temperatures of 32 to 38°C (Ginetsinkaya, 1988). Cercarial production is strongly affected by the temperature under which snails are being maintained during the prepatent period of infection (Lee *et al*., 1995). During years with high precipitation, the prevalence of infected snails is considerably higher probably because of improved snail feeding conditions. Development of intramolluscan stages and cercarial shedding depend in part on the amount of glycogen in snail tissues. Although snails balance parasite-induced losses of glycogen by increased food consumption, the snails exposed to additional stress, e.g. desiccation, or heat shock, are unable to compensate for glycogen losses (Ginetsinkaya, 1988). In general, infected snails are more sensitive to changes in environment than are non-infected ones (Roberts and Suhardono, 1996). Low glycogen levels result in arresting of cercarial shedding, and retardation of larval development. However, these changes are reversible. Redial burden of *L. truncatula* was considerably lower in snails exposed to desiccation for 10 days; the decrease was particularly apparent in the number of first generation rediae (Rondelaud, 1994). Eight days after cessation of experimentally induced desiccation, there was no difference in the redial burden between stressed and control snails (Rondelaud, 1994). Consequently, during the summer the number of released cercariae is low or none; however, after rains cercarial shedding immediately increases (Rondelaud, 1994).

Control of Snail Transmission of *Fasciola hepatica*

Fasciola hepatica is dependent for its transmission on a limited subset of species of the genus *Lymnaea* within the basommatophoran family Lymnaeidae. Control of snail transmission of *F. hepatica* is based on understanding the patterns of seasonal transmission, intermediate host–pathogen interactions, and reliable methods for identification of infected snails. Snail control by strategic and tactical draining programmes or fencing of wet areas is an essential component in controlling fasciolosis (Roberts and Suhardono, 1996). However, drainage or fencing is not cost effective on grazing land in developed countries (Wilson *et al*., 1982) and may not be feasible in developing countries (Roberts and Suhardono, 1996) (see also Chapter 4 of this volume).

Chemical control

Aquatic pulmonate snails can be controlled by broadcasting of molluscicides on snail-infested waters. A variety of molluscicides based on inorganic or

organic compounds and plant-derived substances have been used to reduce populations of intermediate hosts of *F. hepatica* (Haseeb and Fried, 1997). Molluscicides have been used successfully and cost effectively in short-term control of fasciolosis (Roberts and Suhardono, 1996). Significant reduction of snail populations (over 90%) was achieved when molluscicide broadcasting was synchronized with adverse environmental conditions that limit growth of snail population (Crossland, 1976). Environmental stress, e.g. cold weather, drought periods, negatively impact on snails and particularly on those infected with the pathogen (Crossland, 1976; Roberts and Suhardono, 1996).

The levels of molluscicide sublethal for *F. hepatica*-susceptible snails impact negatively on the development of intramolluscan stages of the pathogen (Rondelaud, 1995). The effect of redial burden and the prevalence of infections were studied in *L. truncatula* sublethally poisoned with cupric chloride (Rondelaud, 1995). Redial burden and infection prevalence were almost twice as high in control snails than in snails exposed to sublethal concentration of the molluscicide (Rondelaud, 1995). The decrease in rediae was particularly marked in the first generation of rediae (Rondelaud, 1995). However, the effect of snail chemical toxicity on prevalence and intensity of infection disappeared after 8 days (Rondelaud, 1995) indicating that snail survivors can transmit *F. hepatica* to the same extent as the untreated snails (Rondelaud, 1995).

Satisfactory results on temporal control of transmission of *F. hepatica* can also be obtained when anthelmintic medication of animals in enzootic areas (that reduce egg contamination in pastures) is applied during adverse weather conditions (Zukowski *et al.*, 1993; Roberts and Suhardono, 1996).

The continued survival of liver flukes in the enzootic areas indicates that snail control is not sufficient and impractical in eliminating the pathogen and eradicating the disease. This is because areas without non-agricultural areas connected by streams with the pastures and grazing lands are not amenable for chemical snail control. Multiple molluscicide applications are required to achieve substantial reduction in snail populations (Roberts and Suhardono, 1996). Also, as lymnaeid snails have a high biotic potential they rapidly repopulate aquatic environments after molluscicide treatments. Many molluscicides are toxic to humans and leave residual toxicity for non-targeted aquatic fauna (Haseeb and Fried, 1997). In some situations, e.g. irrigated rice fields, application of molluscicides has gained little acceptance (Roberts and Suhardono, 1996).

Biological control

Competitors and predators of lymnaeid snails

Marisa cornuarietis successfully competed with *Lymnaea cailliaudi* eliminating the latter species from aquatic habitats (Roberts and Suhardono, 1996). Snail competitors may feed on eggs or juveniles of pulmonate snails (Haseeb and Fried, 1997). As lymnaeid snails have a thin shell and do not have an operculum, they are vulnerable to predation by crustaceans, birds, fish and aquatic rodents. Usually, populations of lymnaeid snails coexist in equilibrium

with their predators; however, intense commercial duck and goose husbandry may completely eliminate lymnaeid snails from aquatic habitats (Levine, 1970).

Parasites and commensals of lymnaeid snails

Under natural conditions, lymnaeid snails are heavily infested with the ectocommensal oligochaete, *Chaetogaster limnaei* (see Rajasekariah, 1978). This oligochaete ingests cercariae of *F. hepatica* emerging from the surface of the snail (Rajasekariah, 1978). Concurrent infection of juvenile pulmonate snails harbouring *F. hepatica* larvae with a prostrongylid nematode, *Muellerius capillaris*, resulted in considerably high mortality of the infected snails (Hourdin *et al*., 1993). Mortality of *F. hepatica*-infected snails was strongly related to the events of reinfection with *M. capillaris* (Hourdin *et al*., 1993). Larvae of *F. hepatica* or other trematode species can sterilize snails or reduce their fecundity by damage to the gonads (Roberts and Suhardono, 1996).

Utilization of snails

In some regions, lymnaeid snails are collected from the wild and used as protein supplement to feed domestic animals, particularly poultry (Roberts and Suhardono, 1996). Incorporation of pulmonate snails into the food of scavenging village chickens eliminated fasciolosis from some regions in Sri Lanka (Gunaratne *et al*., 1993).

Detection of Snail Infection and Transmission Foci, and Defining of Enzootic Areas

As snails infected with *F. hepatica* can be difficult to recover from their natural habitats in enzootic areas (Malone *et al*., 1984), a wide spectrum of techniques for the detection of snail infection have been developed. These techniques are incorporated into the tactical strategy for defining the enzootic areas of *F. hepatica*. The term 'transmission foci' refers to a snail reservoir (of *F. hepatica*) that exists at certain locations irrespective of prevalence and intensity of snail infection with *F. hepatica*. The term 'enzootic areas' refers to the presence of the pathogen that is continuously maintained in certain territory by the components of the ecosystem, e.g. snails, wildlife, domestic animals and humans. Transmission foci of *F. hepatica*, characterized by the temporal transmission of the pathogen, are smaller than the enzootic areas in which pathogen transmission is continuous throughout the year (although with different intensity).

Microscopic techniques

Microscopic techniques include observation of cercarial release, tissue sectioning and snail crushing to isolate rediae or cercariae. These techniques, although practical and relatively inexpensive, do not have the high sensitivity or specificity of the molecular techniques.

Molecular techniques

The most advanced molecular methods include a variety of polymerase chain reactions (PCR) in which specific fragments of *F. hepatica* RNA or DNA are amplified. The sensitivity of these techniques is extraordinarily high, as positive PCR signals can be obtained from the tissue of a snail penetrated by a single miracidium. Although specificity is close to 99.9%, in some cases *F. hepatica* primers can also produce positive reaction with the RNA of other Fasciolidae species (Rognlie *et al.*, 1994). Detection of infected snails by PCR is particularly useful if several species of lymnaeid snails are present in large numbers in the aquatic environment (Kaplan *et al.*, 1995; Rognlie *et al.*, 1996).

Snail infection with *F. hepatica* can be detected by PCR that amplify fragments of *F. hepatica* ribosomal RNA; this approach was successfully used to detect intermediate snail hosts in Montana (USA) (Rognlie *et al.*, 1996). Further modifications of this technique include the reverse-transcriptase (RT) PCR used to amplify the region of *F. hepatica* small subunit RNA (Rognlie *et al.*, 1994). The RT PCR does detect *Fascioloides magna* RNA and *Fascioliopsis buski* RNA (Rognlie *et al.*, 1994). Reverse-transcriptase PCR detects *F. hepatica* RNA in snail tissue immediately after miracidial exposure and throughout the parasite prepatent and patent periods (Rognlie *et al.*, 1994). A 124 bp fragment of *F. hepatica* repetitive DNA has been cloned and sequenced; the fragment hybridizes specifically with *F. hepatica* DNA when tested versus a variety of DNA probes originating from a broad spectrum of pulmonate snails susceptible to *F. hepatica* (Kaplan *et al.*, 1995). Using the 124 bp repetitive DNA fragment it was possible to detect *F. hepatica* DNA in snail tissue immediately after miracidial penetration (Kaplan *et al.*, 1995).

Although not directly related to molecular techniques used to diagnose *F. hepatica* in snails, it is interesting to note that PCR techniques have been used to elucidate on the systematic status of species in the family Lymnaeidae where approximately 1800 species have been described (Remigio and Blair, 1997). Many of these species serve as actual or potential intermediate hosts of *F. hepatica*. Hopefully, molecular techniques along with the more conventional biological approaches will shed light on the confusion that exists in the systematics of lymnaeid snails.

Animal tracer method

Transmission foci of *F. hepatica* are detected by tracer sheep that pasture for various time periods on different pasture-land sites. The tracer sheep method is usually combined with snail surveys that utilize molecular techniques to determine snail infection with *F. hepatica* (Rognlie *et al.*, 1996). This approach was successfully used to identify the foci of seasonal transmission of *F. hepatica* in Montana (USA) (Rognlie *et al.*, 1996). Tracer animals are also used to determine the efficacy of molluscicide treatment of pastures and grazing lands (Crossland, 1976).

Enzootic areas are usually defined based on country- or state-wide snail surveys carried out in the areas from which *F. hepatica* infections in cattle,

sheep or wildlife have been reported (Dunkel *et al*., 1996). Information on infected animals is collected utilizing a questionnaire survey of veterinarians (McKown and Ridley, 1995; Dunkel *et al*., 1996). The snail monitoring covers large geographical regions (Dunkel *et al*., 1996). Defining of enzootic areas allows for prediction of the potential development of *F. hepatica* transmission foci within an enzootic area. Defining of the potential transmission foci is associated with collection of all species of pulmonate snails and testing in the laboratory their susceptibility to *F. hepatica* (McKown and Ridley, 1995). The model for estimating the risk of fasciolosis in domestic animals present in the enzootic areas of *F. hepatica* has been developed based on soil-type maps incorporated into the Geographic Information System (GIS) (Zukowski *et al*., 1993). The model is based on GIS typing of snail habitats and prediction of the presence of pulmonate snails susceptible to *F. hepatica* and the size of their population (Zukowski *et al*., 1993). The model has been successfully used in estimating site-specific differences in the risk of *F. hepatica* transmission to cattle grazing at Louisiana coastal marsh rangeland (Zukowski *et al*., 1993).

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3 Development of *Fasciola hepatica* in the Mammalian Host

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Introduction

The Common Liver Fluke by Pantelouris was published in 1965, at a time when much basic work on the morphology of the fluke had been carried out, but before electron microscopy had made a major impact. The aim of this chapter is to demonstrate the advances that have taken place in our understanding of the fine structural organization of the major organ systems in the fluke. The systems to be considered are the tegument, the parenchyma, the musculature, the nervous system, the cytoskeleton, the gut, the excretory system and the reproductive system. Each system will be examined in turn and additional emphasis placed on the changes that it undergoes during the development of the fluke in the mammalian host (Plate 1).

Tegument

The tegument is the layer in most intimate contact with the host's tissues and body fluids. As such, it represents a site where considerable biochemical, physiological and immune interplay takes place between the fluke and its host. Our understanding of the nature and roles of the tegument has come a long way since the late 1950s when it was considered to be a metabolically inert cuticle secreted by cells below the muscle layers. It is now appreciated that it is a syncytial, protoplasmic layer connected by cytoplasmic strands to nucleated cell bodies located in the general body parenchyma below the main somatic muscle layers. Moreover, it is a metabolically active layer specialized to carry out a number of functions: synthesis and secretion of various substances; absorption of nutrients; osmoregulation; protection against the host's (and parasite's) digestive enzymes and the surfactant properties of bile; and protection against the host's immune response. Furthermore, it possesses a sensory role. In this section, the basic organization of the tegument will be described first, then the developmental changes, before discussing the different functions.

Fasciolosis (ed. J.P. Dalton)

Surface features

The adult fluke has a leaf-like shape, is dorsoventrally flattened and has fluted margins. At the anterior end is the oral cone, with the oral sucker at its tip and the ventral sucker at the oral cone–main body junction (Fig. 3.1). The genital pore, which is the common opening for the male and female reproductive systems, lies just anterior to the ventral sucker (Fig. 3.1). The two suckers are the main organs of attachment, used to grip the lining of the bile duct and to assist the migratory movements of the fluke. The general body surface is covered by spines, which are closely packed, dorsoventrally flattened and point posteriorly (Fig. 3.1). The spines help to maintain the position of the fluke in the bile duct; they also serve to erode the epithelium and puncture blood vessels, in this way playing a role in feeding. On the oral cone, the spines occur in a regular pattern of 60 rings, each with 60–70 spines, whereas those on the posterior body are scattered without any pattern of rings (Bennett, 1975a,b). The number of spines in the posterior region of the body doubles (from 3000 to 6000) during the first week post-infection (pi) and increase up to eightfold by 3 weeks pi. The spines also elongate during development of the fluke to maturity – the anterior spines approximately 8 times, the posterior spines approximately 24 times. Between 2 and 3 weeks pi, all the spines on the body surface metamorphose from being single-pointed to multi-pointed, by division at the spine tips. The anterior spines have between 10 and 15 points, whereas the posterior spines have up to 30 points, but the overall shape comes to resemble a child's mitten (Bennett, 1975b).

Also scattered over the body surface are groups of sensory papillae, which are most numerous in the anterior region, around and inside the suckers (where spines are missing) and on the ventral surface. The papillae are conical and have a shallow pit into which the tip of a cilium projects. In the suckers there are low, conical papillae which do not have an exposed ciliary tip; they may be mechanoreceptors, whereas the ciliate type may be either mechano- or chemoreceptors (Bennett, 1975a).

Fine structure

The following account is based on the studies by Threadgold (1963, 1967). The tegument comprises a surface syncytial layer of cytoplasm, joined by cytoplasmic connections to nucleated tegumental cell bodies situated beneath the fibrous basal lamina and the circular and longitudinal muscle layers (Fig. 3.2). The syncytium is 15–20 μm thick and the surface is folded into a series of broad plateaux separated by deep valleys. The surface area is further increased by small, flask-shaped intuckings of the apical plasma membrane, known as apical invaginations (Fig. 3.3). The apical membrane is covered by a thick glycocalyx (see below). The basal plasma membrane is invaginated to form long, parallel-sided structures known as basal infolds which reach almost to the apical plasma membrane (Fig. 3.3). Associated with the basal infolds are ill-defined, membrane-less vacuoles of relatively low electron density which contain mucopolysaccharide (Threadgold and Brennan, 1978).

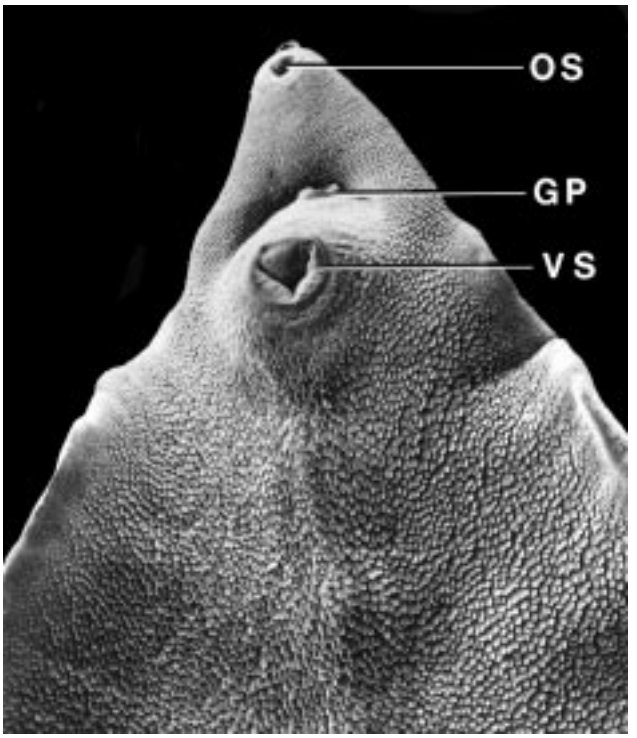

Fig. 3.1. Scanning electron micrograph of the anterior ventral surface of an adult liver fluke showing the oral sucker (OS), ventral sucker (VS) and genital pore (GP). The general body surface is covered with backwardly directed spines.

The cytoplasm contains numerous mitochondria, concentrated basally and which are often arranged in vertical chains parallel to the basal infolds (Fig. 3.3). In the adult fluke, the syncytium also contains two types of secretory body, designated the T1 and T2 secretory bodies. The T1 bodies are round and uniformly electron-dense and occur in a gradient within the syncytium, with the greatest number situated basally. The T2 bodies have the shape of a biconcave disc with a less dense centre and have a gradient opposite to that of the T1 bodies, being concentrated apically, where they often lie with their long axis at right angles to the apical plasma membrane. A third type of secretory body, the T0 body, is present in the newly excysted juvenile, but once the juvenile enters the liver the T0 cells metamorphose into T1 cells. The T0 bodies are very dense biconcave discs (Bennett and Threadgold, 1973, 1975). The glycoprotein nature of the secretory bodies, developmental changes in their production and their antigenic roles are discussed below. The other major component of the syncytium are the spines which, although they project well above the general surface of the tegument, are completely enclosed within the apical and basal plasma membranes. The spines are firmly attached to the basal plasma membrane by dense bodies resembling

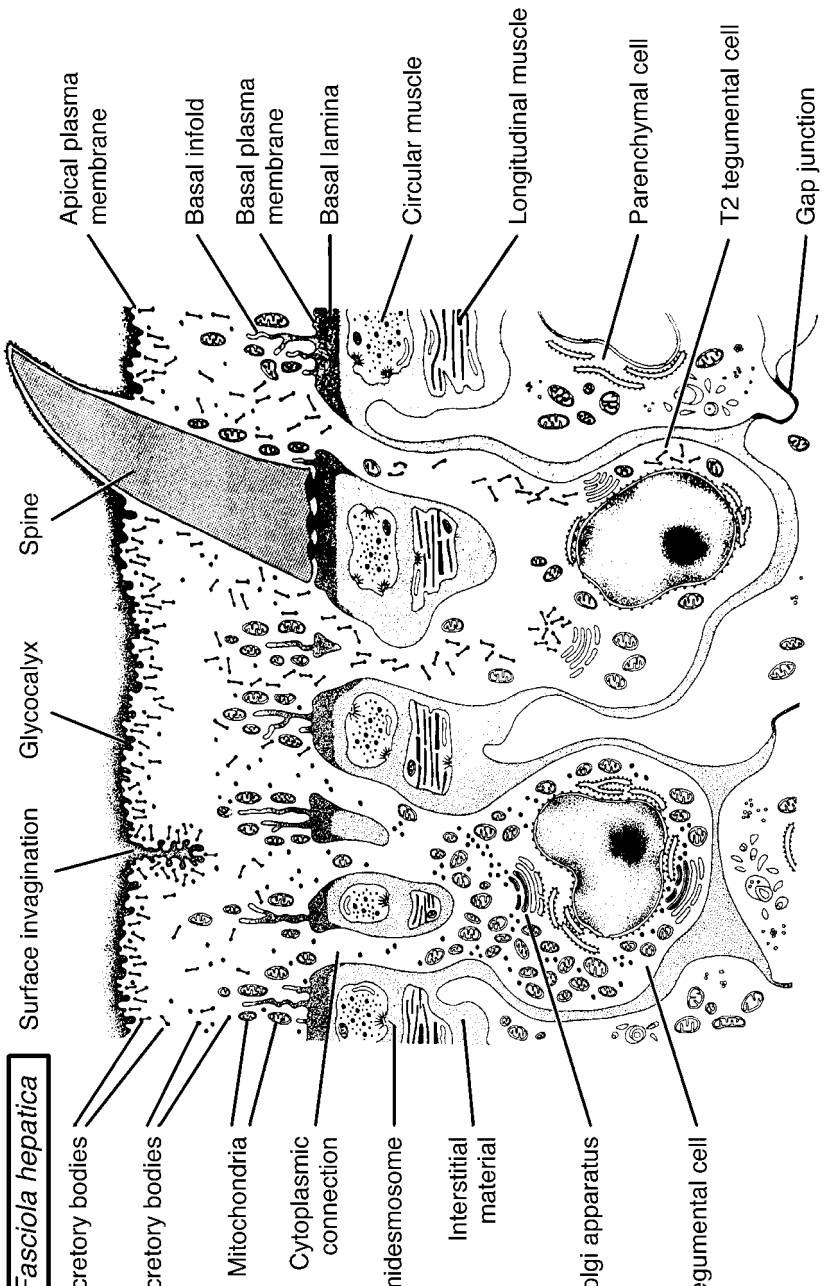

Fig. 3.2. Diagram showing the fine structural organization of the tegument and related structures in *Fasciola hepatica* (Smyth, 1994, based on Threadgold, 1963, 1967).

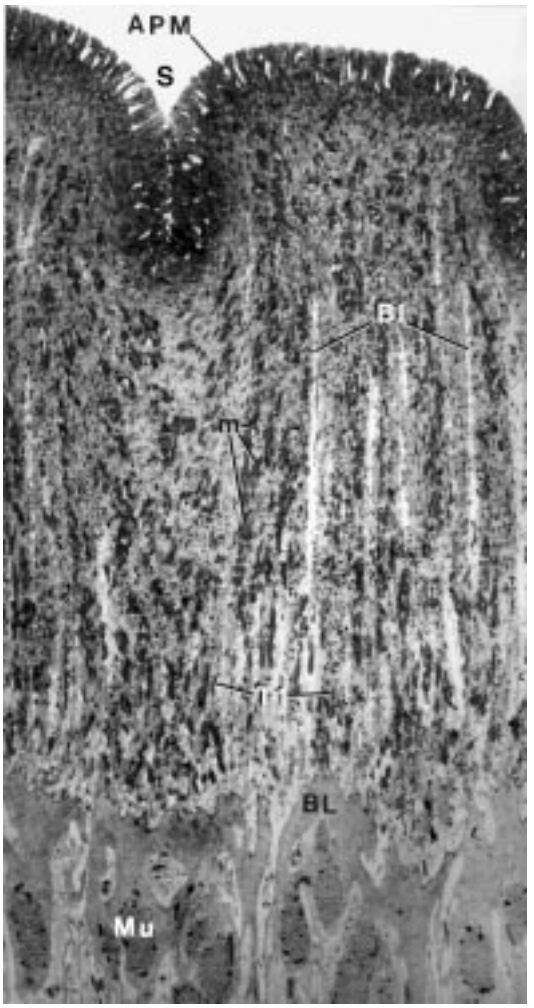

Fig. 3.3. Transmission electron micrograph of the tegument of an adult liver fluke showing the characteristic arrangement of surface pits (S) and plateaux. Note the invaginations of the apical plasma membrane (APM) and the long basal infolds (BI) formed by invaginations of the basal plasma membrane, with their associated chains of mitochondria (m). Note also the basal lamina (BL) and the underlying subtegumental muscle blocks (Mu).

hemi-desmosomes. They have a crystalline structure and have been shown to be composed of a form of actin with a molecular mass of 15 kDa (Stitt *et al.*, 1992a).

The nucleated tegumental cells are of two types, the T1 and T2 cells, corresponding to the type of secretory body they produce. The cells occur in groups, with the T1 cells being more numerous: the T1:T2 ratio is approximately 4:1. The T1 cells contain a large nucleus with a prominent nucleolus,

numerous mitochondria, a network of GER cisternae and a number of distinct Golgi complexes which produce the secretory bodies – all features typical of an active secretory cell (Fig. 3.4). The T2 cells are similar but have a less extensive GER system, fewer and less well-defined Golgi complexes, fewer mitochondria but greater numbers of secretory bodies in the cytoplasm.

A number of enzymes have been demonstrated in the tegument, including acid phosphatase, Na^+/K^+-ATPase, esterase, peroxidase, cytochrome oxidase and succinic dehydrogenase (Barry and Mawdesley-Thomas, 1968; Thorpe, 1968; Threadgold and Read, 1968; Threadgold and Brennan, 1978; Fujino *et al*., 1983; Skuce *et al*., 1987). Acid phosphatase activity, for example, occurs in the syncytium and is mainly associated with the apical plasma membrane, either close to or in contact with its inner component; greater activity is evident in the dorsal than in the ventral tegument (Threadgold, 1968; Fujino *et al*., 1983). Na^+/K^+-ATPase activity is particularly associated with the invaginations of the apical plasma membrane and the infoldings of the basal plasma membrane; this is indicative of the presence of ion pumps on the tegumental membranes. The enzyme is inhibited by ouabain, a known inhibitor of Na^+/K^+-ATPase activity, and there is greater activity in the ventral than in the dorsal tegument (Threadgold and Brennan, 1978; Skuce *et al*., 1987).

While the tegument of *F. hepatica* is a syncytial layer, there is evidence to suggest that there are regional differences within the tegument and its associated structures. Anterior/posterior and dorsal/ventral differences in the morphology and distribution of spines and the distribution of acid

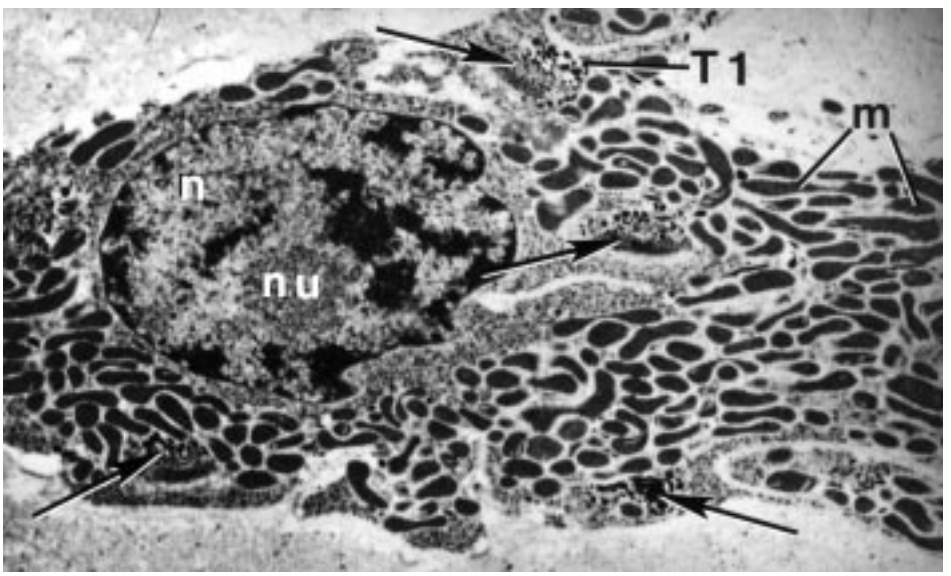

Fig. 3.4. Transmission electron micrograph of a T1 tegumental cell showing the nucleus (n) with its nucleolus (nu) and in the cytoplasm a number of Golgi complexes (arrows) giving rise to T1 secretory bodies (T1). Note also the numerous mitochondria (m) in the cell.

phosphatase and Na^+/K^+-ATPase activities have already been mentioned. The sensory receptors associated with the tegument also exhibit regional differences in their distribution; these differences and the fine structure of the three types of receptor will be described later. There are regional differences, too, in the susceptibility of the tegument to anthelmintic-induced damage; this point is discussed in Chapter 7 of this volume. While the available evidence is limited, it suggests that the tegument does not function as a uniform entity throughout the body of the fluke. It is an aspect of tegumental architecture and function that needs to be examined in greater depth.

Developmental changes

The tegument of newly excysted juveniles (NEJ) contains only one type of secretory body, the T0 body, and the cells are packed with T0 bodies (Bennett and Threadgold, 1973). The tegument of flukes recovered from the abdominal cavity of mice 12 h pi contains greater numbers of T0 bodies in the syncytium, but fewer bodies in the T0 cells; the glycocalyx on the apical plasma membrane is more prominent than at 0 h (Bennett and Threadgold, 1975). At 1 day pi, the numbers of T0 bodies in the cells recover, and at 2 and 3 days pi – with the flukes still within the abdominal cavity – differentiation of T2 cells from embryonic cells in the parenchyma begins, although connections with the syncytium are not yet established (Bennett and Threadgold, 1975). The connections are complete by 4–5 days pi, soon after penetration of the liver by the juvenile flukes and T2 bodies are observed in the syncytium. Between 5 days and 3 weeks, the synthesis of T0 bodies gradually declines, being replaced by the formation of T1 bodies, and the metamorphosis of T0 cells into T1 cells is complete by 3 weeks pi. By this time, the numbers of T1 bodies predominate over T0 bodies, the basal infolds and their associated vacuoles in the syncytium are increased in number and size, and there are greater numbers of mitochondria (Bennett and Threadgold, 1975). These changes continue after entry into the bile duct (4 weeks onwards), being accompanied by invagination and folding of the apical plasma membrane to adopt the morphology typical of the adult. Only T1 and T2 bodies are present at this stage, being produced by the T1 and T2 cells, respectively (Bennett and Threadgold, 1975).

Developmental changes in the tegument have been linked to immune responses by the host to fluke infection and have thus highlighted a potential role for the secretory bodies. The contents of the latter have been shown to be released at the apical plasma membrane and added to the glycocalyx, in this way presenting an antigenic stimulus to the host's immune system. Immunofluorescent studies using sera from infected sheep have shown that the level of T1 antigen in the tegument peaks at 3–5 weeks pi (in rat fluke infections), while that for T2 reaches a plateau after 10–12 weeks, T2 antigen being first detected only after 6 weeks pi (Hanna, 1980b). The results suggest that although T2 bodies begin to appear in the syncytium 1–2 weeks pi (in mice), they do not express their contents at the surface until flukes are in the bile ducts (Hanna, 1980b) (Fig. 3.5). In separate fluorescent antibody-labelling

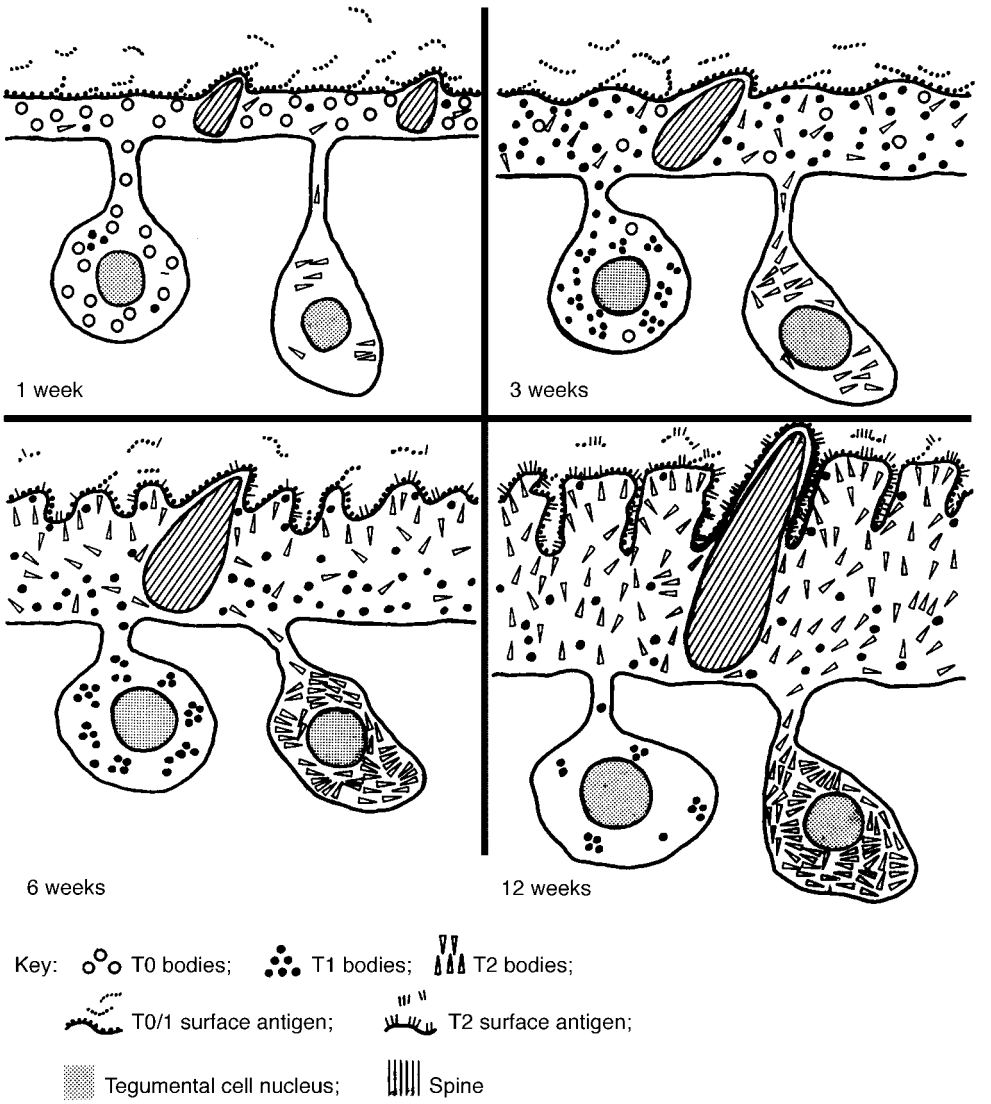

Fig. 3.5. Diagram summarizing the proposed changes in the antigenicity of the tegument of *Fasciola hepatica* during development in the mouse as related to ultrastructure (grossly simplified, not to scale) (Hanna, 1980b).

experiments, it has been shown that newly excysted juveniles become coated with host IgG when incubated in immune serum. This is sloughed when the flukes are transferred to medium lacking antibody, although when re-exposed to immune serum they acquire a new layer of IgG (Hanna, 1980a). This suggests that the glycocalyx had been replaced by an antigenically similar coat, by the release of (T0) secretory bodies at the apical plasma membrane.

This ability of the fluke to continually replace the surface glycocalyx represents an important mechanism whereby it can evade the host's immune response – that is, it can slough off attached host antibody and replace damaged surface membrane via addition of the membrane of secretory bodies following exocytosis (Hanna, 1980a; see also Chapter 10 of this volume). The evasion mechanism is particularly important in the early, migratory phase of the life cycle and the large stores of T0 bodies in the tegumental cells of the metacercaria may be a pre-adaptation, enabling the newly excysted juvenile to counteract immune attack by the host (Hanna, 1980a). The stores are mobilized rapidly after excystation, but then replaced (Bennett and Threadgold, 1975). The immunoprotective role of T0 bodies is taken over by T1 bodies in the liver parenchyma, because they contain similar antigenic determinants although they are morphologically distinct (Hanna, 1980b) (Fig. 3.5). Once in the immunologically 'safe' environment of the bile duct, protection is no longer required and the rate of glycocalyx turnover declines, although it continues throughout the life of the fluke. The role of the T2 bodies is uncertain, but may be concerned with maintaining the structural integrity and nutritive function of the apical plasma membrane. Their expression at the tegumental surface coincides with the entry of the fluke into the bile duct and may be related to structural reorganization of the apical membrane required following the switch from a tissue to a fluid (bile) environment. The accumulation of T2 bodies in the tegument during migration through the liver parenchyma may be a pre-adaptation to the new environment (Hanna, 1980b) (Fig. 3.5). Therefore, the combined morphological and immunological data have illustrated an intriguing and important functional link between tegumental secretory body production, migration of the fluke and evasion of the immune response. Other radiolabelling studies have shown that different proteins and glycoproteins are expressed at the surface of flukes at different developmental stages and may well correlate with the morphological changes described. However, definitive proof will require the production of antisera specific to each of the surface molecules (Dalton and Joyce, 1987).

Glycocalyx

The glycocalyx is a fairly labile structure that is not fully preserved by conventional fixation techniques. It comprises two layers: a continuous inner layer, approximately 25 nm thick, lying next to, and tightly bound to, the apical plasma membrane, and an outer fibrillar layer, 25–35 nm thick (Threadgold, 1976). The glycocalyx is largely composed of glycoprotein, with projecting side-chains of oligosaccharides containing terminal sialic acid residues. It is polyanionic throughout its thickness, and has a net negative charge (Threadgold, 1976). Lectins have been used to characterize the carbohydrate components of the glycocalyx, since individual lectins bind to specific sugars. For example, incubation of adult flukes in concanavalin A led to a number of tegumental changes, including surface blebbing, formation of microvillus-like projections, deepening of the apical invaginations, increased numbers of T2 secretory bodies in the apical cytoplasm, evidence of accelerated release of

secretory bodies and swelling of the basal infolds. The effects of concanavalin A could be prevented by addition of α-methyl-D-mannoside to the incubation medium. Similar but less pronounced changes were induced by wheat germ agglutinin binding. The combined data indicate the presence of mannose, glycosamine or glucose moieties and of *N*-acetylglucosamine in the adult fluke glycocalyx (Rogan and Threadgold, 1984). Stereological analysis of the morphological changes observed by SEM and TEM confirmed the increase in apical plasma membrane surface area, the apical concentration of T2 bodies and greater numbers of 'open' bodies (which are indicative of the release of the contents of the secretory bodies) (Rogan and Threadgold, 1984).

The importance of an intact glycocalyx to the normal functioning of the tegument has been shown in experiments involving the use of poly-L-lysine, to neutralize its net negative charge on the adult fluke glycocalyx (Threadgold, 1985). Pre-incubation with poly-L-lysine decreased the normal ability of the tegument to resist the action of enzymes such as pepsin and α-amylase. Treatment with poly-L-lysine then amylase had the most drastic effect, resulting in the total loss of the tegument, perhaps due to the action of the enzyme on the glucoside linkages of the oligosaccharide components of the glycocalyx (Threadgold, 1985). The changes induced by poly-L-lysine plus pepsin were less severe: surface blebbing, formation of microvilli and deep surface invaginations, swelling of the basal infolds and limited breakdown of the surface. The swelling of the basal infolds may be due to an osmotic effect, pepsin attacking the protein component of the apical plasma membrane and glycocalyx and thus altering the semipermeable properties of this combined structure (Threadgold, 1985). Surface blebbing, formation of microvilli and accelerated release of the secretory bodies appear to be features of the response of the tegument to a stress situation. They have been observed following lectin binding (as described above), following enzyme attack (as just described), following immunological attack (see below) and in response to anthelmintics (see Chapter 7 of this volume). Poly-L-lysine pretreatment did little to potentiate the action of bile, which had minimal effect on the tegument on its own (Threadgold, 1985).

Synthetic activity

The secretory activity of the tegumental cells has been studied by a number of techniques: autoradiography, stereology, immunocytochemistry, cytochemistry and inhibitor studies. Incubation of adult fluke slices in [^{3}H]leucine for up to 3 h showed that the label in the tegumental cells was initially incorporated into protein being synthesized by the GER, then moved into the Golgi complexes, becoming associated with the T1 secretory bodies being packaged by the complex. Labelled secretory bodies moved into the cytoplasmic connections leading to the syncytium and reached the base of the syncytium after 60 min. Here they remained for some time, for label did not appear in the apical cytoplasm until after 180 min: at this time the label was associated with the apical plasma membrane and its glycocalyx, and also occurred throughout the tegumental syncytium (Hanna, 1980c). The results demonstrate that the

process of protein synthesis in the tegumental cells of *F. hepatica* follows the typical GER–Golgi complex–exocytosis pathway observed in other secretory cells and that the entire process of secretory body synthesis, transport and release requires approximately 3 h for completion. Moreover, transport appears to be a three-step process, with initial movement through the cytoplasmic connections to the basal region of the syncytium, where the secretory bodies are stored for some time before undergoing a rapid transit to, and discharge from, the apical surface where the glycoprotein content of the vesicles is incorporated into the glycocalyx. Radiolabelled galactose has also been shown to be incorporated into the glycoprotein being packaged into secretory bodies by the Golgi complexes in the T1 cells (Hanna, 1976b). Labelled secretory bodies were also observed in the cytoplasmic connections, but over the 60 min incubation period, no label was observed in the syncytium, a result which emphasizes the relatively slow nature of the transport process. Evaginations of the parenchymal cells which penetrate the muscle layers and end close to the base of the tegument were often heavily labelled with [^{3}H]glucose; this observation supports the theory that the tegument is the main route for glucose transport into the fluke (Hanna, 1976b).

The effects of certain metabolic inhibitors on the synthesis of secretory bodies by the T1 cells have been analysed by stereological methods. Cycloheximide, an inhibitor of protein synthesis, inhibits the synthesis of secretory bodies but has no effect on the transport and discharge of those already present prior to drug treatment (Hanna and Threadgold, 1976). However, with longer incubation periods, the number of secretory bodies in the syncytium declined, due to the fact that they could not be replaced by synthesis in the cell bodies (Hanna and Threadgold, 1976). Treatment with iodoacetate (an inhibitor of glycolysis) and 2,4-dinitrophenol (an inhibitor of oxidative phosphorylation) did not lead to a drop in number of secretory bodies in the tegumental cells; instead the numbers stayed relatively constant at a level above normal. The numbers of secretory bodies in the syncytium dropped less in treated material than in the controls (Hanna and Threadgold, 1976). The results were interpreted as demonstrating an inhibition of the synthesis, transport and release of the secretory bodies (Hanna and Threadgold, 1976).

Monoclonal antibodies have been raised against an antigen present in the tegumental syncytium and glycocalyx of juvenile flukes and used in immunolocalization studies (Hanna and Trudgett, 1983). Gold labelling occurred over the T1 secretory bodies in the syncytium and over the glycocalyx, the latter being the result of exocytosis of the contents of the secretory bodies, replacing the glycocalyx as it sloughs from the surface. In the T1 cells, the secretory bodies were heavily labelled, whether lying free or forming in the Golgi complex. Antibody binding also occurred over the GER and ribosomes to some extent. Labelling was confined to T1 bodies in adult and juvenile flukes (Hanna and Trudgett, 1983). The monoclonal antibodies also bound to T0 secretory bodies in the tegumental syncytium and cell bodies of metacercariae, suggesting that the T0 and T1 bodies share a common antigen. Labelling also occurred in the gut and excretory system,

being associated with the glycocalyx lining the lamellae in both sites. In the gut cells, labelling was associated with the secretory vesicles, Golgi complex and GER. The results indicate that the gut and excretory system have antigenicity in common with the tegument (Hanna and Trudgett, 1983). The epitope bound by the monoclonal antibody has been shown to be present in the polypeptide component of the glycoprotein and is not altered by glycosylation in the Golgi complex. The protein has a molecular mass of 50 kDa and may be linked to smaller entities with molecular masses of 25–40 kDa (Hanna and Trudgett, 1983).

Movement of secretory bodies from the tegumental cells to the apex of the syncytium is blocked by the microtubule inhibitors colchicine and tubulozole-C, indicating a role for microtubules in the transport process (Stitt and Fairweather, 1993). This idea is supported by immunolocalization studies involving a monoclonal antibody raised against β-tubulin: staining occurred in the syncytium, the tegumental cell bodies and the connections between the two (Stitt *et al.*, 1992b). The effects of tubulozole-C are more rapid and severe than the changes induced by colchicine. Thus, tubulozole-C inhibits the synthesis of secretory bodies, an action stemming from a marked vacuolation of the GER cisternae, which retract towards the cell nucleus, and the migration of Golgi complexes to the cell periphery, followed by their gradual disappearance. These changes are classic cell responses to microtubule inhibition, because microtubules are responsible for the organization, distribution and movement of the GER, Golgi complexes and other cell organelles (for references, see Stitt and Fairweather, 1993). Inhibition of secretory body formation and transport, not unexpectedly, have serious effects on the tegument (Stitt and Fairweather, 1993). Microfilaments may also play a role in the movement of secretory bodies, because the microfilament inhibitor cytochalasin B causes a block of the two transport phases involved in this process. There is a gradual decline in their production too, and this may exacerbate the situation (Stitt and Fairweather, 1991). Microfilaments have been demonstrated in the tegumental cell bodies of *F. hepatica* and their connections with the surface syncytium, and this supports their postulated role in secretory body transport (Stitt *et al.*, 1992a).

The sodium ionophore monensin is known to block the transport of secretory products through the Golgi complex, causing an osmotic swelling of the *cis* and medial cisternae, although it has no effect on their initial synthesis. It induces similar changes in the tegumental cells of *F. hepatica*, although the changes are more rapid in tissue-slice material than in whole flukes (Skuce, 1987; Skuce and Fairweather, 1989). So, the studies on secretory mechanisms in the tegument of the fluke show that the basic processes and pathways involved and the responses to established inhibitors of individual elements of the process are very similar to those observed in other secretory cell types.

Functions of the tegument

The synthetic activity of the tegument has been discussed above. It is also responsible for the uptake of small molecules – amino acids solely by diffusion

(Isseroff and Read, 1969), whereas monosaccharide uptake is via a carrier-mediated process that has been interpreted as facilitated diffusion rather than active transport (Isseroff and Read, 1974). The importance of an intact glycocalyx in protecting the fluke against digestive enzymes has already been discussed. The tegument is also the first line of defence against anthelmintics, for both adult and juvenile flukes. This is because the tegument of adults will be exposed to anthelmintics as they are being excreted in the bile, while that of juveniles is bathed in blood as they are migrating through the liver parenchyma. The interaction of the tegument with anthelmintics is discussed in Chapter 7 of this book.

Immune protection

The importance of the tegument to the fluke in resisting immune attack from the host has been demonstrated by the extent of immune-related damage observed in a number of experimental situations. For example, incubation of juvenile (16-day-old) flukes in immune serum *in vitro* has been shown to cause surface changes leading to complete destruction of the tegument; the damage was dependent on IgG_1 and IgG_2 antibodies (Eckblad *et al.*, 1981). Attachment of eosinophils to the surface of NEJ *in vitro* occurs in the presence of immune serum. The binding is independent of complement and does not affect the viability of the fluke, as measured by its ability to infect naive rats (Doy *et al.*, 1980; Doy and Hughes, 1982). Attachment of eosinophils and neutrophils to NEJ is mediated by IgG_1 and IgG_2 antibodies and is dependent on F_c receptors. In the presence of excess antibody, aggregations of antigen/antibody complexes build up over the surface of the parasites; they are shed into the medium. Again, no damage was observed and the viability of the flukes was unaffected (Duffus and Franks, 1980). In contrast to these light microscope studies, in which no damage was detected, more detailed electron microscope studies have shown that, in the presence of immune serum *in vitro*, eosinophils become attached to NEJ in those regions not covered by antigen/antibody precipitates. Subsequent degranulation of the eosinophils leads to vacuolation of the syncytium in these regions, thus showing that damage does take place (Glauert *et al.*, 1985).

Coating of NEJ with antibody occurs *in vivo* as well as *in vitro*. It can occur within the lumen of the intestine of sensitized (that is, resistant) rats. All classes of antibody are involved, but especially IgG and IgM (Burden *et al.*, 1982). The coating did not appear to cause any damage (Burden *et al.*, 1982, 1983) and became shed when the flukes were incubated in culture medium *in vitro* for 3 h (Burden *et al.*, 1982). The antibody coating is 'wiped off' as the fluke penetrates through the gut wall but, on entering the peritoneal cavity, the fluke is coated with antibody and host cells – initially eosinophils, but later neutrophils and macrophages. Degranulation of the eosinophils created pits in the tegument, leading to its erosion (Burden *et al.*, 1983). The results were confirmed by a separate study involving injection of NEJ into the peritoneal cavity of sensitized rats. The flukes were dead within 6 h of entry. Once the tegument had been breached by eosinophils, neutrophils were seen to attach to the internal tissues (Davies and Goose, 1981).

Introduction of adult flukes into the peritoneal cavity of sensitized rats led to their death between 9 and 12 h (Bennett *et al.*, 1980). In contrast to the mechanism of tegumental destruction described above for the NEJ, the host cells appear to penetrate the syncytium of the adult fluke and prise it off. The fluke attempts to replace the tegument with the formation of a new one underneath. In the early stages after transfer, there is an accelerated movement of T2 secretory bodies to the surface, followed by their release. In addition, there is evidence of blebbing of the surface membrane and formation of microvilli. These features are all indicators of a stress response by the fluke, to shed and replace damaged membrane, as described previously. The number of secretory bodies eventually declines and host cells start to penetrate the syncytium, but this is not seen until the supplies of T2 bodies become depleted (Bennett *et al.*, 1980).

Therefore, it should be apparent that an intact glycocalyx and syncytium are vital to the survival of the fluke. Once they are breached, this leads to more widespread damage to the internal tissues, and eventually the death of the fluke. The mechanism by which the fluke protects itself against immune attack is via the continual turnover and replacement of the apical plasma membrane and glycocalyx as a result of the synthesis and release of secretory bodies, particularly the T0/T1 bodies in the early migratory phases of development. The mechanism allows the fluke to shed antigen/antibody complexes and resist attack by immune effector cells; details have already been given.

Osmoregulation

Fasciola hepatica appears to carry out little or no water regulation in media of various concentrations, suggesting that it behaves like an osmoconformer. It is able to tolerate a fairly wide range of osmotic pressures *in vitro* (Δ 0.40 – 0.81°C) (Knox and Pantelouris, 1966). *Fasciola gigantica* acts in a similar way (Siddiqi and Lutz, 1966). The fluke's natural environment is bile, which has a relatively high osmolarity: 290–320 mosmol l^{-1} in sheep. Bile contains ions such as Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^- at concentrations that are believed to be 2–6 times those within the fluke itself (Threadgold and Brennan, 1978). Consequently, the fluke will be subject to either an influx of ions or efflux of water. In addition, the flame cells of the excretory system are continuously operating, resulting in the extrusion of large volumes of fluid.

These facts suggest that the fluke has a high requirement for water, as well as a need to reduce levels of certain ions. Evidence suggests that the tegument plays a role in ion and water control. As described previously, the tegument is characterized by amplification of both the apical and basal plasma membranes and the Na^+/K^+-ATPase activity associated with them is indicative of the presence of ion pumps (Threadgold and Brennan, 1978; Skuce *et al.*, 1987). Mitochondria are associated with the basal infolds, as are polymorphic masses of mucopolysaccharides. The basal infolds are particularly long and respond to changes in external osmolarity by swelling in hypotonic, and collapsing in hypertonic, media. The responses are not simply passive ones, because the infolds return to near normal configuration

within 1 h in either medium, even though the whole fluke may be shrunken or turgid depending on the osmolarity of the medium (Threadgold and Brennan, 1978). Thus, the tegument has many of the features of a transporting epithelium and a model has been put forward to account for its role in osmoregulation (Fig. 3.6). According to this idea, ions from the bile would enter the tegument by diffusion and could be pumped out again by the ion pumps associated with the apical plasma membranes and its ATPase. Ions which escaped the pumps would enter the cytoplasm of the syncytium and could be pumped out into the lumen of the basal infolds by their associated ion pumps. This could result in a lumen which was hypertonic to the surrounding cytoplasm, thus forming a standing gradient of the forward type. The energy required for the pumping would be supplied by the many mitochondria closely associated with the basal infolds and orientated along their long axes. Because of the standing gradient in the lumen of the infolds, water would be drawn from the tegumental cytoplasm and presumably be replaced by water movement into the tegument from the bile. Some of this bile-derived water could be trapped and held by the mucopolysaccharide masses associated with the basal infolds. These masses would then function as a water sink and so act as a buffer against sudden changes in fluid content of the bile which might either dehydrate or flood the tegument. The establishment of such a standing gradient, down which water could flow, would result in the drag or entrainment of solutes such as monosaccharides and amino acids through the tegument and into the fluke. If small organic molecules and ions were swept into the flask-like invaginations of the apical plasma membrane, this would increase their chance of being 'trapped' by the glycocalyx or of being transported. A further gradient for water, ions and small molecules could be established between the base of the tegument and the excretory system. This gradient could cause water solutes and metabolites to diffuse through the parenchyma and other organ systems, and also transport wastes or fluids into the excretory system (Threadgold and Brennan, 1978). As indicated above, this hypothesis as originally proposed was based on the assumption that the levels of ions are higher in the bile than in the fluke. However, more recent studies have shown that, of Na^+, K^+, Ca^{2+} and Mg^{2+} ions, only Na^+ ions are greater in bile than in the fluke; this was true of both bovine and ovine bile and their respective flukes (Caseby *et al*., 1995). Consequently, some revisions of the tegumental osmoregulatory model may be required.

Sensory perception

The tegument contains three types of sensory receptor, suggesting that it plays a role in sensory perception. There is a ciliated type which occurs between the spines on the anterior ventrolateral surfaces; a pair of these receptors also occurs in the mid-anterior dorsal surface. Each consists of a bulbous body containing mitochondria and vesicles, and is joined to the surrounding cytoplasm by means of a septate desmosome. Within the bulb, adjacent to the desmosome, are two electron-dense collars. The bulb also contains a basal body with striated rootlet, from which arises the single cilium

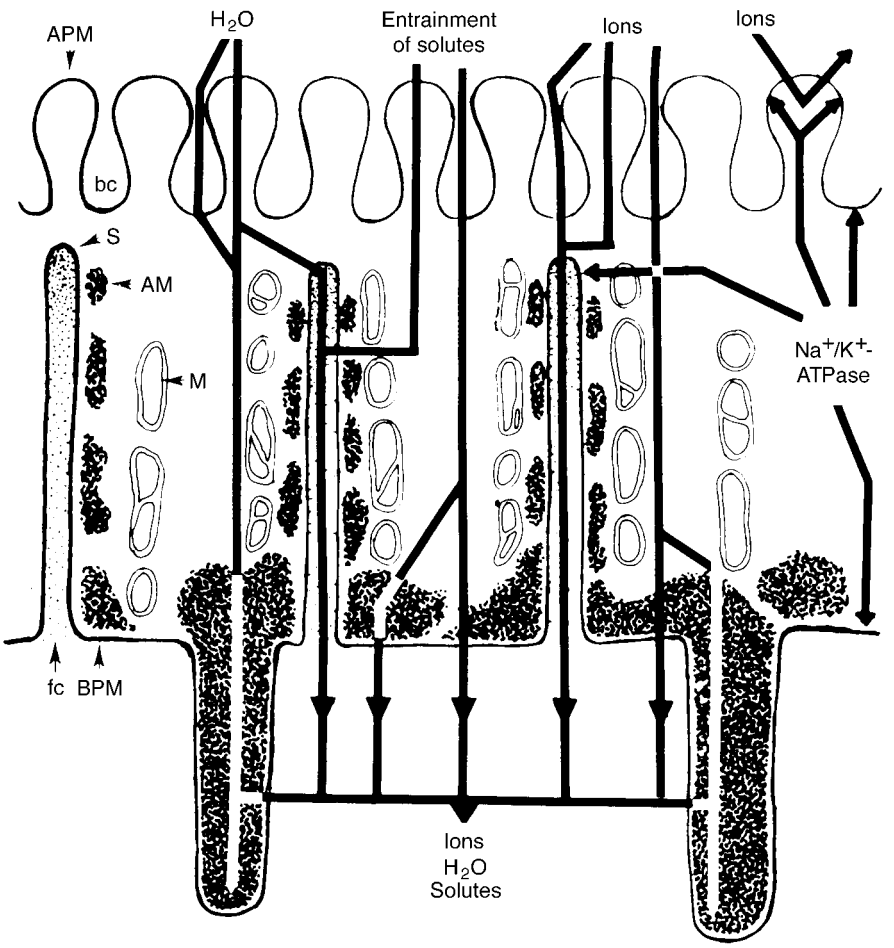

Fig. 3.6. Diagrammatic representation of the ion, water and solute flow through the tegument of *Fasciola hepatica*. AM, associated acidic mucopolysaccharide (vacuoles); APM, apical plasma membrane; bc, backward channel; BPM, basal plasma membrane; fc, forward channel; M, mitochondria; S, basal invagination (Threadgold, 1979).

which projects to the same height as the surrounding spines. This type of receptor presumably serves as a tangoreceptor (Bennett and Threadgold, 1973; Bennett, 1975a). A second non-ciliated and 'domed' type occurs within the spine-free tegument overlying the oral and ventral suckers: a pair of these receptors occurs on the dorsal lip of the oral sucker and six more are regularly spaced around the ventral sucker. Each comprises a bulb containing two electron-dense collars and a basal body with striated rootlet, although no cilium is present. Again, it is connected to the surrounding cytoplasm by means of a septate desmosome. It may serve as a pressure or contact receptor (Bennett, 1975a). The third type of presumed receptor consists of a

spiral of tegument apparently shielding a pit. A group of three of these receptors occurs on each side of the oral sucker. This type has not been observed by transmission electron microscopy so its internal structure is not known. It may serve as a chemoreceptor, allowing the fluke to 'taste' the substrate over which it is moving (Bennett, 1975a). The complement of receptors enables the fluke to detect changes in the external environment and pass on this information to more central regions of the nervous system to formulate an appropriate response. The data on receptors is limited to the newly excysted juvenile; later stages have not been examined.

Parenchyma

The parenchymal cells fill the spaces between the organ systems in the fluke. The cells are separated from each other and from other organ systems by interstitial material composed of fibres in a homogeneous matrix. It is believed that the parenchymal cells are responsible for the synthesis and secretion of the interstitial material, even though they do not show a great capacity for synthetic activity as evidenced by ultrastructure (Threadgold and Gallagher, 1966; Gallagher and Threadgold, 1967). The interstitial material probably functions as a flexible cytoskeleton, the elastic fibres allowing considerable distortions of body shape (Threadgold and Gallagher, 1966). It also provides anchorage for muscle fibres.

The parenchymal cells contain a few narrow cisternae of GER, numerous mitochondria that are arranged in groups near the nucleus and scattered throughout the cytoplasm of the cell, small Golgi complexes, a few acid phosphatase-positive primary lysosomes and a number of other inclusions that will be discussed below (Threadgold and Gallagher, 1966; Threadgold and Arme, 1974). In addition, the cells contain considerable quantities of α- and β-glycogen, thus serving as a main storage site for carbohydrate food reserves in the fluke (Threadgold and Gallagher, 1966). Incorporation of [^{3}H]glucose and [^{3}H]galactose into glycogen storage areas of the parenchymal cells has been demonstrated by Hanna (1976b). Particularly dense labelling occurred in the terminals of parenchymal cell entensions that pass between the muscle layers to lie close against the base of the tegument, reinforcing the view that glucose enters the fluke mainly across the tegument (Hanna, 1976b).

Originally viewed as a simple packing tissue, the parenchyma is now considered to carry out an important transport function and to be involved in carbohydrate metabolism, as well as performing a skeletal function via the interstitial material. In places, the interstitial material between the parenchymal cells is absent, allowing very close contact between the membranes of adjacent cells; the cells are joined by desmosome-like structures (Threadgold and Gallagher, 1966). Similar contacts are made between the parenchymal cells and the gut, excretory system, tegument and reproductive system. For example, pseudopodia-like processes from the parenchymal cells pass between the muscle blocks surrounding the gut, penetrate the interstitial material and basement membrane and extend for a short distance into the base of the gut

cells. Similar projections from parenchymal cells make contact with the flame cells and larger ducts of the excretory system and with the tegumental cell bodies and their connections with the surface syncytium (Gallagher and Threadgold, 1967). In the reproductive system, parenchymal cells form junctional complexes with the nurse cells within the vitelline follicles and with the seminal vesicle and ejaculatory duct (Irwin and Threadgold, 1970; Threadgold, 1975a). The junctional complexes permit the exchange of ions, metabolites and excretory products and, in the absence of a circulatory system, the parenchyma could serve as a means of transporting substances around the body of the fluke (Threadgold and Gallagher, 1966; Gallagher and Threadgold, 1967).

Starvation and refeeding experiments carried out by Threadgold and Arme (1974) have confirmed a role for parenchymal cells in carbohydrate metabolism. During starvation, mobilization of glycogen reserves (glycogenolysis) occurs (at least in part) by a process of autophagy, although the internal membranes involved are derived from the mitochondria, rather than from the endoplasmic reticulum which is more typical. Protrusions from the mitochondria become pinched off to form M bodies; the M bodies fuse to form smooth membranous cisternae (SMC) which engulf portions of cytoplasm containing glycogen, giving rise to β bodies. The β bodies fuse with primary lysosomes derived from the Golgi complexes to form secondary lysosomes and the glycogen is broken down by lysosomal enzymes to release glucose. Refeeding of starved flukes leads to replenishment of glycogen stores, although the mechanism involved is uncertain since no morphological correlates of glycogenesis have been observed. It has been suggested that the enzymes required for glycogen synthesis and the intermediates involved exist in soluble form in the cytoplasm (Threadgold and Arme, 1974).

In the NEJ, the parenchymal cells are filled with large stores of α- and β-glycogen and contain numerous mitochondria and some lipid droplets (Bennett and Threadgold, 1973; Bennett, 1977). The limiting plasma membranes of the cells are much invaginated. By 12 h pi, the cells are greatly increased in volume, and the glycogen stores have become depleted. The latter show signs of an increase by 24 h pi and are replenished by 2–3 days pi (Bennett, 1977). The increase in volume of the parenchymal cells is most likely to be due to rehydration after excystment during the metacercarial stage, and the initial mobilization of glycogen reserves may be linked to the migration of the NEJ across the gut wall (Bennett, 1977). Initially, the parenchymal cells only form junctional complexes with the excretory system and tegument; contacts with the caecal cells of the gut do not develop until 24 h pi (Bennett, 1977). Morphological evidence of glycogenolysis, as described above, is present at all stages of juvenile development, although in this study (Bennett, 1977) the parenchymal cells were never seen to synthesize or secrete any substances; this does not support the previous suggestion that these cells are responsible for production of the interstitial material (Threadgold and Gallagher, 1966).

Muscle

The main somatic musculature is situated beneath the tegument. It comprises two main layers – an outer circular muscle layer and an inner longitudinal layer. The antagonistic actions of these two layers are responsible for the rhythmic waves of contraction and relaxation that take place along the lateral margins of the fluke. The oral and ventral suckers are highly muscular organs too, of vital importance to the adult fluke in maintaining attachment to the lining of the bile duct and (for the oral sucker) in the suctorial feeding movements of the fluke. In more juvenile stages, both the suckers and the somatic muscles are important in the migratory movements from the gut lumen, through the gut wall, across the peritoneal cavity and through the liver parenchyma to the bile duct.

Little is known about the fine structural organization of the muscle cells in *F. hepatica*. The morphology of the cells is believed to be similar throughout the body of the fluke, in both the somatic and sucker musculature and to be identical in the NEJ and the adult fluke (Bennett and Threadgold, 1973). Essentially, the muscle is of the invertebrate smooth type, with the muscle cell body separated from the contractile portion of the cell, but joined to it by a cytoplasmic connection. The cell body contains the nucleus which is surrounded by a narrow layer of cytoplasm. The latter is packed with α- and β-glycogen; free ribosomes are also present, together with mitochondria that have few cristae (Bennett and Threadgold, 1973). The contractile region of the muscle cell contains thick and thin myofilaments, with thin being more numerous. Up to 12 thin myofilaments surround each thick filament, but there is no regular ratio between them (unpublished observations; also Ishii and Sano, 1980). The thick filaments have tapered ends and the thin filaments show evidence of branching (unpublished observations). Immunostaining for actin has been localized to the thin myofilaments within the muscle fibres of the somatic muscle layers (Stitt *et al*., 1992a). Dense bodies anchor the thin filaments to the sarcolemmal membrane and to the surrounding interstitial material (Bennett and Threadgold, 1973). A paramyosin-like protein has been extracted from fluke muscle (Ishii and Sano, 1980). Its molecular mass (98 kDa) and amino acid composition are similar to those of paramyosins from other invertebrates, although the structural pattern of the paracrystal exhibits some differences. The paramyosin is believed to be localized in the thick muscle filaments (Ishii and Sano, 1980).

The myofilaments are surrounded by a thin layer of cytoplasm which contains a number of mitochondria, glycogen granules and a poorly developed system of sarcoplasmic reticulum cisternae which lies beneath the outer sarcolemma (unpublished observations). Deposits of Ca^{2+}-ATPase activity have been localized to the sarcoplasmic reticulum and the sarcolemma, indicating the presence of Ca^{2+} pumps on these membranes (Skuce, 1987). The pumps may be involved in the control of the movement of Ca^{2+} ions into the cytoplasm of the muscle (across the sarcolemmal membrane and out of the sarcoplasmic reticulum) which will bring about muscle contraction; also in the opposite direction (involving sequestration in the sarcoplasmic

reticulum or exit from the cell) to promote muscle relaxation. However, the mechanism of muscle contraction in the fluke is poorly understood.

Nervous System

Following its excystment in the small intestine, the fluke penetrates through the gut wall into the abdominal cavity. It spends some time wandering within the cavity before locating and penetrating the liver. It then burrows through the liver parenchyma before reaching the bile duct, where sexual maturation takes place. Such a complex migration suggests that the fluke is able to detect specific environmental cues and respond to them with the appropriate behaviour pattern. There is some experimental evidence to support this idea. For example, bile is essential for excystment of the metacercaria, but is harmful to the long-term survival of the fluke (Tielens *et al*., 1981; Sukhdeo, M.V.K. *et al*., 1988). Indeed, it has been suggested that bile provides a negative chemotactic stimulus for the fluke to leave the gut and enter the abdominal cavity (Tielens *et al.*, 1981). Different bile salts have different effects on the movement of NEJ: dehydrocholic acid increases activity, whereas taurine- and glycine-conjugated chenodeoxycholic acids decrease locomotory cycles (Sukhdeo, M.V.K. *et al.*, 1988). The emergence response to the bile salt, glycocholic acid, is dose dependent, indicative of a receptor-mediated response (Sukhdeo, M.V.K. and Mettrick, 1986). Bovine bile has been shown to increase locomotory movements of the NEJ, as do duodenal extracts, although the latter response is lost within 2 days of development (Sukhdeo, M.V.K. and Mettrick, 1986; Sukhdeo, M.V.K. *et al*., 1987, 1988). In orientation assays, NEJ showed aversion responses to duodenal and liver extracts, high pCO_2 and acid pH, indicating an ability to orient in a directional manner to specific stimuli (Sukhdeo, M.V.K. and Mettrick, 1986). However, location of the liver by juvenile flukes is not believed to be the result of any chemotactic or orientation response; rather, a passive or random movement along the body wall, the fluke recognizing the liver only after contact and penetration.

The adult fluke is also able to respond to chemical cues in the form of hormones that will be ingested with the blood meal. For example, cholecystokinin increases the rate of ventral sucker activity *in vitro* (Sukhdeo, M.V.K. and Sukhdeo, 1989). Cholecystokinin acts to stimulate bile flow in the host and, in stimulating sucker activity and attachment behaviour, may serve to prevent the flukes from being flushed out of the bile duct *in vivo*. The related peptide, caerulein, inhibits oral sucker activity and the frequency of contraction of the longitudinal muscles; motilin, another gut hormone, inhibits ventral sucker activity and the frequency and amplitude of contractions of the longitudinal muscles (Sukhdeo, M.V.K. and Sukhdeo, 1989). The results suggest that the fluke can respond in different ways to individual hormones and can discriminate between closely related peptides.

While the data described above are limited, they do indicate that *F. hepatica* can respond in various ways to a range of 'environmental' cues within its host. As indicated in the section dealing with the tegument, the

fluke possesses a number of different types of sensory receptors, enabling it to respond to these cues. The cues elicit an extensive repertoire of behaviour patterns, the full extent of which we can barely appreciate at present. The release of a particular behaviour pattern at the appropriate time (and in the correct sequence to complete the life cycle) argues for a high degree of nervous coordination and a fairly complex nervous system. The more so when it is remembered that it is not just the metacercaria to adult transformation that we are concerned with here. Somatic continuity extends back to the cercarial stage, so the morphological changes, behaviour patterns and changes of environment with their associated cues that are involved are even more complex. The control of the ontogenetic changes resides in the nervous system, so it needs to be correspondingly sophisticated. Recent work on the neurochemistry, pharmacology and developmental changes in the nervous system suggests that this is so.

The gross neuroanatomy of *F. hepatica* was established towards the end of the last century, on the basis of histological studies at the light microscope level (e.g. Havet, 1900). The central nervous system contains a pair of anterior (so-called 'cerebral') ganglia situated either side of the pharynx just posterior to the oral sucker. The ganglia are connected by a transverse commissure that crosses over the dorsal surface of the pharynx (Figs 3.7 and 3.8). Nerves pass anteriorly from the ganglia to the oral sucker, but the main nerve cords run posteriorly along the body. Three nerve cords arise from each ganglion: ventral, dorsal and lateral, of which the ventral longitudinal nerve cord is the best developed (Figs 3.7 and 3.9). The peripheral nervous system comprises plexuses of cell bodies and nerve fibres beneath the tegument (in association with the subtegumental musculature), in the oral and ventral suckers and in association with the various reproductive organs and ducts.

The fine structure of the anterior ganglia in the adult fluke has been described by Sukhdeo, S.C. *et al.* (1988a). Their structure is atypical compared with that of other invertebrates in that nerve cell bodies are not confined to the periphery of the ganglia but are scattered within the neuropile as well. Each cell body has a large nucleus and a small amount of cytoplasm. The cytoplasm contains numerous mitochondria, Golgi complexes, endoplasmic reticulum, microtubules and a variety of vesicles (both electron-dense and clear vesicles). Two types of unmyelinated nerve process have been identified in the neuropile: small processes, less than 12 µm in diameter, and large or 'giant' processes, with a diameter greater than 12 µm. The small nerve processes contain four types of vesicle (one electron-lucent and three with electron-dense cores), while the giant processes contain electron-dense vesicles only. The giant nerve processes form the bulk of the nervous tissue in the commissure and longitudinal nerve cords and are characterized by extensive invaginations of their cell membranes. Two types of synapse occur between the small nerve processes: simple synapses with associated pre- and post-synaptic specializations, and wedge-shaped synapses (or divergent diads) with one presynaptic process synapsing on to two postsynaptic processes. No synapses have been observed between giant processes or between the small and giant processes (Sukhdeo, S.C. *et al.*, 1988a).

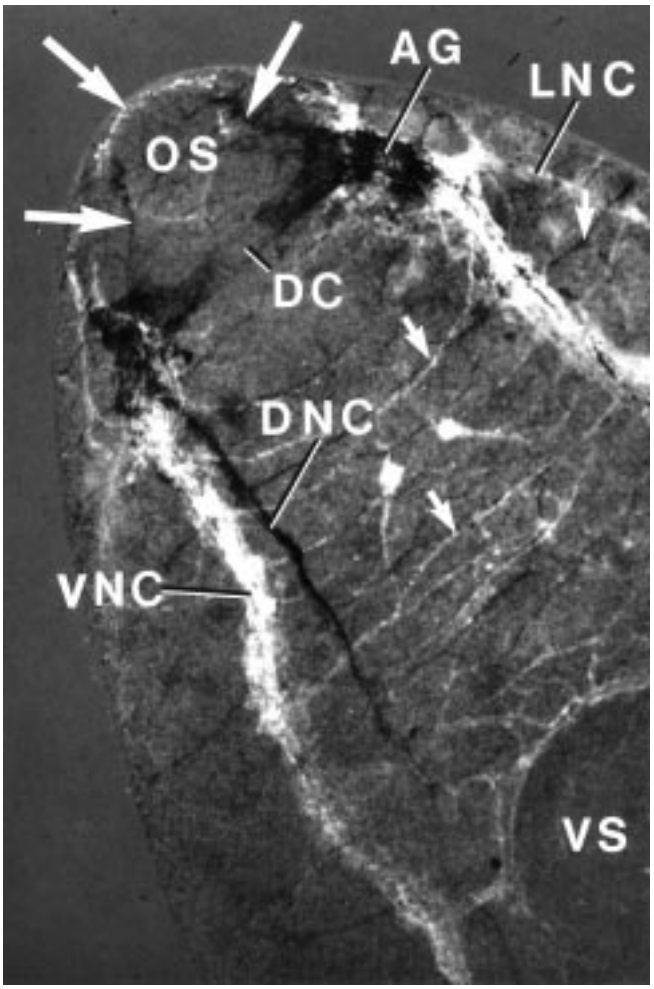

Fig. 3.7. Summation of two confocal microscope images through the forebody of an adult liver fluke showing the distribution of pancreatic polypeptide immunoreactivity in the central nervous system. The anterior ganglia (AG) are connected by a dorsal commissure (DC). Immunoreactive nerve fibres (large arrows) innervate the oral sucker (OS). From each ganglion arise three nerve cords: the dorsal (DNC), lateral (LNC) and ventral (VNC). Small arrows indicate transverse connections between the nerve cords. Note that the dorsal part of the nervous system is black while the ventral part is white. VS, ventral sucker.

The invaginations of the giant nerve processes are filled with a second, mesenchyme cell type, whose processes contain a large number of mitochondria and opaque inclusion bodies (Sukhdeo, S.C. *et al*., 1988a; Sukhdeo, S.C. and Sukhdeo, 1994). The mesenchymal cell bodies also contain mitochondria and inclusion bodies, together with Golgi complexes; they occur both around the periphery of the ganglion and within the neuropile. Their

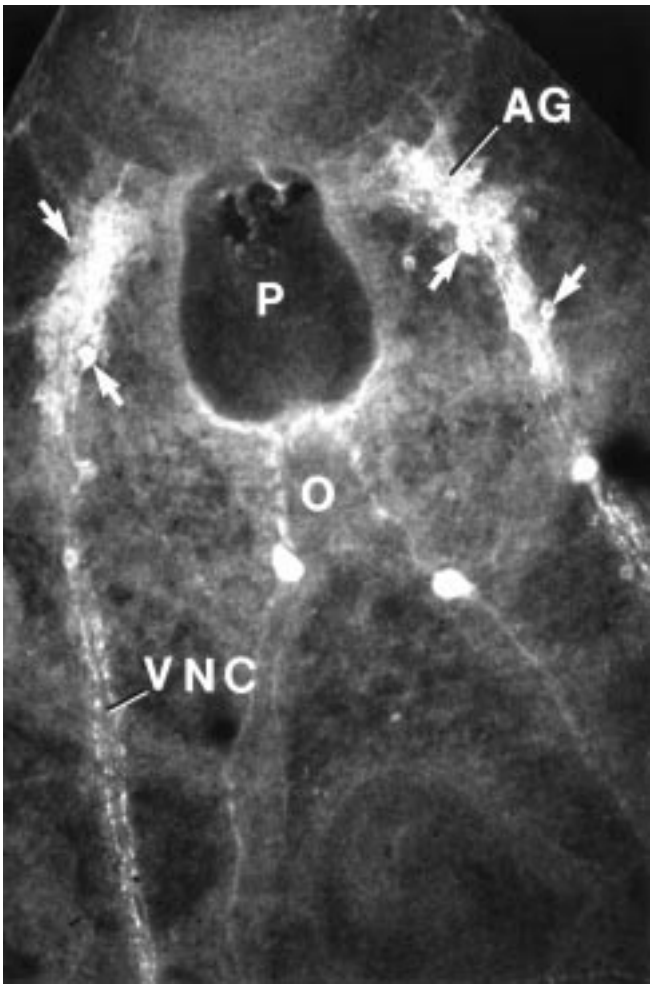

Fig. 3.8. Confocal microscope image showing substance P (SP) immunoreactivity in the anterior ganglia (AG) and ventral nerve cord (VNC) of an adult liver fluke. Nerve cell bodies (arrows) are associated with the anterior ganglia. Immunoreactive nerve fibres are present around the pharynx (P) and oesophagus (O).

processes encircle the ganglion, separating the neuronal processes from the surrounding parenchymal cells, and invaginate into the cell bodies of the giant nerve cells, in addition to their processes. It has been suggested that the mesenchyme cells represent primitive glial-like cells, serving a role in nutrient transfer or acetylcholinesterase production (Sukhdeo, S.C. and Sukhdeo, 1994). The mesenchyme cells and giant nerve cells only appear in the ganglia following entry of the fluke into the liver and by the time the fluke has reached sexual maturity, these structures occupy up to 60% of the ganglia (Sukhdeo, S.C. and Sukhdeo, 1990).

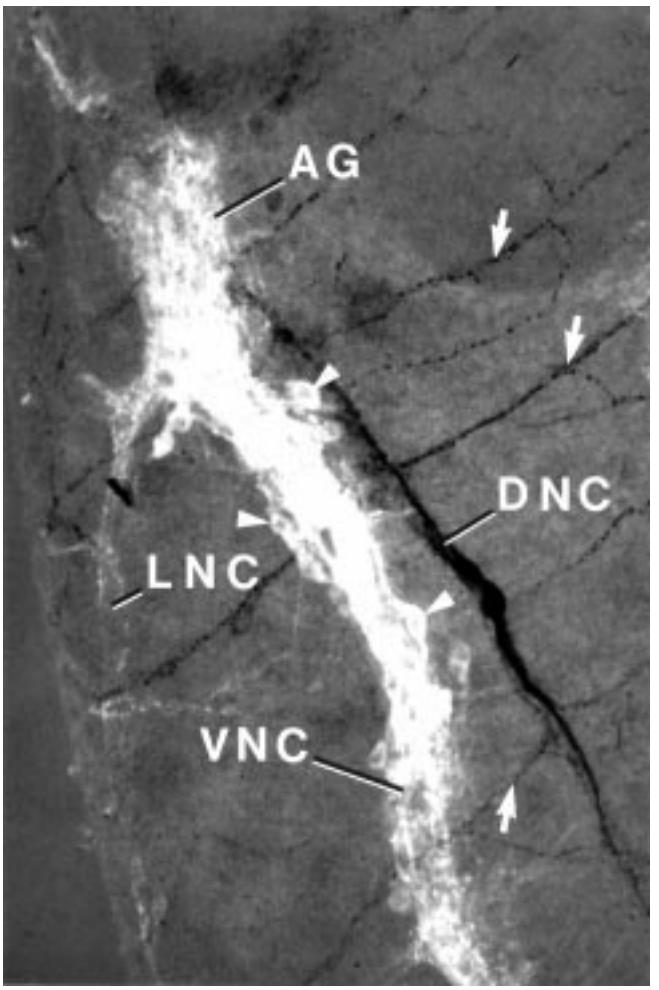

Fig. 3.9. Summation of two confocal microscope images showing pancreatic polypeptide (PP) immunoreactivity in the anterior ganglion (AG) and in the dorsal (DNC), lateral (LNC) and ventral (VNC) nerve cords. Immunoreactive nerve cells (arrowheads) are associated with the ventral nerve cord. Arrows indicate transverse commissures between the nerve cords. Note that the dorsal part of the nervous system is black while the ventral part is white.

The organization of the ganglia undergoes other ontogenetic processes. In 5-day-old flukes, each ganglion is surrounded by a complete rind of cell bodies, one cell thick, that envelops the central neuropile. In intrahepatic juvenile flukes, the integrity of the rind of cell bodies is absent, with the lining of the cell bodies being discontinuous. There is little evidence for any kind of rind in fully mature flukes – cell bodies occur within the neuropile, as well as around it, and mesenchyme cells are also observed within the neuropile (Sukhdeo, S.C. and Sukhdeo, 1990). The volume of the ganglia

increases enormously during development. This is due largely to an increase in the neuropile as a result of the appearance of giant nerve processes and the insinuation of mesenchymal cell elements into the giant cells and their processes. It is not certain whether the growth of the ganglia is accompanied by a corresponding increase in nerve cell bodies (Sukhdeo, S.C. and Sukhdeo, 1990). However, the appearance of giant nerve cells suggests that this is likely.

The variety of neuronal vesicles observed in ultrastructural studies suggests that the fluke possesses a number of transmitter molecules. The electron-lucent vesicles probably represent cholinergic vesicles, hence the presence of acetylcholine. Acetylcholine is also indicated by staining for acetylcholinesterase activity, acetylcholinesterase being the enzyme responsible for the inactivation of acetylcholine. Staining for cholinesterase activity in whole mount preparations has been used to delineate the gross anatomy of the cholinergic component of the nervous system (e.g. Ramisz and Szankowska, 1970). At the electron microscope level, acetylcholinesterase activity has been localized in the cisternae of the endoplasmic reticulum, in the Golgi complex (especially in the *trans* Golgi cisternae where secretory vesicles are budding off) and in vesicles near the Golgi complex – that is, in sites associated with the synthesis, packaging and transport of the enzyme (Sukhdeo, S.C. *et al*., 1988b). Acetylcholinesterase is also associated with the outer surface membrane of nerve cell bodies and nerve processes in the neuropile, especially between nerve processes and with synaptic clefts involving presynaptic endings characterized by the presence of clear synaptic (that is, presumed cholinergic) vesicles – both the simple type of synaptic contact and the wedge-shaped synapse. These are sites where one would expect the enzyme to be situated on the basis of its role in the breakdown of acetylcholine (Sukhdeo, S.C. *et al*., 1988b). Pharmacological studies have shown that acetylcholine inhibits the motility of *F. hepatica*, suggesting that it acts as an inhibitory transmitter in the fluke (Holmes and Fairweather, 1984).

The electron-dense neuronal vesicles indicate the presence of aminergic or peptidergic (neurosecretory) transmitters. Among potential aminergic transmitters, 5-hydroxytryptamine (or serotonin), dopamine and noradrenaline (norepinephrine) have been demonstrated in the fluke. The distribution of serotonin has been elucidated by immunocytochemical methods and the synthetic pathways established (see Fairweather *et al*., 1987). It causes a stimulation of fluke motility, suggesting that it is an excitatory transmitter (Holmes and Fairweather, 1984; Tembe *et al*., 1993). In addition to its role as a neurotransmitter, serotonin serves a wider metabolic role in the regulation of various aspects of carbohydrate metabolism, operating via a cAMP second messenger system (for references, see Fairweather *et al*., 1987). Dopamine and noradrenaline have also been localized in the nervous system (Bennett and Gianutsos, 1977; Gianutsos and Bennett, 1977) and they exert stimulatory and inhibitory effects, respectively, on fluke motility (Holmes and Fairweather, 1984).

More recent immunocytochemical studies on the peptidergic component of the nervous system have indicated a potentially greater number of

peptidergic than classical neurotransmitters. Thus, immunoreactivities to a number of vertebrate peptides have been localized to the nervous system: they are pancreatic polypeptide (PP), peptide tyrosine tyrosine (PYY), peptide histidine isoleucine (PHI), gastrin releasing peptide (GRP), substance P (SP), human chorionic gonadotropin (hCG) and pancreastatin (PST) (Figs 3.7–3.9). Immunostaining for the molluscan peptide FMRFamide and the flatworm peptides, neuropeptide F (NPF) and GNFFRFamide has also been demonstrated (Basch and Gupta, 1988; Gupta and Basch, 1989; Magee, 1990; Magee *et al*., 1989, 1991a; Marks *et al*., 1995). These fluorescent and confocal microscope studies at the light microscope level have been reinforced by immunogold labelling studies at the electron microscope level. Labelling for PP and FMRFamide has been localized to electron-dense vesicles in nerve cell bodies and processes within the main elements of the CNS. Double labelling demonstrated apparent co-localization of FMRFamide and PP immunoreactivities in the same dense-cored vesicles, although separate populations of vesicles labelling solely for FMRFamide were also evident (Brownlee *et al.*, 1994). The immunocytochemical evidence for the presence of neuropeptides has been supported by radioimmunoassay data, which have revealed interesting differences in the levels of peptides in flukes from different hosts (Magee *et al*., 1991a). However, no endogenous peptide has been isolated from *F. hepatica*, so the true identities of the peptides present in the fluke and which bear some relation to peptides present in other organisms are unknown. Having said that, the partial sequence of a PP-like peptide has been resolved (Magee *et al*., 1991b).

Peptide immunoreactivities represent the equivalent of what, in slightly older studies, would have been described as neurosecretions, on the basis of histochemical stains and the presence of large, dense-cored vesicles. The presence of neurosecretory vesicles and cells in *F. hepatica* has been described by a number of workers: Gresson and Threadgold (1964); Grasso (1967a,b); Grasso and Quaglia (1972, 1974); Radlowski (1975); Shyamasundari and Rao (1975). The neurosecretory (peptidergic) component of the nervous system assumes greater significance in the liver fluke and other flatworms because it functions as an endocrine system in the absence of true endocrine and circulatory systems. A gonad-stimulating role for neurosecretions in *F. hepatica* has been proposed by Grasso and Quaglia (1972). In support of a morphogenetic role for peptides in the fluke, NPF has been shown to inhibit protein and nucleic acid synthesis, while FMRFamide is stimulatory and GNFFRFamide has no effect (Fairweather *et al*., 1995). The effects of host gut peptides on fluke motility have already been mentioned and it may be that the fluke possesses their equivalents. A number of molluscan, nematode and platyhelminth FMRFamide-related peptides have been examined for their impact on fluke motility. Of the flatworm peptides tested, GYIRFamide had the most excitatory effect, stimulating motility at concentrations as low as 50 μM. RYIRFamide was slightly less potent, while NPF and GNFFRFamide were without effect (Graham *et al*., 1997). The role of peptides (and serotonin) in egg formation will be discussed in the section dealing with the reproductive system.

In addition to cholinergic, aminergic and peptidergic components of the nervous system, there is evidence for a fourth component incorporating amino acids. Thus, glutamate-like immunoreactivity has been localized in the CNS and PNS of *F. hepatica* (Brownlee and Fairweather, 1996).

The studies described above have shown that the fluke possesses a neurochemical complexity that belies its relatively simple neuroanatomy. The number of transmitters identified to date is small and probably represents the tip of the iceberg – more await discovery, especially neuropeptides. The transmitters underpin the wide range of behaviour patterns displayed by the fluke and it seems that their control will be even more complex than appreciated hitherto. The control of muscle activity, too, is likely to be more complex than the straightforward antagonism between cholinergic and aminergic elements that used to be presumed. Superimposed on these actions are important endocrine-like roles in controlling the development of the fluke – many of these await clarification (see Chapter 9, this volume).

Cytoskeleton

In a previous book on *Fasciola* (Pantelouris, 1965), the cytoskeleton of the fluke was not even mentioned – an indication of the fact that techniques (such as immunocytochemistry) for the visualization of the system were not well developed at that time. The intervening years have seen major advances in our understanding of the organization of the cytoskeleton and its role in many important cellular processes. Actin and tubulin, the major protein components of microfilaments and microtubules, respectively, have been isolated and their genes identified. Multiple forms of each are known, with different forms existing in different cell types. An ever-expanding number of cytoskeletal 'motors' and other proteins associated with microfilaments and microtubules have been isolated and their roles in cytoskeleton-based movements determined. The situation in 1965 is reminiscent of a statement made almost 20 years later by Birchmeier (1984) in a review of the cytoskeleton to celebrate the hundredth issue of *Trends in Biochemical Sciences*:

> It is reasonable to assume that in 1976 around 95% of experimental biologists were not aware of the fact that living cells have cytoskeletons and that such networks are somehow involved in cell motility! In 1984, however, 95% seem to know, and many of them now even consider the cytoskeleton to play a fundamental role in quite a wide variety of biological phenomena both in prokaryotes and eukaryotes.

The cytoskeleton can be divided into three components: microtubules (based on tubulin), microfilaments (based on actin) and intermediate filaments. In terms of helminth parasites, intermediate filaments have attracted little study. Most attention has focused on microtubules because they are the target for benzimidazole anthelmintics (see Chapter 7 of this volume). For *F. hepatica*, knowledge of the cytoskeleton has only increased in the last few years, thanks largely to the studies of Alan Stitt and colleagues.

Microfilaments play important roles in many cellular processes, including the maintenance of cell shape, movement of single cells (e.g. amoeboid movement), muscle contraction, cytoplasmic streaming, movement of secretory vesicles and cytokinesis (for references, see Stitt *et al.*, 1992a). Indirect immunofluorescence techniques using monoclonal and polyclonal anti-actin antibodies have shown that actin is present in the subtegumental and gut musculature of the fluke, as might be expected from its familiar role in muscle contraction. Actin is also present in the vitelline cells, in the spermatogenic cells, in the tegumental cell bodies and their connections with the surface syncytium, and in the tegumental spines (Stitt *et al.*, 1991, 1992a).

Incubation of fluke material in the microfilament inhibitor, cytochalasin B, leads to a number of changes. They include the movement of secretory vesicles in the tegumental and vitelline cells, cell shape and cytokinesis. There is a block in transport of tegumental secretory bodies from the cell body to the base of the tegumental syncytium and from the base of the syncytium to the apical plasma membrane prior to their release (Stitt and Fairweather, 1991). This leads to disruption of the apical plasma membrane which can be visualized by scanning electron microscopy (Stitt and Fairweather, 1991). Given the important roles that the tegument has and that the integrity of the apical plasma membrane is dependent on the continual movement of secretory vesicles and glycocalyx turnover, such disruption would have serious consequences for the fluke. Treatment with cytochalasin B also leads to disruption of the transport of shell protein globules in the vitelline cells and their aggregation to form shell globule clusters. Furthermore, deposition of shell protein material on the surface of newly formed eggs becomes uneven and abnormal (Stitt and Fairweather, 1991).

Evidence for inhibition of cytokinesis, the final separation phase in cell division, comes principally from studies involving the spermatogenic cells. During spermatogenesis, normal cell division and accompanying cytokinesis is complete up until the four-cell stage. Subsequent to this, although mitosis and meiosis continue, cytokinesis is incomplete, resulting in rosettes of 8, 16 and 32 cells (Stitt and Fairweather, 1990). Cytochalasin B treatment leads to formation of bi- and multinucleate cells and the typical rosettes of spermatocyte and spermatid cells are replaced by syncytial masses of cells: nuclear divisions continue but not cytoplasmic division. The results are consistent with the distribution of microfilaments in these cells. Moreover, spermatozoon formation becomes abnormal (Stitt *et al.*, 1991).

The combined results of immunostaining with actin antibodies and experiments involving cytochalasin B lend support to the suggestion that microfilaments are involved in a number of processes within the fluke. They include the movement of secretory vesicles in the tegumental and vitelline cells, muscle contraction, the maintenance of cell shape and cytokinesis. These processes are typical of established functions of microfilaments in other cell types. Immunoblotting studies of different fluke preparations using a monoclonal actin antibody indicate that there are at least three different forms of actin in the fluke: a 43 kDa form, probably associated with muscle; a 28 kDa form, not localized to any particular tissue; and a 15 kDa form in the

tegumental spines (Stitt *et al.*, 1992a). The latter result rejects a previous assertion that the spines of *F. hepatica* were not composed of actin (Pearson *et al.*, 1985). The idea that the fluke possesses multiple forms of actin with different tissue distributions and different functions is not unreasonable, because it is known to occur in other cell types (e.g. Otey *et al.*, 1987). Among other platyhelminth parasites, several different actin genes have been isolated from the tapeworm *Taenia solium* (Campos *et al.*, 1990) and a number of actin mRNAs demonstrated in the blood fluke, *Schistosoma mansoni* (Davis *et al.*, 1985). The expression of the actin genes varies during the schistosome life cycle (Davis *et al.*, 1985) and other work has shown that the different forms of actin are expressed in different tissues of the fluke (Abbas and Cain, 1989). Schistosome spines, like those of the liver fluke, are composed of actin (Cohen *et al.*, 1982; Davis *et al.*, 1985; Abbas and Cain, 1987; Matsumoto *et al.*, 1988; MacGregor and Shore, 1990).

Like microfilaments, microtubules are involved in many important cell processes, including the spatial organization and intracellular movements of organelles, the formation of the spindle apparatus and chromosome movements during mitosis and meiosis, the maintenance of cell shape and cell motility (via ciliary and flagellar movement) (for references, see Stitt *et al.*, 1992b). Using a monoclonal antibody raised against β-tubulin, tubulin has been localized in the tegumental syncytium, associated cell bodies and the connections between the cells and the surface syncytium. Immunostaining was also evident in the nerve fibres innervating sensory receptors in the tegument, in the nerve plexus innervating the subtegumental musculature and in the cytoplasmic extensions of the nurse cells within the vitelline follicle (Stitt *et al.*, 1992b). Immunoblotting of a whole fluke homogenate has shown that fluke tubulin has a molecular size of approximately 54 kDa, which is consistent with that of tubulin from other helminth parasites and eukaryotes in general (Stitt *et al.*, 1992b).

Incubation of fluke material in the microtubule inhibitors colchicine and tubulozole-C results in a number of changes within the fluke. In many respects the changes are similar, but show some differences and, in general, the changes induced by tubulozole-C are more severe and occur more quickly. Both drugs cause a block in transport of tegumental secretory bodies, but with colchicine accumulations of vesicles occur in the cell body (around the Golgi complexes) and at the base of the tegumental syncytium, whereas with tubulozole-C the accumulations occur at the base of the syncytium and in the cytoplasmic connections between the tegumental cells and the surface syncytium (Stitt and Fairweather, 1993). Tubulozole-C also induces a dramatic coalescence and vacuolation of the GER cisternae and their retraction towards the cell nucleus; the migration of the Golgi complexes to the periphery of the cell and their gradual disappearance from the cell; and a change in cell shape (Fig. 3.10). These are classic cell responses to microtubule inhibition, because microtubules are know to be responsible for the organization, cellular distribution and movement of these organelles (for references, see Stitt and Fairweather, 1993). The disruption of the GER and Golgi complexes leads to inhibition of secretory body synthesis and this, together with the block in

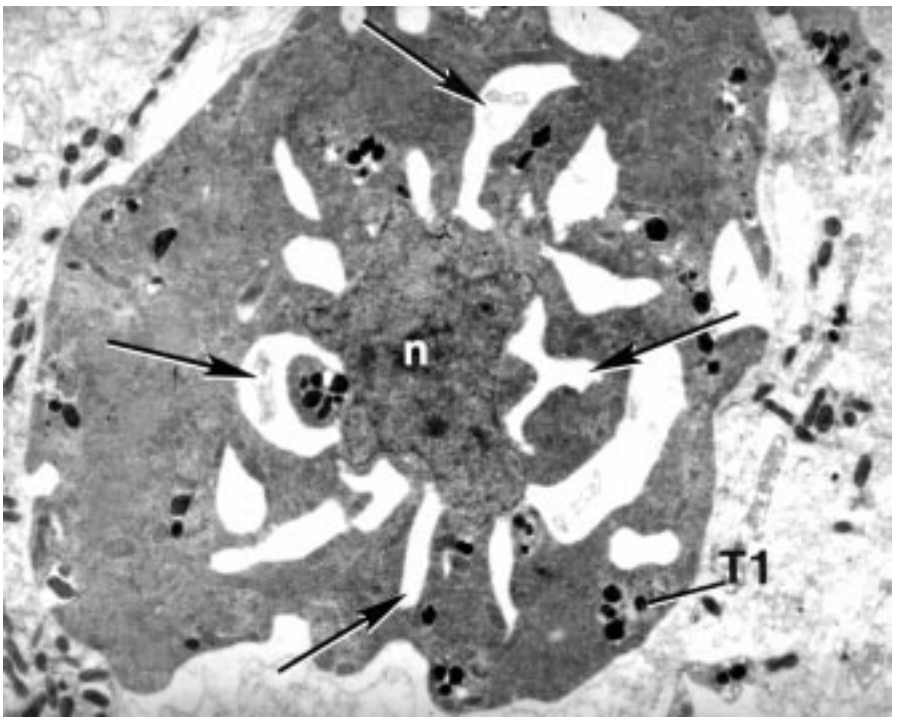

Fig. 3.10. Transmission electron micrograph of a T1 tegumental cell following treatment with tubulozole (1×10^{-6} M; 6 h). The cell contains a number of vacuole-like structures corresponding to swollen GER cisternae (arrows). In the cytoplasm there are only a few T1 secretory bodies (T1). n, Nucleus.

transport of existing vesicles, has a deleterious effect on the tegumental surface. In the case of tubulozole-C, the damage is so severe that it culminates in the sloughing of the syncytium (Stitt and Fairweather, 1993).

The two inhibitors prevent division of the stem vitelline cells, leading to relatively greater numbers of stem cells, but fewer intermediate-type cells (especially the It1-type) in the follicle. Less shell protein is synthesized, resulting in smaller and more loosely packed shell globule clusters and the movement of the globules to the cell periphery is also blocked. In the mature vitelline cells the production of 'yolk' globules and glycogen declines and this is accompanied by an increase in autophagy. The nurse cell cytoplasm becomes disrupted giving the follicle a disorganized appearance (Stitt and Fairweather, 1993). A more dramatic inhibition of mitotic activity by tubulozole-C has been observed in the spermatogenic cells in the testis. The spermatogonial cells are prevented from undergoing cell division and the spermatocyte and spermatid rosettes become disrupted and break apart. With longer periods of time the tubule becomes almost devoid of cells and some of the spermatozoa that remain show abnormalities in the organization of their microtubules (Stitt and Fairweather, 1992).

As was the case for microfilaments, the combined data from tubulin localization studies and inhibitor experiments indicate that microtubules are involved in a number of important processes in the fluke. They include the movement of secretory vesicles, the spatial organization of cellular organelles, maintenance of cell shape and cell division. These functions are typical of the roles played by microtubules in other cell types. *Fasciola* appears to be more susceptible to the action of tubulozole-C than colchicine, high concentrations (1×10^{-3} M) of the latter being required to bring about any effects. Tubulozole-C is known to be a more potent inhibitor than colchicine (De Brabander *et al.*, 1986). The two inhibitors have different modes of action and the relative insensitivity to colchicine may have a bearing on the limited efficacy of most benzimidazole anthelmintics against *Fasciola*. These drugs are known to act by binding to the colchicine-binding site on microtubules, thus disrupting microtubule-based processes in a variety of helminth parasites. In contrast, *F. hepatica* is susceptible to a benzimidazole derivative, triclabendazole, which lacks activity against other helminths. It is possible that triclabendazole acts in a tubulozole-C-like manner; this point is discussed in more detail in Chapter 7 of this volume.

In the last ten years or so there has been a marked interest in, and awareness of the significance of, the fluke's cytoskeleton. We now know that the fluke does possess a cytoskeleton (in common with other cells), that disruption of cytoskeleton-based processes has serious consequences for the fluke, and that the cytoskeleton has a prominent role in mechanisms underlying many important processes in cells belonging to different organ and tissue systems in the fluke. In the future, it is to be hoped that these roles will become clarified, that the genes for the cytoskeletal proteins will be identified and their stage and tissue expression be determined. Elucidating cytoskeletal mechanisms has a relevance to fluke chemotherapy and may be important in determining mechanisms and monitoring the spread of potential drug resistance problems.

Gut

The alimentary tract can be divided into two distinct regions – the foregut, comprising mouth, pharynx and oesophagus, and the paired intestinal caeca, which end blindly and whose lateral diverticula are highly branched in the adult fluke. Most of the observations on the morphology of the gut are confined to the intestinal caeca. The epithelial lining of the caeca consists of a continuous single layer of cells of one basic type, although they show considerable variation in fine structure (Robinson and Threadgold, 1975). The differences in structure reflect different functional states, the cells undergoing cyclical transformations between absorptive and secretory phases. Neighbouring cells are at different stages in the cycle so that secretion, absorption and digestion are occurring more or less simultaneously and continuously throughout the diverticula. Cells in the secretory phase (group I cells of Robinson and Threadgold, 1975) are characterized by the presence of many dense secretory vesicles, abundant and active Golgi complexes, an

extensive network of GER cisternae and numerous mitochondria, features typical of an actively secreting cell (Fig. 3.11). The absorptive cells (group II cells of Robinson and Threadgold, 1975) bear much longer and more numerous apical lamellae, between which lie exocytosed secretory granules and membranous whorls, possess few and largely inactive Golgi complexes, show a lack of secretory vesicles and contain numerous cytoplasmic bodies (indicative of endocytosis and autophagy) (Fig. 3.11). The main gut caeca are lined by a third form of the cell (group III cells of Robinson and Threadgold, 1975). The group III cells show some signs of secretory and autophagic activity, but their main function appears to be associated with the movement of material back and forth within the lumen of the main caeca and their diverticula and with the mixing of the released secretion with this material; this role is aided by the subepithelial musculature which is more developed along the main caeca than the diverticula (Robinson and Threadgold, 1975).

Development of the fluke gut in the mouse host has been described by Dawes (1962). Initially in the NEJ, the caeca are short and show signs of elongation in the first day following excystment. Lateral diverticula begin to develop in day 3 pi, following invasion of the liver. By day 8 pi, there are 13 diverticula on each side of the body and they are club shaped. Secondary and tertiary branches are present by day 11 pi, the intestine displaying much of the complexity of the fully developed system (Dawes, 1962). In the NEJ, the gut cells are filled with secretory vesicles produced and stored during the metacercarial phase. The cells appear to be specialized for secretion only, not undergoing the cycles of secretion and absorption seen in the adult fluke. A dramatic reduction in numbers of secretory vesicles is evident post-excystment, leading to the suggestion that they contain hydrolytic enzymes for use in excystment, penetration through the gut wall, migration and penetration of the liver capsule (Bennett and Threadgold, 1973). Secretory activity, as evidenced by the presence of Golgi complexes, begins during day 1 pi. The development of apical lamellae indicates limited absorptive capacity, mainly of small molecules in solution (Bennett, 1975c). A second, smaller type of secretory vesicle appears in day 3 pi following penetration of the liver capsule and the cells start to assume a more adult-like morphology, but true cyclical activity is not evident until 2 weeks pi (Bennett, 1975c).

Secretory activity in the gut cells of the adult fluke has been studied by pulse-chase autoradiography involving tritiated amino acids (tyrosine, methionine, leucine and phenylalamine) (Hanna, 1975). Initially (following a 0–10 min chase period) the radioactive labels were incorporated into protein being synthesized by the GER in the base of the cell. After a 20 min chase period, label had moved, via the transition vesicles, into the Golgi cisternae and was associated with the secretory bodies packaged by the Golgi complex. In longer chase periods (30–45 min), label was predominantly located in the apical region of the cell, being associated with the secretory bodies, with material being released at the apical surface and with the lamellae. Little activity was detected in material following a 60 min chase period, indicating that the entire process of synthesis, transport and release of secretory proteins takes less than 1 h (Hanna, 1975). The results also suggest

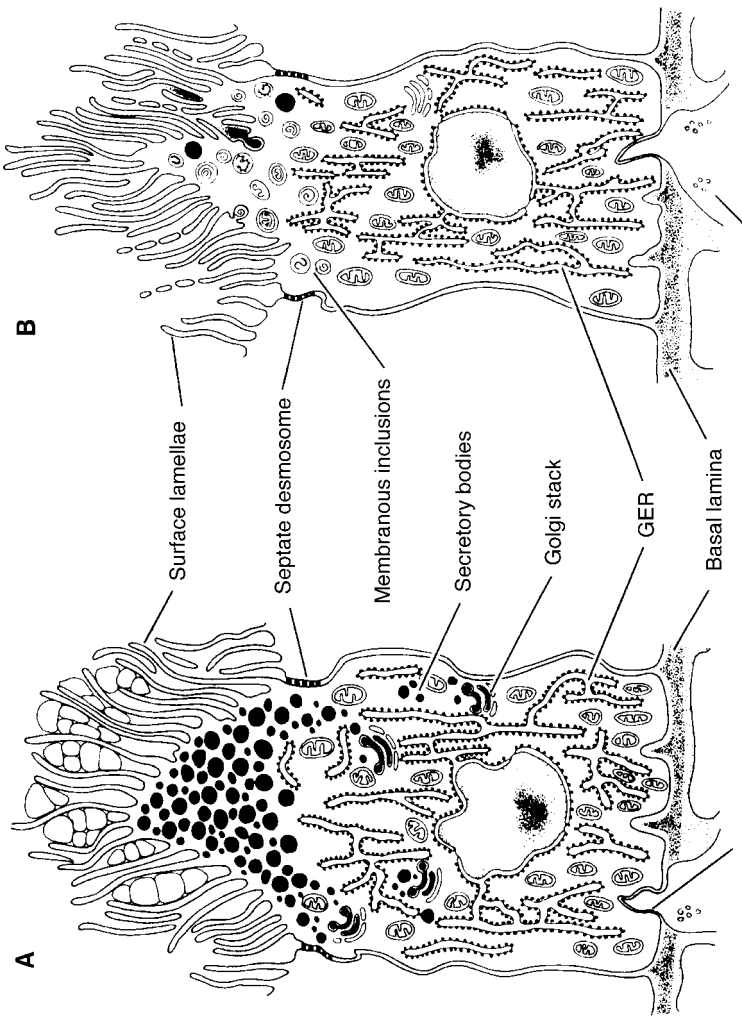

Fig. 3.11. Diagrams showing the fine structure of the gut cell in *Fasciola hepatica* in (A) the secretory phase (group I-type cell) and (B) the absorptive phase (group II-type cell) of its cycle (Smyth and Halton, 1983; after Robinson and Threadgold, 1975).

that the entry of label occurs across the basal and lateral membranes, rather than the apical membrane, and probably via the parenchymal cells and their inpushings into the gut cells, as described previously.

As is typical of digenetic trematodes, digestion in *F. hepatica* is predominantly an extracellular process, taking place in the caecal lumen and completed intracellularly. The eccrine release of secretions by the epithelial cells is associated with disruption of the apical plasma membrane and lamellae. This membranous material, together with any substances adsorbed on to its glycocalyx or trapped between the lamellae, is then endocytosed and gives rise to cytoplasmic bodies in the cell. The bodies are reactive for acid phosphatase, which is derived from the ingested lamellar membrane (see below), and develop into secondary lysosomes. Their contents undergo further enzymic breakdown and the soluble products diffuse into the surrounding cytoplasm; the indigestible remnants accumulate in residual bodies which are extruded from the cell. The diet of the fluke changes during its migration within the mammalian host. Initially, during the gut penetration phase, the gut is predominantly secretory and any ingestion of tissue debris resulting from penetration is of minor importance. Feeding on abdominal viscera is probably limited and insufficient for growth and development, since flukes which become lost in the abdominal cavity remain stunted (Dawes, 1963). Following entry into the liver, the juvenile fluke feeds largely on hepatic cells together with some ingestion of blood. The adult fluke, resident in the bile duct, is primarily a blood-feeder although it ingests hyperplastic bile duct epithelium as well (for a more detailed discussion of the feeding habits of the adult fluke, see Smyth and Halton, 1983).

A number of potential digestive enzymes have been demonstrated in *F. hepatica*. For example, acid phosphatase activity has been localized to the apical plasma membrane lining the lamellae and to the GER within the gut epithelial cells (Threadgold, 1968; Fujino *et al*., 1983; Yamasaki *et al*., 1992). The enzyme associated with the lamellae may be involved in the initial stage of digestion, the partly digested material adhering to the lamellar membrane being endocytosed and broken down further in the cytoplasmic bodies referred to above by enzymes (including acid phosphatase) derived from lysosomes. The fluke possesses a range of proteinase enzymes that are capable of degrading various protein substrates, including collagen, haemoglobin and immunoglobulins. The immature, migratory fluke possesses a collagenase enzyme of use in penetration of the gut wall, the liver capsule and the bile duct (Howell, 1966). Proteolytic enzymes capable of cleaving haemoglobin and immunoglobulins have been isolated and partially characterized from excretory/secretory (E/S) products (Rupova and Keilova, 1979; Simpkin *et al*., 1980; Chapman and Mitchell, 1982; Dalton and Heffernan, 1989; Rege *et al*., 1989). The fluke also possesses proteinase enzymes capable of cleaving trypsin and chymotrypsin substrates at alkaline pH (Hadjú *et al*., 1979). The substrate specificity of the enzymes indicates roles in nutrition (in the digestion of ingested red blood cells) and in immune evasion (by preventing antibody-mediated attachment of immune effector cells). Using immunocytochemical methods, a cysteine proteinase with

properties similar to those of cathepsin-like enzymes has been localized to secretory vesicles within gut epithelial cells and to host blood cells in the intestinal lumen, supporting a role in digestion for this enzyme (Yamasaki *et al*., 1992). More recently, a proteinase has been isolated from *F. hepatica* that possesses considerable sequence homology with cathepsin L-type enzymes (Smith *et al*., 1993). The enzyme has been localized to secretory vesicles in gut epithelial cells and possesses the ability to cleave immunoglobulin and inhibit antibody-mediated attachment of eosinophils to newly excysted juveniles (Carmona *et al*., 1993; Smith *et al*., 1993). It has been suggested that the latter may supplement the continual turnover of glycocalyx as part of the strategy used by the fluke to avoid the immune response of the host. Subsequent cloning of cysteine proteinase genes has revealed sequences for several enzymes, most showing homology to cathepsin L though some have homology to cathepsin B (Heussler and Dobbelaere, 1994; Wijffels *et al*., 1994; Dalton and Brindley, 1997).

The complement of enzymes present in the fluke may vary with its stage of development. For example, three cysteine proteinases have been demonstrated in intrahepatic juvenile flukes and four in the adult: only one was common to both and possessed substrate specificity similar to mammalian cathepsin B (McGinty *et al*., 1993). In a separate study, four cathepsin L-like proteinases identified in the E/S products of NEJ juveniles were not present in later stages and the 5-week intrahepatic stage possessed an enzyme that was not produced by the 3-week or adult fluke (Carmona *et al*., 1993). The results suggest that the fluke produces different enzymes at different stages of development and this may be associated with changes in environment and diet during the migration of the fluke and can be linked with the morphological changes in the gut accompanying development that have been described above. The potential use of cysteine proteinases to vaccinate host animals is discussed in Chapter 15 of this volume.

Excretory System

The excretory system consists of flame cells which are connected to fine tubules. In turn, the tubules lead into primary ascending or descending ducts which feed into the main, paired ascending, then descending, ducts which drain into a single bladder that opens posteriorly via a median excretory pore. The terminal flame cell contains a large oval or kidney-shaped nucleus, mitochondria, a few cisternae of GER and vesicles (Pantelouris and Threadgold, 1963; Gallagher and Threadgold, 1967). It bears a bunch of 120–135 hexagonally arranged cilia which form the 'flame' and project into the lumen of the associated tubule (Pantelouris and Threadgold, 1963) (Fig. 3.12). The beating of the flame creates a movement of fluid within the system. It has been suggested that the axial filaments of individual cilia within the flame are organized in such a way as to enable alternate rows of cilia to beat in opposing directions, resulting in equal propulsion for both directions of the stroke (Smyth and Halton, 1983). The cilia are anchored in the cytoplasm of the cell by means of basal bodies, and fibres from the basal

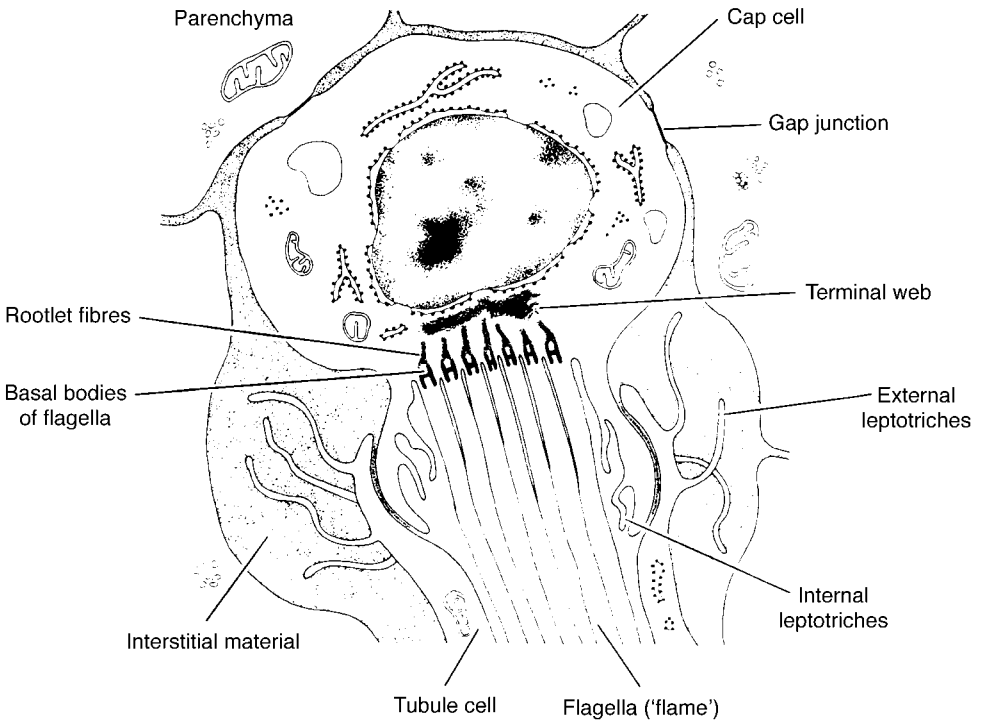

Fig. 3.12. Diagram showing the fine structural organization of a flame cell and associated structures in *Fasciola hepatica* (Smyth and Halton, 1983).

bodies and their rootlets penetrate more deeply into the cell to form an extensive network (Pantelouris and Threadgold, 1963).

The proximal part of the tubule leading away from the flame cell is composed partly of the cytoplasm of the flame cell and partly of the cytoplasm of the tubule cell. The two cells are connected by interdigitations of ribs of cytoplasm, which are connected to each other by fibrous elements. The ribs occurring internally to the sheet originate from the flame cell and those occurring externally arise from the tubule cell (Pantelouris and Threadgold, 1963; Gallagher and Threadgold, 1967). Ultrafiltration across the fibrous sheet is believed to occur in the spaces between the ribs. Additional microvillus-like projections, termed internal leptotriches, extend from the flame cell cytoplasm into the lumen around the flame. Similar structures, termed external leptotriches, extend from the tubule cell into the surrounding interstitial material (Pantelouris and Threadgold, 1963; Gallagher and Threadgold, 1967) (Fig. 3.12). The leptotriches presumably serve a structural role. While the lumen of the proximal region of the small tubule leading from the flame cell is intracellular, that of more distal regions of the tubule and of the primary ascending and descending ducts is intercellular. The lining is

formed of one or two cells surrounding the lumen and joined to themselves or adjacent cells by septate desmosomes. The cytoplasm contains some mitochondria, a few smooth-membraned inclusions and the luminal plasma membrane is thrown into short irregular microvilli (Pantelouris and Threadgold, 1963; Gallagher and Threadgold, 1967; Bennett and Threadgold, 1973; Bennett, 1977). Some of the cells bear groups of cilia in the newly excysted juveniles (Bennett and Threadgold, 1973) but the cilia are absent in the adult (Bennett and Threadgold, 1973; Bennett, 1977). The function of the cilia may be to assist fluid movement against capillary drag, as the ascending ducts of the juvenile are half the diameter of adult ducts. Another possibility is that the cilia specifically help to pump fluid through the excretory system of the metacercaria while enclosed in the cyst (Bennett, 1977).

The lining of the main collecting ducts and bladder is syncytial, the cytoplasm containing mitochondria, ribosomes, GER, Golgi complexes, β-glycogen granules and large lipid droplets. The apical surface bears long, narrow lamellae and the basal plasma membrane forms invaginations (Gallagher and Threadgold, 1967; Bennett and Threadgold, 1973). The lining of the excretory bladder has a morphology similar to that of the ducts, although the lamellae may be longer. Near the excretory pore is a long septate desmosome connecting the bladder wall to the distal tegument, which appears to line the pore opening itself (Bennett and Threadgold, 1973).

The primary ascending ducts are not present in the NEJ but arise and grow early during development, as the posterior region of the body grows (Bennett, 1977). The number of flame cells doubles during the first 10 days of development in the mouse host, again mainly in the posterior region of the body (Kawana, 1940) (Fig. 3.13). A 'reserve bladder' is not present in the metacercaria but begins to develop 3 days after feeding in the juvenile (Kawana, 1940). The lumen of the main descending duct and bladder of the NEJ is filled with large round concretions, built up of concentric rings of granular or fibrous material, but they disappear within 24 h (Bennett and Threadgold, 1973; Bennett, 1977). The concretions resemble calcareous corpuscles of cestodes and may serve roles in carbon dioxide fixation, in buffering of excretory fluids and in concentrating metabolic waste during the metacercarial stage when removal of waste products would be difficult (Bennett and Threadgold, 1973; Bennett, 1977). Lipid droplets appear in the syncytial lining and lumen of the main ducts and bladder by 12 h pi, suggesting that the excretory system comes into operation very quickly, ejecting the concretions and becoming involved in lipid metabolism (Bennett, 1977). The number of lamellae lining the main ducts and bladder increases in the 12 h following excystment, as does the number and length of the invaginations of the basal plasma membrane. It is evident, then, that there are a number of gross changes in the excretory system that take place during the early stages of development in the final host, but the basic ultrastructure and functioning of the adult system is considered to be established by 12 h pi (Bennett, 1977).

The excretory system is surrounded by interstitial material. In places around the circumference of the flame cell and along the ducts, the layer is

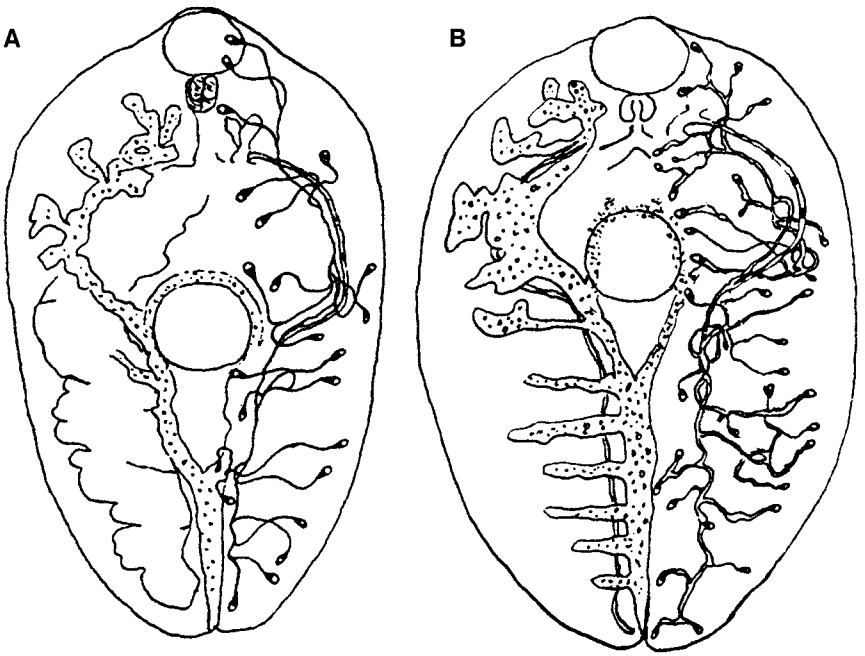

Fig. 3.13 (*and opposite*). Diagram showing the development of the excretory system of *Fasciola hepatica* in the mouse host. A, 5 days post-infection; B, 10 days post-infection; C, 11 days post-infection; and D, 12 days post-infection.

penetrated by processes from the parenchymal cells. Junctional complexes form at the points of contact between the excretory and parenchymal cells, as previously described (Gallagher and Threadgold, 1967). Acid and alkaline phosphatase activity is associated with these complexes, the enzyme being located on the parenchymal side of the complex (Threadgold, 1968; Fujino *et al*., 1983). This observation supports the idea that the complexes are sites where intercellular exchange of excretory products takes place, emphasizing the role of the parenchyma as a transport system (Threadgold and Gallagher, 1966; Gallagher and Threadgold, 1967). Deposits of phosphatase activity are present within and between the surface lamellae and are associated with the basal invaginations of the syncytial epithelium. Enzyme activity appears to be confined to the smaller- and medium-sized ducts of the system, perhaps being related to the resorptive and excretory functions of these ducts, as compared with the purely excretory function of the large ducts (Threadgold, 1968). Moreover, it is indicative of transport activity, an activity that is aided by the amplification of the apical and basal plasma membranes. Pinocytotic activity has been observed on the luminal surfaces of the flame cells, tubules and primary ducts of juvenile flukes, but does not occur in the adult. The process may be of benefit to the rapidly growing juveniles by increasing the resorption of potentially metabolizable molecules from the fluid drawn into the system by the flame cells (Bennett, 1977).

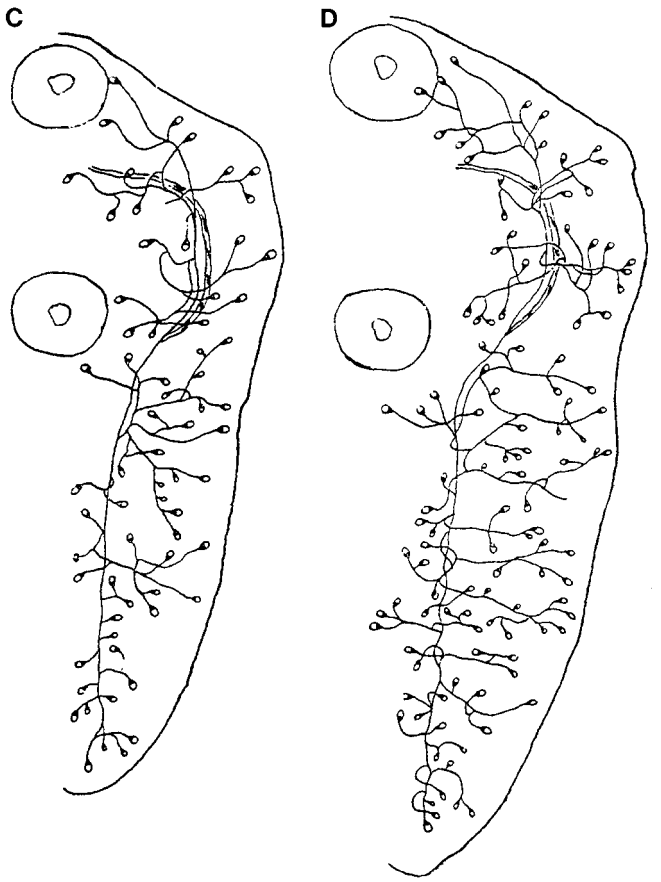

Fig. 3.13 (*continued*)**.**

The fluid in the lumen of the excretory system contains nitrogenous compounds, such as ammonia and urea, and several amino acids, including proline, alanine and histidine (Lutz and Siddiqi, 1971). Both neutral (including cholesterol and its esters) and polar lipids are excreted (Burren *et al*., 1967), being released from the syncytial epithelium by an apocrine mechanism (Bennett, 1977).

In conclusion, the excretory system of *Fasciola* appears to play a role in the regulation of body-fluid composition and in the excretion of waste. However, it shows little evidence of any response to ionic or osmotic stress; it acts as an osmoconformer, being in osmotic balance with its host's body fluids. There is no regulation of water content in hypo- or hypertonic media (Knox and Pantelouris, 1966; Siddiqi and Lutz, 1966). In contrast, the tegument has some control over its osmotic state (Threadgold and Brennan,

1978) and this aspect of tegumental biology has been discussed in the section dealing with the tegument.

Reproductive System

The reproductive system of *F. hepatica* is hermaphrodite. In the male system, there are two much-branched testes, one behind the other, which occupy much of the space in the middle of the body. From each testis a vas deferens leads forwards, the two vasa deferentia uniting to form the seminal vesicle which lies within the cirrus sac. The seminal vesicle passes into the ejaculatory duct which in turn leads into the protrusible cirrus; the latter opens to the exterior via the common genital pore, situated anterior to the ventral sucker. The ejaculatory duct is surrounded by cells belonging to the prostate gland.

In the female system, there is a single ovary, situated on one side of the body more anteriorly than the testes. Like the testes, the ovary is branched, but the branches are thicker. A short oviduct leads into the midline, close to where the ducts from the right and left vitelline glands converge to form the vitelline reservoir. The vitelline glands are composed of numerous follicles and are extensive, lying along the lateral margins of the body. A short duct from the vitelline reservoir joins the oviduct to give rise to the common ovovitelline duct and this leads into the egg-producing chamber, or ootype. The latter passes into the convoluted uterus which runs forwards to the common genital pore, from whence the eggs are released to the exterior. The ootype is surrounded by cells of Mehlis' gland.

Development

The development of the fluke reproductive system in the mouse host has been described in detail by Dawes (1962) and only the most significant features will be repeated here. The genital rudiment is present in the metacercaria and NEJ. It has an hour-glass shape: the anterior portion gives rise to the cirrus, cirrus pouch and terminal part of the uterus; the posterior region forms the gonads, Mehlis' gland complex and related ducts; and the intervening region forms the main parts of the uterus and vas deferens. By 22 h pi the posterior end of the rudiment has become U-shaped, the two limbs developing into the testes. The rudiment grows larger in the next two days and shows signs of separation; separation is complete by day 8 pi and the testes are separated from the rudiment of the ovary and Mehlis' gland complex. At this stage, the anterior end of the genital rudiment shows some indication of duality, the larger portion representing the rudiment of the cirrus pouch. By day 11 pi, the testes are lobed and the ovarian rudiment is growing out from the rudiment of the Mehlis' gland complex. The testes are much branched by day 13 pi and the ovarian rudiment is more elongate and shows three or four branches. The Mehlis' gland complex stands out sharply and in front of it the uterus displays slight folds. The rudiment of the cirrus, which is clearly developed at day 11 pi, becomes more prominent at day 18 pi and the branching of the testes is more extensive. The testes approach the follicular stage by day 21 pi; at this

stage the ovary is longer and its outgrowths more branched, the cirrus forms an S-shaped bend and the uterus is longer and more folded. These trends continue through day 24 pi to day 28 pi when the lateral fields of the body are filled with vitelline follicles, although the vitelline cells are immature. Full maturity is reached by day 37 pi in the mouse (although it may be attained earlier) and several hundred eggs are present in the uterus. Secretory activity in the vitelline cells appears to be switched on a few days before maturity.

Male system

Testes and spermatogenesis

The most complete account of spermatogenesis and the fine structure of the mature spermatozoon of *Fasciola hepatica* has been provided by Stitt and Fairweather (1990). The following description is based on this account; other references will only be included where they illustrate specific points not in that account. During spermatogenesis, the primary spermatogonium undergoes three mitotic and two meiotic divisions to give rise to 32 spermatids, which become morphologically transformed into spermatozoa. Cell development begins at the periphery of the tubules and, as cells proceed through spermatogenesis, the later stages become increasingly located towards the centre of the lumen of the tubule. The primary spermatogonia are highly basophilic and have a high nucleo-cytoplasmic ratio. The cytoplasm is packed with free ribosomes and contains many small mitochondria, typically grouped at one pole of the cell. Sections through primary spermatogonia which are nearing the end of interphase often contain a centriole pair, located close to the nucleus.

The secondary spermatogonia differ little from the primary spermatogonia. They divide to produce four tertiary spermatogonia which lie grouped together but are not joined. However, their inner faces begin to push out conical protrusions towards the centre of the four-cell cluster. Mitochondria move into and accumulate within the protrusions. The protrusions display a strong reaction for actin, suggesting that microfilaments are involved in their formation, a process that bears some analogy to the development of growth cones in other cell types, particularly nerve cells (Stitt *et al.*, 1991).

Mitosis of the tertiary spermatogonia results in a rosette of eight primary spermatocytes, joined together by means of cytoplasmic bridges connecting a central cytoplasm or cytophore. The latter is presumably formed as a result of fusion of the conical processes observed in the tertiary spermatogonia and subsequent incomplete cytokinesis of the cells. Actin filaments are concentrated in the cytophore region of this and later rosette stages, as might be expected from the established role of actin in the cytokinesis phase of cell division (Stitt *et al.*, 1991). The nuclei of the primary spermatocytes contain one or more synaptonemal complexes, reflecting the pairing of homologous chromosomes at zygotene during meiotic prophase. Golgi complexes and long cisternae of GER are present in the cytoplasm of these cells. The first meiotic division of the primary spermatocytes produces a rosette of 16 secondary spermatocytes, which appears to be a short-lived stage. Upon

completion of meiosis, there are 32 fusiform spermatid cells, still connected by a fairly large cytophore.

It is at this stage that spermiogenesis begins and each cell undergoes a number of morphological changes during its development into a mature spermatozoon. Differentiation of the spermatid commences with elongation of the cell and its nucleus. A number of nuclear pores appear along the length of the nuclear envelope as it elongates. The chromatin becomes increasingly condensed and granular, the granules eventually forming lamellar-like structures, lying along the longitudinal axis of the nucleus. The lamellae become tightly folded into a reticulum of scroll-like structures, which appear as a honeycomb structure when cut transversely. Accompanying the nuclear changes is the formation of the zone of differentiation at the distal end of the cell with respect to the cytophore. Initially, this takes the form of a conical projection from the cell, the plasma membrane of which is underpinned by a single row of microtubules. It contains a pair of centrioles, which divide to form four such structures, two of which come together to form the 'central body'. The two remaining centrioles lie on either side of the central body and become basal bodies for the development of the axonemes. The central body together with the basal bodies probably represent a microtubule-organizing centre, the basal bodies being responsible for the formation of the axonemes, while the central body may be responsible for the cortical microtubules which provide support for the zone of differentiation as it elongates to form the median process. Initially, the axonemes develop at right angles to the central body but later rotate through 90° to lie parallel to the median process. The next stage is characterized by the migration of the nucleus into the median process: it migrates to the distal end of the process and, as it does so, the two axonemes and median process fuse together to form a single structure. Along with the nucleus, two mitochondria (formed by fusion of a larger number) migrate into the median process. The fully developed spermatozoon is pinched off from the residual cytoplasm at a point marked by a groove-like collar at the proximal end of the median process and is released into the lumen of the testis tubule.

Living spermatozoa are very long, thread-like structures, approximately 400 µm in length. They exhibit active undulatory movements, especially along the anterior and middle regions, whereas the posterior (nuclear) region is relatively immotile.

The structure of the mature spermatozoon has been reconstructed from transverse sections along its length. It possesses two axonemes which are slightly staggered, so that only one axoneme is present at each end of the spermatozoon. The axonemes have a 9 + '1' pattern typical of flatworm sperm, the central element being a rod-like element rather than a microtubule. The anterior region of the spermatozoon (which is proximal in relation to its formation from the zone of differentiation) contains the first of the two mitochondria. The two mitochondria are separated by a short space, the second mitochondrion occupying the middle region and overlapping slightly with the nucleus which resides in the posterior (i.e. distal) region of the spermatozoon. Cortical microtubules lie beneath the outer plasma

membrane, principally in the anterior and middle regions, being scarce or absent in the posterior region. In the region posterior to the nucleus, dynein side-arms are missing from the outer doublet microtubules of the axonemes and this, together with the lack of cortical microtubules, may explain why this region of the sperm is immotile. Finally, very little if any glycogen has been observed in the spermatozoon of *F. hepatica*, although in separate radio-labelling experiments incorporation of [^{3}H]glucose into glycogen granules in the spermatozoon has been demonstrated (Hanna, 1976b).

For processes so heavily dependent upon microtubule action, it is scarcely surprising that spermatogenesis and spermiogenesis are disrupted by micro-tubule inhibitors such as tubulozole-C. Mitosis is inhibited and this leads to a sharp decline in the number of cells in the testis tubule. The spermatocyte and spermatid rosettes become disrupted and the cytophore regions become frag-mented. Abnormalities of the microtubular organization of the spermatozoa were also observed (Stitt and Fairweather, 1992).

Cytokinesis is the final, separation phase of cell division and is dependent on microfilament action. Treatment with the microfilament inhibitor, cytochalasin B leads to severe disruption of spermatogenesis. Bi- and multinucleate cells increase in frequency with time, and the typical rosettes of spermatocyte and spermatid cells are replaced by syncytial masses of cells, since nuclear, but not cytoplasmic, divisions continue. Spermatozoon formation becomes increasingly abnormal, the spermatozoa containing variable numbers of axonemes and an altered distribution of peripheral microtubules (Stitt *et al*., 1991).

Accessory ducts and glands

The seminal vesicle consists of two spherical bodies, filled with mature spermatozoa, and the two lobes lie within the cirrus sac. The epithelium comprises a single layer of squamous to cuboidal cells and the apical plasma membrane either bears thin lamellae or is invaginated to form deep, pit-like structures. Both the pits and bulbous endings of the lamellae contain spermatozoa. The cells contain mitochondria, a moderate amount of GER and Golgi complexes which produce lucid secretory vesicles. Projections from the parenchymal cells penetrate into the base of the epithelial cells with which they form typical junctional complexes. While the majority of the spermatozoa in the seminal vesicle lie in the lumen, many lie close to the apical surface or are enclosed within the lamellae or pits. This may be indicative of a nutritive and/or maturation function, the lucid secretory bodies perhaps supplying the appropriate substances (Threadgold, 1975a).

The ejaculatory duct is a small duct which takes a sinuous course through the cirrus sac. The epithelium is composed of cuboidal to columnar cells, although it may be a syncytium. The situation is complicated by the presence of the openings of the prostate gland cells; the terminal parts of the ducts of the latter penetrate between/through the cells/syncytium to open into the duct lumen and are connected to the epithelium by septate desmosomes. The apical plasma membrane of the epithelium is extended to form long, thin lamellae which may branch and unite to form a complex system of projections.

The amplification of the apical surface is indicative of a role for the duct in absorption or reabsorption of the breakdown products of the prostate gland secretions or other fluids. The cytoplasm contains mitochondria, a small amount of GER and Golgi complexes which synthesize a dense ovoid secretion. Extensions from parenchymal cells form junctional complexes with the basal plasma membrane (Threadgold, 1975a).

Both the cirrus sac and cirrus are covered by a thin modified tegument; that of the cirrus is especially so, which may be related to its need to evaginate as the thick tegument covering the general body surface would hinder such a process. There are some differences between the tegument of the body surface and of the cirrus and cirrus sac, but they are small. For example, there are fewer basal invaginations in the cirrus and cirrus sac, and fewer mitochondria; the cirrus sac has very few spines whereas the cirrus has many; and type 2 secretory bodies predominate over T1 bodies in the cirrus sac, while there is a normal ratio of T1 to T2 bodies in the cirrus. Processes from the parenchymal cells form junctional complexes with the tegumental cells and their connections to the surface syncytium (Threadgold, 1975a).

The prostate gland consists of numerous unicellular glands grouped around the ejaculatory duct. The individual cells show a high level of secretory activity, containing an extensive system of GER cisternae and numerous Golgi complexes – up to 18 complexes have been observed in one section of a cell. The Golgi complexes form large, electron-lucid secretory vesicles whose content is carbohydrate rich. Moderate numbers of mitochondria occur within the cell. The plasma membrane is invaginated to form deep invaginations which extend almost to the nucleus at times. Running parallel to the invaginations are cisternae of GER which lack ribosomes on the side facing the plasma membrane. They are the equivalent of subsurface cisternae observed in other cell types (e.g. Rosenbluth, 1962). This arrangement of cisternae is believed to facilitate the passage of precursor molecules into the cell. The necks or ducts of the gland cells are long and run in a random fashion through the parenchyma towards the ejaculatory duct. Close to the latter, the gland ducts are supported by a single ring of peripheral microtubules which probably serve to resist the compressive forces of the muscle layers, interstitial material and ejaculatory duct epithelium around them. It is interesting that no junctional complexes between prostate gland cells and parenchymal cells have been observed. The ducts from the gland cells pass through the epithelium of the ejaculatory duct to release their secretion into its lumen and are connected to the surrounding epithelium by septate desmosomes. The terminal region of the gland duct within the epithelium is swollen and appears to serve as a storage site for the prostate secretions. While the precise role of the secretion is not known, a number of suggestions have been put forward, including stimulation of spermatozoa previously stored in a quiescent state in the seminal vesicle and as an extra energy source for the spermatozoa in addition to the glycogen stored within the spermatozoon (Threadgold, 1975b).

Female system

Ovary and oogenesis

Descriptions of some aspects of oogenesis have been made at the electron microscope level (Björkman and Thorsell, 1964; Gresson, 1964), but full details of the process remain to be clarified. The outer wall of the ovary is a membranous structure and contains muscle tissue. It is lined by a layer of cells, believed to act as nurse cells, and this layer separates the germ cells from the outer wall. The germ cells make intimate contact with the nurse cells, processes from the oogonia and immature primary oocytes penetrating into the nurse cell layer (Björkman and Thorsell, 1964). The nurse cells contain mitochondria and a more extensive system of endoplasmic reticulum cisternae than the germ cells; the cisternae are partly covered with ribosomes (Björkman and Thorsell, 1964).

The oogonia are situated at the periphery of the ovary and are small in size, with a diameter of approximately 10 µm. As is typical of undifferentiated cells, the cells contain relatively little cytoplasm, and the nucleus contains one or two nucleoli. The cytoplasm contains many free ribosomes, a few cisternae of GER and a number of mitochondria that may be grouped at one pole of the cell or may occur in two clumps at opposite ends of the cell, depending on the state of development of the cell (Björkman and Thorsell, 1964; Gresson, 1964).

The oogonia differentiate into primary oocytes, but the number of mitotic divisions is unknown. As development proceeds the cells move towards the centre of the ovary. They increase in size (up to 25 µm in diameter) and change shape, becoming fusiform at first (sending processes between the oogonia to the nurse cell layer) but more rounded later (Björkman and Thorsell, 1964; Gresson, 1964). Within the immature primary oocyte, the nucleus contains a single nucleolus and pores are present in the nuclear envelope. The cisternae of GER are more extensive than in the oogonia. Initially, the mitochondria are present in a loose clump near one pole of the nucleus, but as development proceeds some of the mitochondria move away to form a second clump at the opposite pole of the cell, later becoming distributed throughout the cytoplasm. Typical Golgi complexes have not been observed in oogonia or oocytes, but structures containing vacuole-like areas may represent their equivalent (Gresson, 1964). More mature oocytes contain dense spherical granules, typically in close contact with the plasma membrane, but occasionally they lie deeper in the cytoplasm. The granules are strongly osmiophilic and PAS-positive, indicating a polysaccharide nature (Govaert, 1960; Björkmann and Thorsell, 1964). The granules have been described as 'nutritive bodies' (Gresson, 1964) but are probably equivalent to the cortical granules observed in oocytes of other organisms. The precise function of the cortical granules in *F. hepatica* is not known, but in mammals the discharge of their contents is believed to alter the egg coat in such a way as to prevent polyspermy (Longo, 1987). The primary oocytes reach prophase of the first meiotic division, but do not proceed beyond this until they leave the ovary (Gresson, 1964). According to Govaert (1960), the metaphase of this division takes place in the proximal part of the uterus.

Vitelline cells

A large proportion of the body is taken over for vitelline cell formation, the vitelline follicles occupying the lateral margins of the fluke. Fine ducts from the individual follicles lead into the two main longitudinal vitelline ducts on each side of the body. The latter feed into the two main lateral vitelline ducts that unite medially to form the vitelline reservoir; from the reservoir a short duct enters the ootype, uniting with the oviduct to form the common ovovitelline duct before it does so. The vitelline follicles contain a cluster of cells at different stages of development. The cells synthesize the shell protein material involved in egg formation and also provide the developing embryo with nutrients in the form of glycogen and 'yolk' material.

The fine structure of the vitelline cells of *F. hepatica* has been described by Irwin and Threadgold (1970) and the developmental sequence divided into two major phases: growth and cell synthesis (Threadgold, 1982). Shell protein is produced at first, followed later by glycogen synthesis and storage. Although development is a continuous process, four distinct cell types have been identified as representative of the different stages in the developmental sequences: they have been designated the stem (S) cell, the intermediate types 1 and 2 (It1 and It2) and the mature (M) cell (Threadgold, 1982) (Fig. 3.14). The stem cells occupy a peripheral position in the follicle and give rise to the other stages by mitosis, one daughter cell remaining as a stem cell and the other undergoing development. Each stem cell contains numerous free ribosomes and mitochondria, but no GER or globules of shell protein material. The It1 cell contains cisternae of GER, numerous single shell protein globules and a few small-shell globule clusters at the periphery of the cell. The It2 cell contains an extensive system of GER cisternae, many large-shell globule clusters and a few single protein globules. The shell protein material in the intermediate cells is packaged by Golgi complexes, but the rapid turnover of secretory material by these cells results in the Golgi complex being reduced to a rather diffuse system of cisternae. The mature cells occupy a central position within the follicle. In these cells the GER cisternae are confined to the perinuclear region or the extreme cell periphery. The numerous shell globule clusters lie at the cell periphery, while the intermediate zone of the cell is filled with glycogen granules together with a number of heterophagosomes (the 'yolk' globules) (Threadgold, 1982). A fifth cell type, the nurse cell, is also present in the follicle and occupies a peripheral position within it. Long cytoplasmic processes arise from the cell body and ramify between and around the developing vitelline cells, being connected to them and to the surrounding parenchymal cells by junctional complexes. It is believed that the nurse cell is involved in the uptake of precursor molecules (such as amino acids and sugars) from the surrounding parenchyma and their distribution to the developing vitelline cells (Irwin and Threadgold, 1970).

A series of pulse-chase autoradiographic experiments involving tritiated amino acids and monosaccharides has been carried out to examine shell protein and glycogen synthesis by the vitelline cells (Hanna, 1976a). Following short chase periods (0–20 min), amino acid labels were confined to

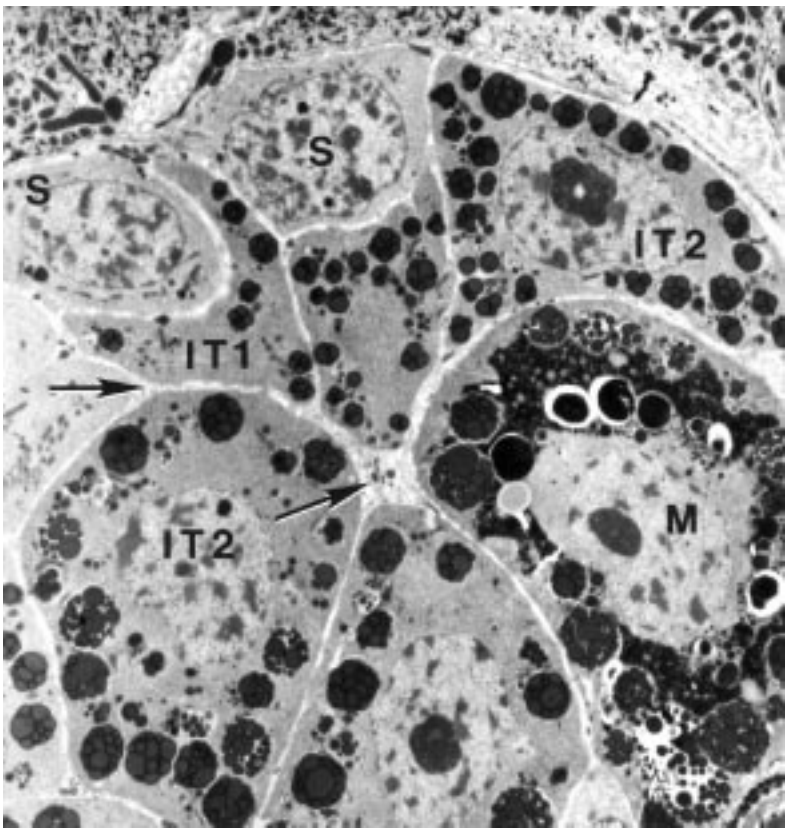

Fig. 3.14. Transmission electron micrograph of a vitelline follicle containing a heterogeneous population of vitelline cells. Present are stem cells (S), intermediate type 1 (It1) and intermediate type 2 (It2) cells and a mature (M) vitelline cell. Cytoplasmic extensions of the nurse cells (arrows) envelop the various vitelline cells.

the It1 cells in which shell protein globules were beginning to appear. Silver grains were present over the GER, then shell protein globules. After longer chase periods (45–60 min), labels were mainly associated with shell protein globules and shell globule clusters and with It2, not It1, cells (Hanna, 1976a). The experiments show that the amino acids (tyrosine, leucine, phenylalanine, not methionine) become incorporated into shell protein material and follow the typical secretory pathway from GER to Golgi complex via transition vesicles and from Golgi complex to secretory vesicle (that is, shell protein globule). The heaviest labelling was obtained with [^{3}H]tyrosine, not surprising given that di-tyrosine links are responsible for the cross-linking reactions involved in shell formation. The experiments also show that the vitelline cells undergo some development during the time scale of the experiment, but do not give a precise indication of the life span of the cell, from stem to mature cell. Egg production data suggest that one vitelline cell is produced by the fluke every 0.115 s (see below), but the time for one cell to complete its

development sequence is clearly much longer than this. Labelling with tritiated monosaccharides was confined to the mature cells, in which glycogen synthesis was occurring and was associated with the glycogen deposits and the 'yolk' globules (Hanna, 1976a). The latter are formed by engulfing portions of cytoplasm containing mitochondria and glycogen granules (Irwin and Threadgold, 1970).

The descriptive account of vitelline cell development has been extended by use of stereological techniques to provide quantitative data on the changes in the whole cell and individual organelles that occur during development (Threadgold, 1982). For example, the volume of the M cell is eight times that of the S cell. The most significant growth of the nucleus occurs between the It1 and It2 cell stages, while that of the nucleolus occurs at an earlier phase, between the S cell and It1 cell stage. The nuclear changes can be linked to the activation of new genes and transcription of new mRNAs that are required for shell protein synthesis and other metabolic activities of the cells. Nucleolar changes are undoubtedly associated with ribosome production required for formation of the GER cisternae and for the synthesis of other proteins during development. The GER increases in volume some 16 times and its surface area 25 times between the stem cell and mature cell. However, these figures mask the real extent of membrane production by this system, since it is transferred to the shell protein globules via transition vesicles and the Golgi complex. In reality, when the surface area of the shell protein globules is added to that for GER, the increase in GER-derived membranes is 42, not 25, times. The quantity of shell protein globules increases steadily during development, although the greatest rise occurs between the It1 and It2 cell stages, and the globules eventually contribute about 21% to cell volume of the M cell. Not unexpectedly, the main phase of glycogen and yolk formation takes place between the It2 and M cell stages, and these two inclusions contribute approximately 20% and 14%, respectively, to final cell volume. The mitochondrial data show some interesting patterns. On the one hand, the volume and surface area of individual mitochondria and their cristae peak at the It1 cell stage, suggesting that the capacity for energy production is potentially at its greatest at this stage. However, one would expect that energy supplies would continue to rise beyond this stage to cope with the demands of shell protein synthesis and glycogen and 'yolk' formation, for example. This is borne out when the data for total numbers of mitochondria per cell and the total volume and surface area of mitochondria and their cristae are considered. The peak for these parameters occurs at the It2, not It1, stage, and even at the M cell stage the values are up to three times those in the S cell stage (Threadgold, 1982). The results from the stereological study show that this kind of analysis can provide significant information on changes in individual organelles that is not given by more qualitative methods. Moreover, it can provide an insight into the pattern of development and interrelationships between particular organelles during the cell cycle. Unfortunately, stereology has not found any wider use on *F. hepatica* or other flatworm parasites; it could be used, for example, to determine changes in vitelline and other cells resulting from drug action.

Vitelline cells have been shown to be susceptible to the action of drugs known to disrupt specific processes within the cell. For example, monensin, which is a sodium ionophore, is used to selectively block the intracellular transport of secretory products at the level of the Golgi complex (causing an osmotic dilation of the cisternae), but does not interfere with their initial synthesis (for references, see Skuce and Fairweather, 1988). Incubation of whole flukes and tissue slices in monensin (1×10^{-6} M) leads to vacuolation of the Golgi complexes in the intermediate cells that are actively engaged in the synthesis of shell protein material. In addition, there is a block in the normal migration of the shell protein globules to the periphery of the cell, the shell globule clusters becoming very loosely packed and empty and distended single globules accumulate in the perinuclear region of the cell (Skuce and Fairweather, 1988). In these respects, then, the effect of monensin on the vitelline cells follows its classical pattern established with a variety of cell types. However, there is no upstream accumulation of secretory material in the GER, as has been observed in other cells.

Treatment of fluke material with the microtubule inhibitors, tubulozole-C and colchicine, prevented division of the stem cells and this led to a change in the population of cells within the follicle, with relatively more stem cells than normal, but fewer intermediate-type cells, especially It1-type cells (Stitt and Fairweather, 1993). Inhibition of mitosis by the inhibitors is expected because they are known to prevent the formation of the spindle apparatus during cell division (Geuens *et al.*, 1985). The nurse cell cytoplasm, which contains microtubules (Stitt *et al.*, 1992b), became fragmented but only after changes in the vitelline cells, indicating that the cellular effects are the direct consequence of microtubule inhibition. In the intermediate (It1 and It2)-type cells, less shell protein material was synthesized, leading to loosely packed shell globule clusters, and movement of globules from the perinuclear region to the cell periphery was disrupted, a characteristic feature of microtubule inhibition. Less glycogen and fewer 'yolk' globules were produced by the mature cells (Stitt and Fairweather, 1993). The microfilament inhibitor, cytochalasin B, disrupted the movement of shell protein globules and their formation into clusters, suggesting an additional role for microfilaments in these processes. Binucleate stem cells were observed, indicating incomplete cytokinesis or separation of the cells following mitosis, and the eggs produced by treated flukes were abnormal with an uneven coating of eggshell material (Stitt and Fairweather, 1993).

It is evident, then, that the vitelline cells display a high level of secretory activity and undergo a rapid turnover. These two features render them particularly susceptible to the action of drugs such as anthelmintics. Consequently, they represent a good model cellular system for determining the morphological effects of anthelmintics in 'mode of action'-type studies. The results of such studies are discussed in Chapter 7 of this volume.

Ootype/Mehlis' gland complex

The ootype is the site where egg formation takes place. It is lined by a single layer of epithelial cells, rests on a basal lamina and is surrounded by a layer

of muscle (Threadgold and Irwin, 1970). Beyond this, no detailed description of the epithelial lining is available. The ootype is surrounded by the Mehlis' gland and the fine structure of the gland has been described by Threadgold and Irwin (1970). It consists of two types of secretory cell, designated the S1 and S2 types. The cells are arranged radially around the ootype, with the S2 cells relatively close to the ootype and the S1 cells situated further away (Fig. 3.15). Duct-like extensions of the cells converge on the ootype, passing through the muscle layers and epithelial cells to release their secretions into the lumen of the ootype (Fig. 3.15). The extensions are lined by microtubules and are anchored to the ootype epithelium by septate desmosomes. The microtubules provide support, enabling the ducts to resist the compressive forces exerted by the muscular contractions of the ootype wall that assist the movement of ova and vitelline cells through the ootype. Extensions of interstitial material project into the gland cells' cytoplasm and may provide mechanical support for the cells, but no junctional complexes have been observed between the Mehlis' gland cells and the parenchymal cells.

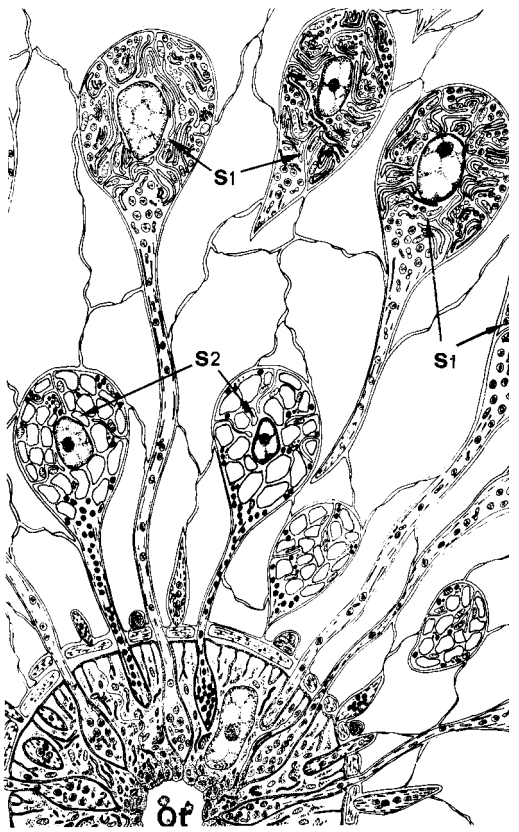

Fig. 3.15. Diagram of the Mehlis' gland showing the relationship of the S1 and S2 cell types to each other and to the ootype (Ot) (Threadgold and Irwin, 1970).

As might be expected of actively secreting cells, the S1 cells contain an extensive network of GER cisternae, numerous mitochondria and many Golgi complexes. The latter produce sausage-shaped secretory bodies which have a filamentous content radiating from the central core. The bodies undergo some morphological changes as they migrate from the cell body to the ootype. The terminal regions of the cell extensions within the ootype epithelium are swollen and this may indicate that the secretory bodies can be stored before their release into the lumen. The S1 cells described by Threadgold and Irwin (1970) correspond to the 'mucous' cells described by Gönnert (1962).

The S2 cells are filled with distended cisternae of GER containing relatively electron-lucid material. This may account for the opalescent or 'serous' appearance of the cells in light microscopy; the S2 cells described by Threadgold and Irwin (1970) correspond to the 'serous' cells described by Gönnert (1962) and the 'large cells' described by Thorsell and Björkmann (1965). Numerous mitochondria and Golgi complexes are present, the latter producing secretory bodies that are spherical with a crystalline or packed fibrous appearance. Unlike the S1 secretory bodies, the S2 secretory bodies do not change shape or form as they move towards the ootype. The secretion contained within the secretory bodies dissociates immediately on entering the lumen of the ootype.

The cells of Mehlis' gland give a positive staining reaction with the periodic acid–Schiff (PAS) technique, indicating the secretion of a mucopolysaccharide or mucoprotein (Johri and Smyth, 1955; Rao, 1959; Clegg, 1965). It has been suggested that the mucous secretion contains lipoprotein and that the lipoprotein may form some kind of template on which eggshell material is deposited (Clegg, 1965), although the latter idea now appears unlikely (see 'Egg formation', below). A number of functions of Mehlis' gland have been put forward. They include lubrication of the uterus for the passage of eggs; activation of sperm; release of shell protein material from the vitelline cells; and a role in the tanning process, in addition to the template idea just mentioned (Threadgold and Irwin, 1970; Smyth and Halton, 1983). A more recent idea on the role of Mehlis' gland secretions in egg formation is discussed below.

Egg formation

Egg production figures for *F. hepatica* are impressively high: in a light infection in sheep (up to 50 flukes), each fluke produces an average of 25,000 eggs per day (equivalent to the release of 500,000 eggs per day by the sheep (Happich and Boray, 1969). This equates to the production of one egg every 3.46 s. Each egg requires approximately 30 vitelline cells (Stephenson, 1947; Rao, 1959). Therefore, the fluke has to produce one vitelline cell every 0.115 s in order to maintain this output. The vitelline cells pass from the vitelline reservoir into the common ovovitelline duct and on into the ootype, together with a single oocyte from the oviduct. Within the ootype, the vitelline cells release the contents of the shell globule clusters and the globules coalesce to form the eggshell around the cell mass.

The eggshell is formed of a sclerotin or quinone-tanned protein. Quinone tanning involves the conversion of DOPA (3,4-dihydroxyphenyl-L-alanine) residues to *o*-quinones by the action of phenol oxidase (phenolase or catechol oxidase). The cross-linking of DOPA-containing proteins forms a very stable, tough, waterproof, resistant and protective capsule around the embryo. The phenol oxidase enzyme needs to be activated (by enzyme action) before it triggers the tanning process. The activating enzyme is presumed to be a protease-type enzyme because phenol oxidases in other organisms are known to be activated by proteolytic cleavage (for references see Wells and Cordingley, 1991). A recent model for eggshell formation in schistosomes makes the assumption that the three components – the eggshell protein, the phenol oxidase 'tanning' enzyme and its activating enzyme – are packaged together in the same membrane-bound vesicle (namely, the shell globule cluster) in the vitelline cells (Wells and Cordingley, 1991). In *F. hepatica*, three different eggshell precursors have been identified and designated vitelline proteins (vp) A, B and C, with sizes of 70, 31 and 17 kDa, respectively (Waite and Rice-Ficht, 1987, 1989, 1992; Zurita *et al.*, 1987, 1989; Rice-Ficht *et al.*, 1992). Each form exhibits heterogeneity due to variations in amino acid sequence and post-translational modification. The precursors are rich in DOPA residues, formed from tyrosine residues during co- or post-translation modification. Precursor vpB is a particularly heterogeneous, though closely related group of proteins (Waite and Rice-Ficht, 1992) and represents 6–7% of total protein in the fluke (Waite and Rice-Ficht, 1987). Moreover, up to seven copies of vpB genes may be present in the genomic DNA of *F. hepatica* (Rice-Ficht *et al.*, 1992). These observations highlight the emphasis placed on reproductive activities (and egg production in particular) within the overall energy budget of the fluke. Also, they suggest that the eggshell is a very complex heteropolymer of proteins, a feature common to other eggshell protein families (see Rice-Ficht *et al.*, 1992). Immunocytochemical studies utilizing an antibody to vpB have localized the protein to the shell protein globules in the vitelline cells (Rice-Ficht *et al.*, 1992). Synthesis of the protein by these cells has been confirmed by use of *in situ* hybridization techniques (Zurita *et al.*, 1989; Rice-Ficht *et al.*, 1992).

Phenol oxidase activity has been demonstrated in the vitelline cells of *F. hepatica* by Johri and Smyth (1956). The enzyme is inhibited by DDC (diethyldithiocarbamic acid), a copper chelator and phenol oxidase inhibitor; inhibition can be reversed by copper sulphate and enzyme activity is stimulated by copper sulphate alone. The results indicate that phenol oxidase is a copper-containing protein (Mansour, 1958). So, two of the three components required for eggshell formation in *F. hepatica* have been localized to the vitelline cells. The third component – the putative phenol oxidase activating enzyme – has yet to be identified in *F. hepatica* or any other digenetic trematode and its site of synthesis remains to be elucidated.

The model for eggshell formation in schistosomes suggests that the release of shell protein material from the vitelline cells is a calcium-dependent process and that the fusion and tanning of the shell protein globules requires alkaline conditions (Wells and Cordingley, 1991). Recent

experiments with *F. hepatica* indicate that a similar mechanism operates in *F. hepatica*. Thus, incubation in the calcium ionophore lasalocid induces premature release of the shell globule clusters from the mature vitelline cells but does not cause any precocious tanning of the shell protein material (Colhoun *et al.*, 1998). Treatment with either monensin or ammonium chloride, which both serve to increase the pH of membrane-bound acidic compartments within cells, leads to fusion of the shell protein globules within the clusters and premature tanning of the shell protein material. The rise in pH serves to abolish the charges on the surface of the globules which prevent their fusion within the clusters. The changes induced by monensin and ammonium chloride can be prevented by DDC (Colhoun *et al.*, 1998).

Translating these results into what happens within the ootype, it can be envisaged that the vitelline cells are induced to exocytose the contents of the shell globule clusters via a calcium-dependent process, although the trigger remains to be identified. The free shell protein globules meet an 'interface' between two liquids of different viscosities and at different pHs: the fluid around the vitelline cells at acid pH and the more viscous Mehlis' gland secretion at an alkaline pH. On contact with the Mehlis' gland secretion, the surface charges on the shell protein globules are lost and the globules can coalesce to form a uniform layer along the interface. The phenol oxidase and its activating enzyme will be released from the vitelline cells along with the shell protein globules and, following their activation, will trigger the cross-linking reactions between DOPA residues in the shell protein precursors that lead to eggshell formation. The mixing movements of the ootype, brought about by contractions of the muscle in the ootype wall, will aid the process. The concept of an interface rather than a lipoprotein template (as suggested by Clegg, 1965) fits in better with the electron microscope observations of Irwin and Threadgold (1972) (Fig. 3.16) and with the schistosome model (Wells and Cordingley, 1991). According to this model, which may be applicable to other trematodes as well, the secretions from Mehlis' gland may play a number of novel roles in egg formation: provision of the alkaline conditions required for fusion of the shell protein globules; triggering the release of the shell protein globules; and activation of the tanning enzyme.

As indicated above, the rate of egg production in *F. hepatica* is extremely rapid: 1 egg every 3.46 s. The combining of one ovum and 30 or more vitelline cells to form an egg in such a short interval of time argues for a sophisticated and highly synchronized mechanism of egg production involving a complex sequence of muscular contractions and relaxations in the proximal portions of the female reproductive tract. Two groups of peptidergic nerve cells have been observed at the junction of the vitelline and ovovitelline ducts and at the entrance of the uterus from the ootype (Magee *et al.*, 1989); these sites correspond to nerve plexuses I and II described by Gönnert (1962) (Fig. 3.17). The cells may control the entry of ova (or secondary oocytes) and vitelline cells into the ootype and exit of newly formed eggs from the ootype into the uterus via the uterine valve (Magee *et al.*, 1989). Other peptidergic cells (together with cells immunoreactive for 5-hydroxytryptamine) lie among the S2 type of Mehlis' gland cells

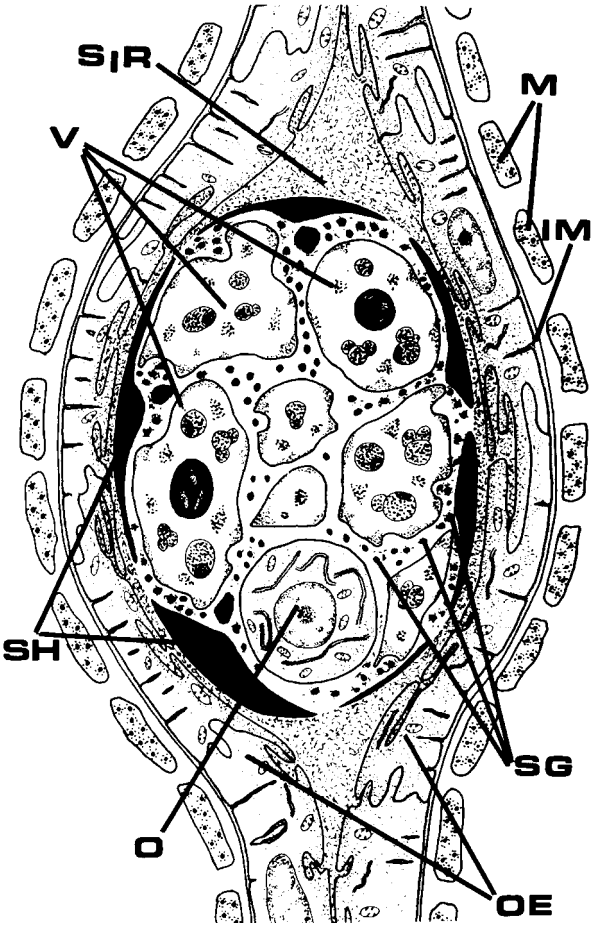

Fig. 3.16. Diagram representing a longitudinal section through the ootype containing a developing egg. IM, interstitial material; M, muscle block; O, ovum; OE, ootype epithelium; SG, shell protein globule; SH, accumulating eggshell; S1R, residue of secretion from the S1 type of Mehlis' gland cell; V, vitelline cell (Irwin and Threadgold, 1972).

(Fairweather *et al.*, 1987; Magee *et al.*, 1989) (Fig. 3.17). Secretions from these cells may exert a paracrine-like influence over the secretory activity of neighbouring Mehlis' gland cells. A plexus of nerve fibres exists within the wall of the ootype and may coordinate the contractions of the ootype musculature, thus controlling the movement of cells through the ootype (Fairweather *et al.*, 1987; Magee *et al.*, 1989). It seems likely that there is a strong neural influence over egg production but a full understanding of the mechanisms involved awaits clarification.

The newly formed egg with its semi-soft shell passes from the ootype into the proximal uterus, where fertilization takes place. The eggshell

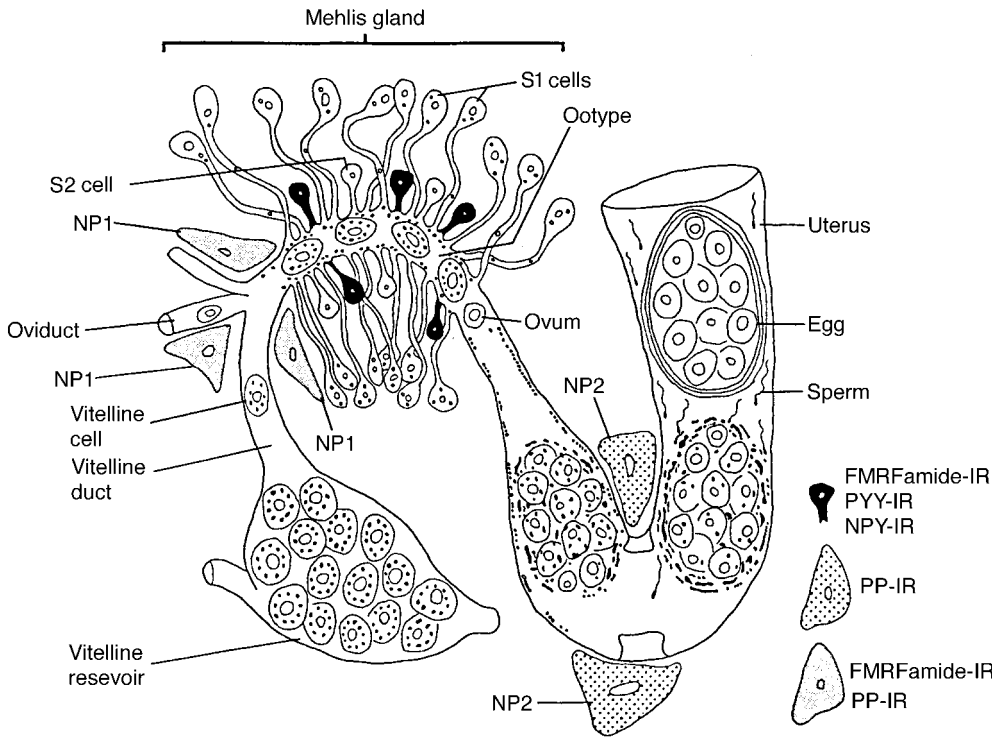

Fig. 3.17. Diagram showing the distribution of PP-, PYY- and FMRFamide-immunoreactive cells in the ootype/Mehlis' gland complex of *Fasciola hepatica*. NP1, cell body in nerve plexus 1; NP2, cell body in nerve plexus 2; S1, S1-type of Mehlis' gland cell; S2, S2-type of Mehlis' gland cell (after Magee *et al*., 1989).

becomes hardened as the egg passes along the uterus. The egg of *F. hepatica* has an oval shape with a smooth surface, and is marked at one pole by the presence of the operculum. The operculum is formed by an unusual process: the ovum apparently pushes out pseudopodia towards the eggshell during its process of formation exactly at the point of rupture in the operculum. The result is that the eggshell is weakened at this point and so readily fractures on hatching (Gönnert, 1962).

Conclusions and Future Perspectives

It is clear that the last 30 years or so have witnessed a dramatic change in our whole perception of the cellular and tissue organization of the liver fluke. We now have at least a basic understanding of the fine structure of virtually all the major organ systems in the fluke. A wide range of techniques and tools available to cell biologists has been used. For example, scanning electron microscopy has provided us with a view of the external architecture of the

fluke, while transmission electron microscopy has enabled descriptions to be made of the fine structural organization of the various internal tissues and organ systems and their interrelationships. Cytochemical and immunocytochemical techniques have been used to localize particular molecules and substances, at the light and electron microscope levels. Further information has been gained recently by the use of confocal scanning laser microscopy. *In situ* hybridization studies have been used to determine the sites of synthesis of certain molecules while pulse-chase autoradiography has yielded information on the intracellular movement of newly synthesized molecules. In some instances, qualitative data have been reinforced by quantitative, stereological analysis. Morphological studies have been allied with physiological experiments to determine the *modus operandi* of various systems and processes. There have been limited attempts to isolate and identify the molecules concerned; this will open up new avenues for study, such as the raising of antibodies for the purpose of localization.

In addition to describing what *is* known about fluke ultrastructure, this review has tried to point out the many gaps in our knowledge. Perhaps the major gap has been created by the tendency to concentrate on the 'adult' (bile duct) and 'juvenile' (liver parenchyma) stages, to the exclusion of other, earlier stages *before* the fluke reaches the liver. After all, the overall development of the fluke, from the metacercaria to the adult, accompanies a complex migration from the gut lumen through the gut wall, into the peritoneal cavity, penetration into the liver and final location in the bile duct. Thus, it has to accommodate a number of changes of habitat. The fluke must be able to detect the appropriate stimuli and respond to them by altering not just behaviour, but diet, respiratory metabolism and morphology. The developmental sequence takes several weeks and the changes involved are subtle and gradual. So there is a need to examine the early stages that are critical in enabling the fluke to become established in the mammalian host. Some studies have been carried out but the data are limited. More than this, such 'two-dimensional' studies on fluke structure need to be put together to build up a more 'three-dimensional' picture of how changes in the fluke are coordinated. The nervous system will play a central role here, through its endocrine function. Once a neglected system, the true importance of the nervous system is beginning to be appreciated and greater knowledge will bring with it major advances in our understanding of the control of processes that underpin the maturation of the fluke. Developmental changes require the switching on of new genes and switching off of others. There is a need to isolate particular genes, unravel the processing of their precursors, determine their sites of synthesis and physiological roles, but more particularly monitor their temporal expression during the development of the fluke. Molecular biology has yet to make a major impact on fluke biology. In many respects, we are in a similar transitional stage with respect to molecular biology as we were with electron microscopy those 30-odd years ago. The next book on *Fasciola* will reveal a far greater contribution of molecular biology, a contribution that will have an impact on novel control strategies.

Acknowledgements

A very real debt of gratitude is due to both Denise Ennis, who somehow managed to interpret and unravel my illegible scrawl and produce an orderly manuscript out of it, and Karen Moore, who supervised the revisions to the original manuscript.

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4 Epidemiology and Control

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Introduction

Epidemiology is the study of disease in populations and the factors that determine its occurrence. In addition it includes investigation and assessment of other health related events in livestock such as productivity. The study of the epidemiology of fasciolosis in livestock encompasses the factors that affect the prevalence and intensity of infection with the parasite and how these impact on animals both in terms of clinical disease and of the economic effects of productivity losses. The epidemiology of the disease depends on factors such as the infection pressure in the environment and the susceptibility of the host species (or individual) through innate or acquired resistance. The infection pressure in turn depends on factors that affect the free living and intermediate stages, such as temperature and moisture. Furthermore, the availability of large numbers of susceptible definitive and intermediate hosts will increase the parasite's ability to reproduce itself and result in a high fluke abundance. Figure 4.1 illustrates the interactions of these epidemiological factors that increase the likelihood of disease due to fasciolosis in livestock. To be effective, control measures depend upon a sound understanding of the epidemiology of the disease so that intervention strategies can be designed to produce the greatest possible benefit in terms of minimizing disease or productivity in animals at risk of infection.

Parasite, Host and Intermediate Host Species

Parasite species

Pathogenic species of *Fasciola* and conditions suitable for the development of all stages of the life cycle need to be present for disease outbreaks to occur. *Fasciola gigantica* and *F. hepatica* both cause disease in domestic animals and, in addition, *F. jacksoni* is recognized to infect elephants

Fasciolosis (ed. J.P. Dalton)

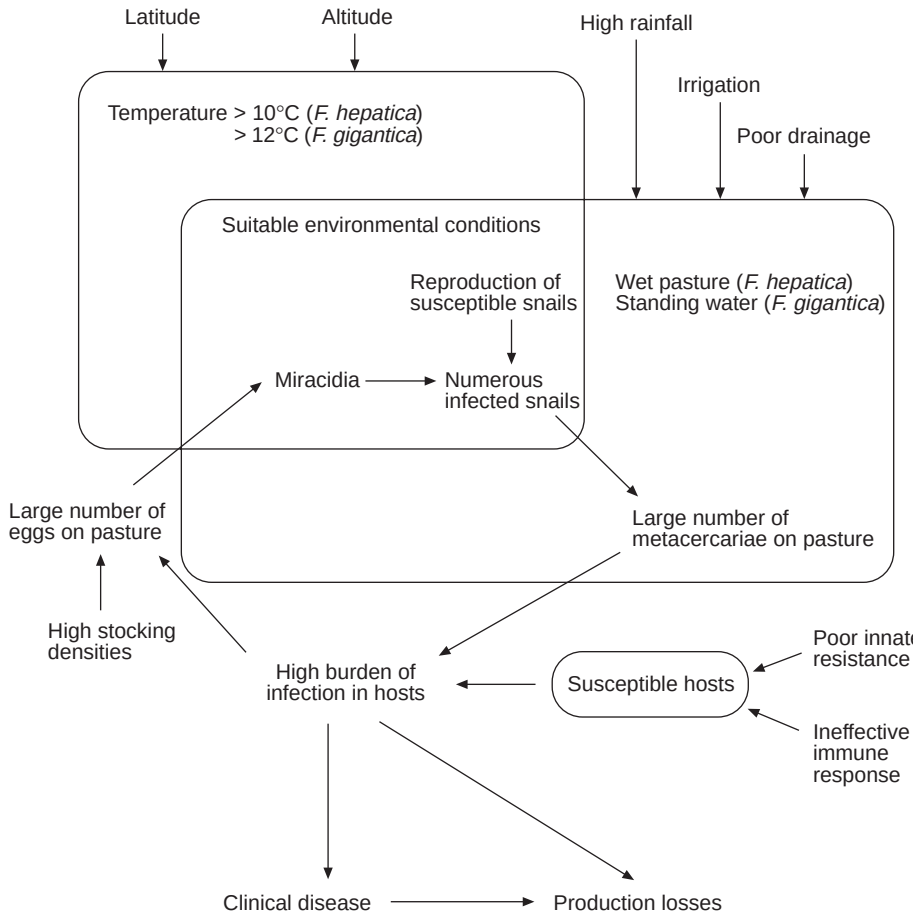

Fig. 4.1. Interactions of epidemiological factors that increase the likelihood of disease due to fasciolosis in livestock.

(Singh *et al*., 1994). In domestic livestock in Japan, diploid ($2n$ = 20), triploid ($3n$ = 30) and chimeric flukes ($2n/3n$) have been described (Sakaguchi, 1980), many of which reproduce parthenogenetically. As a result of this unclear classification, flukes in Japan are normally referred to as *Fasciola* spp.

Fasciola hepatica has a cosmopolitan distribution (Table 4.1, Fig. 4.2) but the distribution of *F. gigantica* is more limited, being restricted to the tropics and having been recorded in Africa, the Middle East, eastern Europe and south and eastern Asia (Table 4.2, Fig. 4.2). Although a number of texts suggest the presence of *F. gigantica* in the southern United States and Hawaii (Soulsby, 1982; Radostits *et al*., 1994; Urquhart *et al*., 1996) there appears to be no confirmation of this parasite in any of the Americas.

Table 4.1. Recorded prevalence of *Fasciola hepatica*.

Country/region	Type of animal	Prevalence (%)	Reference
Africa			
Morocco	Cattle	10.4	Moukrim and Rondelaud (1991), Khallaayoune *et al.* (1991)
	Goats	17.1–23.8	
Americas			
Canada (Quebec)	Dairy cows	up to 68	Bouvry and Rau (1986)
Brazil (state of Parana)	Cattle	0.95	Luz *et al.* (1992)
	Buffalo	19.8	
Chile	Cattle	up to 94	Alcaino (1985)
Jamaica	Cattle	22.2	Bundy *et al.* (1983)
	Goats	17.2	
	Sheep	0.72	
Mexico	Cattle	5.2	Encinas-García *et al.* (1989)
Peru	Cattle	29	Leguía *et al.* (1989)
USA			
California	Beef cattle	52.7	Briskey *et al.* (1994)
Colorado		5.9	
Idaho		36.7	
Nebraska		19	
Texas		15.6–17.3	
Florida		68	Kaplan (1994)
Montana		17.2	Knapp *et al.* (1992)
Asia and Australasia			
Kursk region of the former USSR	Cattle	30.5	Bausov *et al.* (1981)
	Sheep	16.7	
Iraq	Sheep	14.4	A-Al-Bayati *et al.* (1991)
Jammu and Kashmir, India	Sheep	30	Pandit *et al.* (1989)
New Zealand	Cattle	8.5	Mitchell (1995)
	Sheep	4.4	
Queensland, Australia	Cattle	1.1	Baldock and Arthur (1985)
Turkey	Cattle, buffalo and sheep	29.3	Celeb and Ultav (1988)
Europe			
Belgium	Double muscled beef cattle	12.5	Genicot *et al.* (1991)
Germany	Cattle	10.7	Simmank (1987)
Ireland	Cattle	45	Department of Agriculture (Ireland) figures (unpublished)
Italy	Cattle	5	Poglayen *et al.* (1995)
Poland	Cattle	7.2	Konopka (1993)
	Sheep	3.7	
Spain	Cattle	29.5	González *et al.* (1989)
	Sheep	14.7	Manga *et al.* (1990)
United Kingdom	Cattle	10	Taylor (1989)

Table 4.2. Recorded prevalence of *Fasciola gigantica*.

Country/region	Type of animal	Prevalence (%)	Reference
Africa			
Egypt	Cattle	4–6	Lofti *et al.* (1995)
	Buffalo	2–5	
	Sheep	1–2	
	Goats	up to 1	
	Donkeys	4.5	Hasslinger and Elseify (1996)
Gambia	Cattle	20	Ndao *et al.* (1995)
Kenya	Cattle	12	Waruiru *et al.* (1993)
Nigeria	Cattle, sheep and goats	43	Nwosu and Srivastava (1993)
Zimbabwe	Cattle	65.2	Vassilev (1994)
Asia			
Bangladesh	Cattle	44	Chowdhury *et al.* (1994)
India			
Uttar Pradesh	Buffalo	39.0	Bhatia *et al.* (1989)
	Goats	57.3	
	Sheep	81.4	
Maharashtra	Buffalo	7.4	Ratnaparkhi *et al.* (1993)
	Cattle	18.18	
	Sheep	8.87–11.32	
	Goats	25.76	
Iraq	Sheep	3	A-Al-Bayati *et al.* (1991)
Nepal	Buffalo	33.3–71.4	Shrestha *et al.* (1992)
	Cattle	24.7–28.6	
Thailand	Buffalo	47.1	Sukhapesna *et al.* (1990)

Definitive hosts

Cattle, sheep and buffalo are the most important species of farm livestock affected by *Fasciola* spp. Although goats, horses, pigs, deer and many other species of herbivore can also be infected, the parasite is of less economic importance on a global scale in these hosts. Man is also a suitable host and in some areas of the world the human fasciolosis is an important cause of ill health (see Chapter 12 of this volume).

Intermediate host species

Liver fluke is absent in areas where conditions are unsuitable for the development of suitable intermediate-host snails. Suitable snails belong to the phylum Mollusca and class Gastropoda and the species of interest fall into the subclass Euthyneura or Pulmonata, depending on the system of classification (Wright, 1971).

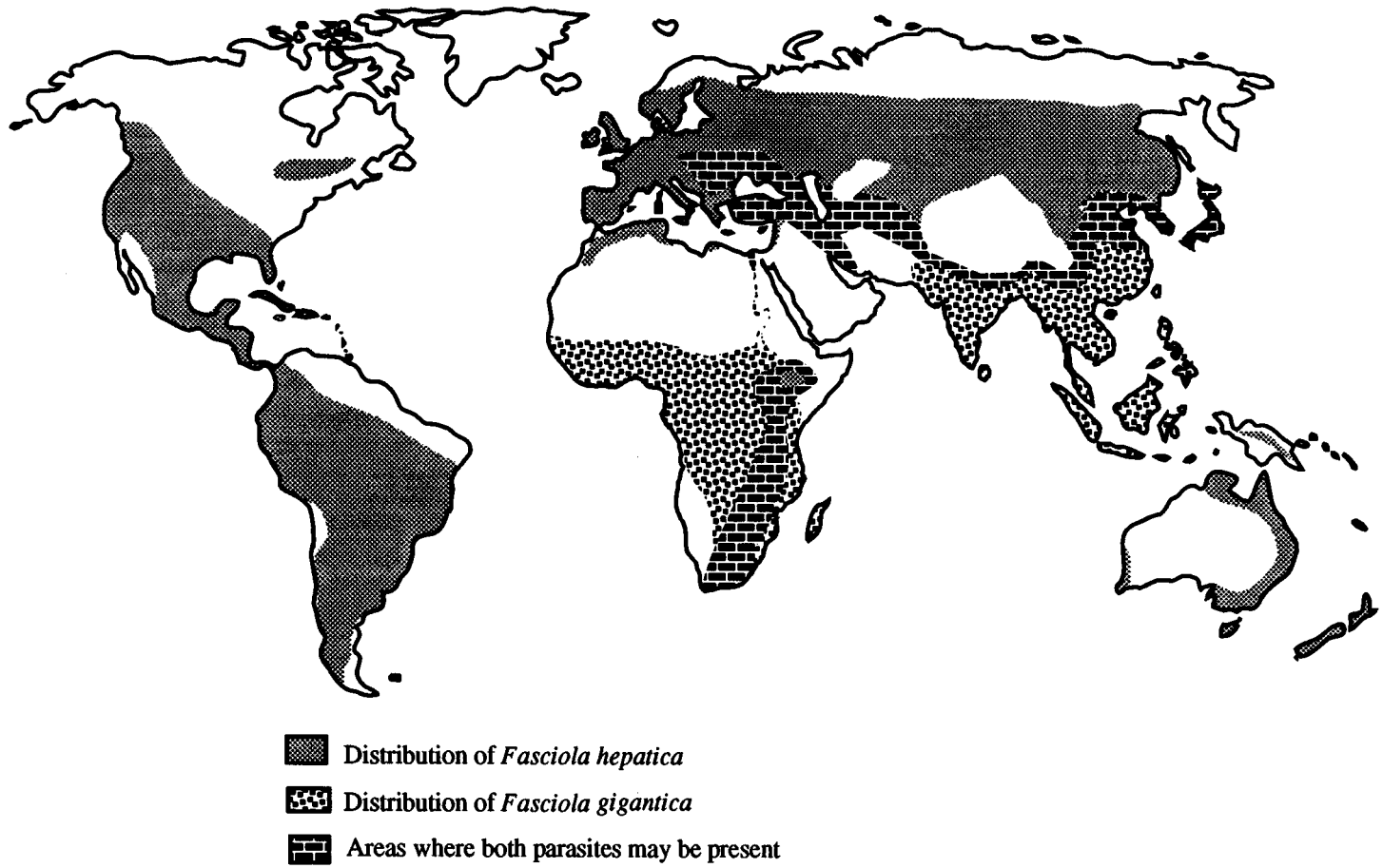

Fig. 4.2. The geographical distribution of *Fasciola hepatica* and *F. gigantica*.

Fasciola hepatica

Lymnaea spp. snails involved in the transmission of *F. hepatica* (Table 4.3; Plate 1) are mud-living and amphibious, living in an environmental niche which is subject to flooding and desiccation (Kendall and Ollerenshaw, 1963; Over, 1982). They are more likely to be found in habitats that are intermittently wet (flush habitat) than in permanently wet sites and in water that is generally slightly acid (Ollerenshaw, 1971; Villegas, 1984) and moving at 15–20 cm s^{-1} (Boray, 1964). Distribution is not uniform because, within each habitat, the snails may be concentrated in small, very wet areas such as

Table 4.3. Summary of species of snail recorded as possible intermediate hosts of *F. hepatica*.

Species	Country/region	Reference
Lymnaea (Fossaria) bulimoides	Australia	Lang (1977)
	USA	McKown and Ridley (1995)
L. (Pseudosuccinea) columella[1]	North and South America	Price (1953) Yong Cong and Perera de Puga (1991)
L. cousini[1]	South America	Over (1982)
L. (Fossaria) cubensis[1]	North and Central America and the Caribbean	Price (1953) Over (1982) Yong Cong and Perera de Puga (1991)
L. ferruginea	North America	Price (1953)
L. humilis	North America	Over (1982)
L. mweruensis (= *L. truncatula*?)	Kenya	Kendall (1965)
L. ollula (= *L. viridis*?)	Japan	Watanabe (1962) Itagaki *et al.* (1988)
	Central Europe	Over (1982)
L. palustris	Europe	Boray (1966)
	USA	Lang (1977)
L. peregra ovata	Europe	Boray (1966)
L. peregra peregra	Europe	Boray (1966)
L. stagnalis	Europe	Boray (1966)
	USA	Lang (1977)
L. traskii	North America	Price (1953)
L. tomentosa[1]	Australia	Boray (1966) Over (1982)
L. truncatula[1]	Europe	Kendall (1950) Boray (1966) Over (1982)
L. (Fossaria) viatrix (viator)[1]	South America	Over (1982)
L. proxima proxima	USA	Lang (1977)
L. modicella modicella	USA	Lang (1977)
Stagnicola montanensis	USA	Rowan *et al.* (1966)
F. (Galba) bulimoides	North America	Price (1953)
G. bulimoides techella	North America	Price (1953)

[1] Species commonly considered intermediate hosts to *F. hepatica.*

ditches and seepages (Kendall and Parfitt, 1975). Snails can travel large distances by drifting in water (Ollerenshaw, 1971) and in Australia large, permanent water areas harbouring a few snails are thought to be important in recolonization of temporary water courses (Kendall, 1949; Boray, 1964). Thirty per cent of snails survive a 12-month artificial drought by aestivation (Soulsby, 1982) and even newly hatched snails can survive 2 months of aestivation (Kendall, 1949). Once water returns, however, snails are able to breed very rapidly.

Fasciola gigantica

Although a range of snail species can be infected, it is generally accepted that the species which play the main role in the epidemiology of infection are in the *L. auricularia/natalensis* group of snails (Kendall, 1965) (Table 4.4).

The Effects of Climate and Environment on *Fasciola* spp.

Parasite distribution in the environment is extremely variable. However, despite this variability, for *Fasciola* spp. to complete their life cycle the environment must provide a consistent set of suitable conditions of moisture and temperature for the development of the larval stages and the development of the intermediate host itself. The majority of work on the development of fluke outside the definitive host has been carried out on *F. hepatica* with a smaller amount of information on *F. gigantica*.

Effect of temperature

On fluke eggs

The minimum critical temperature for development of *F. hepatica* eggs is about 9.5°C (Rowcliffe and Ollerenshaw, 1960) (Plate 1). Development is inhibited above 30°C. The number of degree-days for complete development

Table 4.4. Summary of intermediate hosts of *F. gigantica*.

Species	Country/region	Reference
L. auricularia/natalensis[1]	Africa, Asia	Dacosta *et al.* (1994), Tembely *et al.* (1995), Chartier *et al.* (1993), Chaudhri *et al.* (1993)
L. gedrosiana	Iraq	Massoud and Sadjadi (1980)
L. ollula	Japan	Itagaki *et al.* (1989)
L. peregra[2]	Europe	Boray (1966) Massoud and Sadjadi (1980)
L. stagnalis[2]	Europe	Boray (1966)
L. tomentosa[2]	Australia	Boray (1966)
Biomphalaria alexandrina	Egypt	Farag and El Sayad (1995)

[1] Species commonly considered intermediate hosts to *F. gigantica.*
[2] There is no evidence that these species, infected in the laboratory, have been found infected in the environment.

varies, with estimates of the development of the miracidium in the egg ranging from 60 days at 12°C to 10 days at 30°C (Rowcliffe and Ollerenshaw, 1960; Soulsby, 1982; Gettinby and Byrom, 1991). The variation in the rate of development of the egg with temperature is illustrated in Fig. 4.3. In the Kenyan highlands, where mean temperatures vary between 10 and 22°C, the development time of *F. gigantica* eggs has been reported to be from 52 to 109 days (Dinnik and Dinnik, 1959), while at a constant temperature of 26°C they take 17 days to develop (Dinnik and Dinnik, 1963).

On snail development

A minimum temperature of 10°C is necessary for development of *Lymnaea truncatula* and maximum growth occurs at 18–27°C (Kendall, 1953). *L. truncatula* can grow to sexual maturity in 3 to 4 weeks, depending on the amount of food available and the number of parasites in the snail (Kendall, 1953; Kendall and Ollerenshaw, 1963). *L. viatrix* reaches maturity in 24–27 days (Lara *et al.*, 1988). The number of eggs produced is related to the food

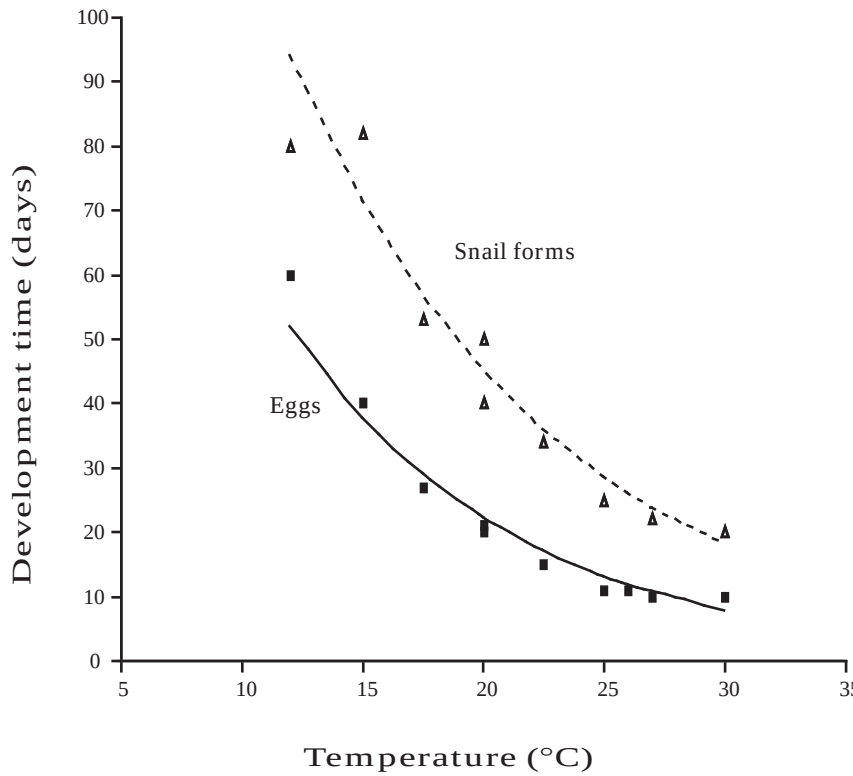

Fig. 4.3. The effect of temperature on the development of *F. hepatica* eggs and intramolluscan forms (data from: Over, 1982; Soulsby, 1982; Gettinby and Byrom, 1991).

supply available and *L. viatrix* may produce up to 5000 eggs in its lifetime of at least 200 to 305 days (Lara *et al.*, 1988). *L. truncatula* may live for 12–14 months (Over, 1982). Under laboratory conditions, *L. viridis* has been shown to grow rapidly, reaching 12 mm in 37 days and producing eggs at 18–24 days old (Lee *et al.*, 1994).

On parasite development in the snail

At 15°C, *F. hepatica* cercariae will complete development in the snail in 80 days, decreasing to less than 20 days at 30°C (Gettinby and Byrom, 1991; Fig. 4.3). A minimum temperature of 10°C is required for shedding but above this minimum, the temperature appears not to be critical (Kendall and McCullough, 1951). There is no development below 10°C and above 20°C mortality of the cercariae increases. Once infected, the snail appears to remain infected for life (Kendall, 1965).

The minimum temperature for the development of *F. gigantica* in *L. auricularia*/*natalensis* is greater than that required for the intermediate stages of *F. hepatica*. Development to cercariae does not occur below 12°C; it takes 73 days at 15°C and 25 days at 30°C. Emergence of the cercariae takes 129 days at 15°C and 43 days at 30°C (Al Habbib and Al Zako, 1981). Dinnik and Dinnik (1963) reported that the development of *F. gigantica* in *L. auricularia*/*natalensis* took 33 days at a constant 26°C maintained experimentally, but required between 69 and 197 days under the prevailing environmental conditions in Kenya.

On metacercarial survival

At 12–14°C, up to 100% of metacercariae can survive for 6 months while only 5% survive for 10 months. Metacercarial survival is reduced in hot conditions. Boray and Enigk (1964) found cysts remained viable for only 3 days at 20°C and a RH of 75–80%, whereas at 10°C and 90% RH they survived 122 days (Fig. 4.4). Cysts have been shown to survive and remain infective for 70 days when alternately frozen to −5°C and warmed to +10°C for 12 hours each day, whereas freezing at −20°C may render the metacercariae non-infective (Boray and Enigk, 1964). For prolonged survival, the relative humidity needs to be above 70%, cysts surviving on moist hay for 8 months and on silage for 57 days. The major cause of loss of infectivity of pasture appears to be death of the metacercariae, except where conditions of rapid pasture growth cause dilution of the infection (Ollerenshaw, 1971).

Implications for the epidemiology of the infection

In countries where there are only 2 months of the year when the mean temperature is greater than 10°C, parasites will have to overwinter in the snail to complete their development. Since snails rarely live longer than one year it can become difficult for the disease to establish (Gettinby and Byrom, 1991). Fasciolosis is therefore absent in Iceland and northern Scandinavia, despite the availability of suitable intermediate hosts.

In temperate climates, where there is year-round rainfall, temperature is still the restricting factor. When there is a mean day and night temperature

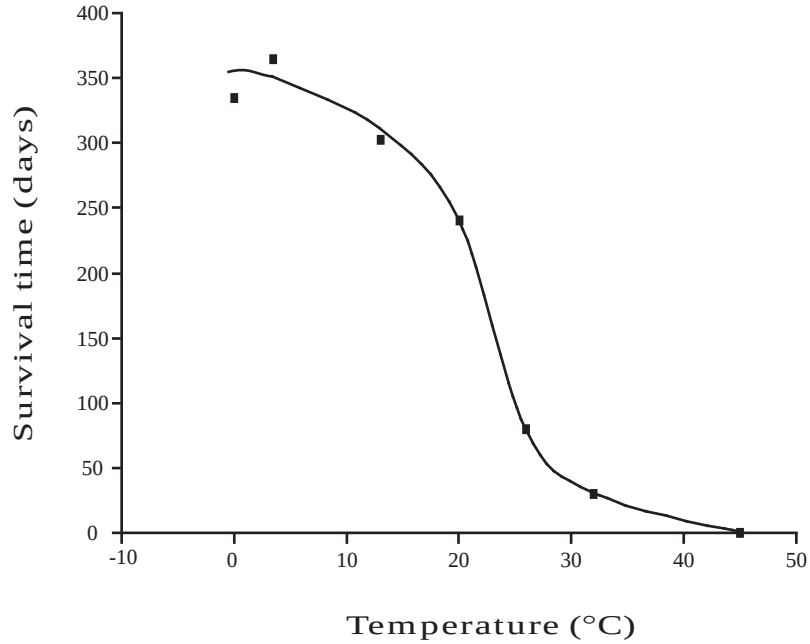

Fig. 4.4. The effect of temperature on the survival of metacercariae of *F. hepatica* (data from: Boray and Enigk, 1964; Boray, 1969).

above 10°C for approximately 6 months of the year the parasite is often endemic. A cycle of summer/winter snail infection has been described for north-western European climates such as Britain, Holland and Denmark (Ollerenshaw and Rowlands, 1959; Ollerenshaw, 1970; Over and Dijkstra, 1975; Shaka and Nansen, 1979; see Chapter 5 of this volume). Most development of the larval stages takes place during the spring and summer months and ceases during the winter. The 'summer' infection of snails results from the hatching of overwintering eggs or eggs passed in the spring, metacercariae appearing on pasture from August to October. A smaller 'winter' infection of snails is due to the infection of snails in the autumn: larval development ceases during the winter months and commences again in spring resulting in pasture contamination with metacercariae in May and June. Disease outbreaks usually relate to the peak time of pasture contamination with metacercaraie.

In climates with moderate winter temperatures parasites can overwinter as eggs, pasture metacercariae or developmental stages in snails, development resuming when the temperature reaches 10°C in the spring. Although Gaasenbeek *et al*. (1992) reported successful overwintering as metacercariae or as developmental stages in snails in The Netherlands, numerous studies have shown that the most important overwintering stage is the egg in similar climates. Several studies in northern Europe support the view that overwintering eggs develop at the same time as eggs that have been shed the following spring (Hope Cawdery, 1975; Over and Dijkstra, 1975; Shaka and

Nansen, 1979; Gaasenbeek *et al.*, 1992). The dominance of the summer infection of snails has also been described in Ireland (Ross, 1967; Ross and Todd, 1968) and Scotland (Armour *et al.*, 1970).

Livestock are infected from late summer onwards as a result of this early summer infection of snails. Acute and subacute disease in sheep is commonly seen in autumn and early winter due to the ingestion of large numbers of metacercariae. More chronic disease in both sheep and cattle is seen in winter and early spring due to the ingestion of lower numbers of metacercariae. Occasionally disease is seen in summer from the winter infection of snails.

The pattern of transmission is similar in north-western United States. In Idaho, peak transmission to sentinel steers occurred between August and November, indicating the summer infection of snails predominating in this region (Hoover *et al.*, 1984); there is little evidence of overwintering metacercariae or larval stages within snails, possibly due to low winter temperatures.

In regions further south, such as central western France, the increasing summer temperature and longer season allow an extra summer cycle (Leimbacher, 1978). In warmer conditions still, stages other than the egg play a much more important role in the overwintering of the parasite. In Australia, because there is often no winter housing of stock, contamination of pasture is continuous. Even in southern areas of Australia conditions may favour the development of eggs and larval stages for 9 months of the year. In these circumstances significant overwintering takes place in the form of infected snails which can result in heavy infection of sheep as early as mid-spring (Boray, 1969).

Fasciola gigantica, having higher temperature requirements for development of intermediate stages, is largely confined to the tropics and warm temperate regions. In some endemic areas, such as eastern Europe and the southern part of the former USSR, seasonal development is likely to follow a similar pattern to *F. hepatica*. Both *F. hepatica* and *F. gigantica* are endemic in many tropical countries; however, *F. hepatica* often predominates at higher altitudes partly because of the lower temperature requirements of the developmental stages.

Effect of moisture

On the parasite

Fasciola spp. require moisture for transmission, proliferation and survival; miracidia need wet surfaces to find snail hosts; snails need moisture to develop; cercariae do not normally emerge from snails unless there has been recent rainfall; and metacercariae need humidity to survive. Where there is seasonal rain, transmission will be limited to the wet season, unless land is irrigated or there are permanent water courses. Ideal conditions, when precipitation exceeds potential evapotranspiration resulting in increased water in the environment, favour both the intermediate-host and intermediate stages.

Fluke eggs will not develop while in the faecal mass. Moisture is necessary to break up the mass and even the presence of soil may reduce the rate

of development of the egg (Rowcliffe and Ollerenshaw, 1960). Eggs in moist faeces can survive for at least 10 weeks in the summer and 6 months in the winter in the United Kingdom. However, if the faecal material dries out, there is rapid mortality of the eggs (Ollerenshaw, 1971). In Spain 55% of over-wintering eggs arrested in wet faeces survived compared to 20% arrested in water (Luzón-Peña *et al.*, 1992).

Free-swimming cercariae, released from snails, encyst by secreting a cyst wall and losing their tails. Encystment usually takes place on a surface (such as vegetation) but some cysts may develop air-filled lacunae in the outer cyst wall and float freely (Esclaire *et al.*, 1989). Between 6.8% and 10% of parasites shed form such free-floating metacercariae (Boray 1969; Esclaire *et al.*, 1989). Once encysted, the metacercariae are thought to be infectious to the definitive host; two-day-old cysts are known to be able to excyst within the host (Ollerenshaw, 1971). The longevity of metacercariae, however, depends on moisture in, and temperature of, the environment (see above).

On the snail

Lymnaea truncatula is a mud snail which, if the environment dries out, may undergo a prolonged state of aestivation, during which transmission of the parasite is suspended. When the rains return, however, there can be rapid recolonization of the environment. *L. (Fossaria) bulimoides*, an important intermediate host in the southern United States (Zukowski *et al.*, 1991), also undergoes aestivation during summer drought (Malone *et al.*, 1984). In many areas, such as in Australia and Spain, the prevalence of fasciolosis is higher than might be expected from the regional climate as a result of increased moisture from irrigation (Meek and Morris, 1979; Uriarte *et al.*, 1985). In such circumstances, summer warmth or winter freezing could affect the viability and development of the parasite and the snail intermediate host.

Although *L. truncatula* can survive periodic droughts through aestivation, other species of lymnaeid snails have different requirements for moisture. *L. tomentosa* and *L. columella* live in habitats that are permanently wet (Mitchell, 1995). The important snail hosts of *F. gigantica* differ from those of *F. hepatica* in that they are aquatic. There is little evidence that they can aestivate (Soulsby, 1982), thus the continuous presence of free water is required for their development.

Implications for the epidemiology of the infection

In Mediterranean climates, such as Spain, moisture can be the limiting factor for overwintering eggs (Luzón-Peña *et al.*, 1992) and, as in northern Europe, these overwintering eggs have an effect on the intensity, but not the timing, of the spring contamination with miracidia. The dry winters of the semi-arid region of the Iberian Peninsula restrict contamination to areas adjacent to ponds, streams and irrigated water courses. Luzón-Peña *et al.* (1995) reported that excessive summer temperatures have a lethal effect on snails and metacercariae in parts of Spain. Similarly, in parts of the southern United States, excessive summer heat and/or drought prevent transmission (Boyce and Courtney, 1990; Zukowski *et al.*, 1991). In Florida, fluke transmission

occurs almost exclusively between December and June with the peak months being February, March and April (Boyce and Courtney, 1990). Despite there being adequate moisture in the summer, a break in the cycle often occurs in April or May when high temperatures and low rainfall cause desiccation of snail habitats. When the summer rains begin a month or two later, excessively high temperatures seem to prevent the snails reappearing. In other southern states, such as Louisiana and Texas, transmission occurs between February and July (Craig and Bell, 1978; Malone *et al.*, 1984). Here, replenishment of soil moisture begins in autumn and by February, the snails are shedding large numbers of cercariae. Provided rainfall is sufficient, this shedding will continue and livestock will become infected. During the first sustained drought of the summer, however, snails aestivate, metacercariae rapidly die and transmission ceases.

Although *L. auricularia/natalensis* is associated with tropical environments which have adequate temperatures for the development of *F. gigantica* all year, a growth cycle has been shown to occur in Malawi where snails were found to reach peak abundance around March/April each year (Tembely *et al.*, 1995). Both the total fluke-counts and the faecal egg-counts are highest at the beginning of the wet season in west Africa (reviewed by Schillhorn van Veen, 1980). This is when snail populations are rising. Large numbers of infected snails are present at the end of the wet season and beginning of the dry season when most ruminant hosts are infected. Acute fasciolosis is then seen towards the end of the dry season. Similarly, there is a cycle of development of snails and infection in cattle and buffalo in India related to the rainy season (Chaudhri *et al.*, 1993).

Other effects

Source of infection

Metacercariae derived from cattle were found to be less infective than those from sheep, although the metacercariae of bovine origin eventually develop into fluke that produced more eggs than those of ovine origin (Dixon, 1964). Similarly, *F. gigantica* from sheep were shown to infect a smaller proportion of *L. auricularia/natalensis* snails than those from buffalo (30% and 82%, respectively), while metacercariae developing from infections derived from sheep have a higher mortality than those of buffalo origin (Al Kubaisee and Altaif, 1989).

Competing infections

The presence of other species of parasites in the snail, such as the pulmonary fluke of frogs, *Haplometra cylindracea*, may reduce development of *Fasciola* spp. (Whitelaw and Fawcett, 1982) (see 'Control options for fasciolosis', below).

Resistance to Fasciolosis in Livestock

Infection with *Fasciola* may result in a degree of acquired resistance, which varies depending on the host species. In addition, some animals show a

degree of innate resistance: horses are less susceptible than ruminants (Nansen *et al.*, 1975); pigs are only significantly susceptible when under 8 weeks old (Nansen *et al.*, 1972).

Resistance in sheep

Numerous studies in sheep have demonstrated that naive animals, sensitized with infections of *F. hepatica*, generate no significant protection to challenge infections (Sinclair, 1971; Smithers, 1976; Sandeman and Howell, 1981; Boyce *et al.*, 1987), though innate resistance between breeds of sheep does vary. Boyce *et al.* (1987) reported that St Croix sheep develop less than half the parasite burden of Barbados blackbelly sheep. Similarly, Gruner *et al.* (1992) demonstrated that Romanov sheep have greater resistance than merinos. There have also been a number of studies documenting variation between individuals within breeds (Meek and Morris, 1979; Sandeman and Howell, 1981; Boyce *et al.*, 1987). Khallaayoune *et al.* (1991) reported a particularly marked variation in the Timahdit breed of sheep in Morocco: in a flock in which the prevalence of infection was only 50%, 10% of untreated lambs died of fasciolosis. This may indicate different degrees of resistance in individual animals and the possibility for selective breeding. Merino sheep have also been shown to develop some resistance to *F. gigantica* (Roberts *et al.*, 1996).

Resistance in cattle

It is often assumed that adult cattle develop resistance to *F. hepatica* and, consequently, such animals are frequently ignored with regard to treatment, particularly since infection is often subclinical. The hypothesis that cattle can develop a degree of acquired resistance is supported by some experimental evidence. For example, Doyle (1972) suggested that the loss of 61.5% of acquired fluke populations between 20 and 24 weeks after infection may have been due to acquired immunity. Furthermore, Doyle (1973) reported a mean of 72.5% protection to reinfection after a sensitizing dose of 750 metacercariae. Hope Cawdery *et al.* (1977) demonstrated that an experimental infection of 600 metacercariae followed by a second infection of 1000 metacercariae leads to fewer flukes becoming established than following a single dose of 1000 metacercariae. However, recent studies by Cleary *et al.* (1996) suggested that cattle with a chronic natural infection remain as susceptible to experimental infection as fluke-naive animals. Variations in age prevalence of fasciolosis provide further evidence for an incomplete protective immune response. The life span of *F. hepatica* in cattle is relatively short and varies from 6 months to about 2 years, depending on the degree of challenge (Ross *et al.*, 1966; Ross, 1968) but is considerably shorter than that of the host. Analysis of the data of González-Lanza *et al.* (1989) of the age-prevalence profile of cattle in Spain (Fig. 4.5) indicates that in older animals the prevalence approaches an asymptote of approximately 40%, suggesting an equilibrium has been reached between rate of parasite acquisition and parasite death. There appears to be little evidence of immunity preventing

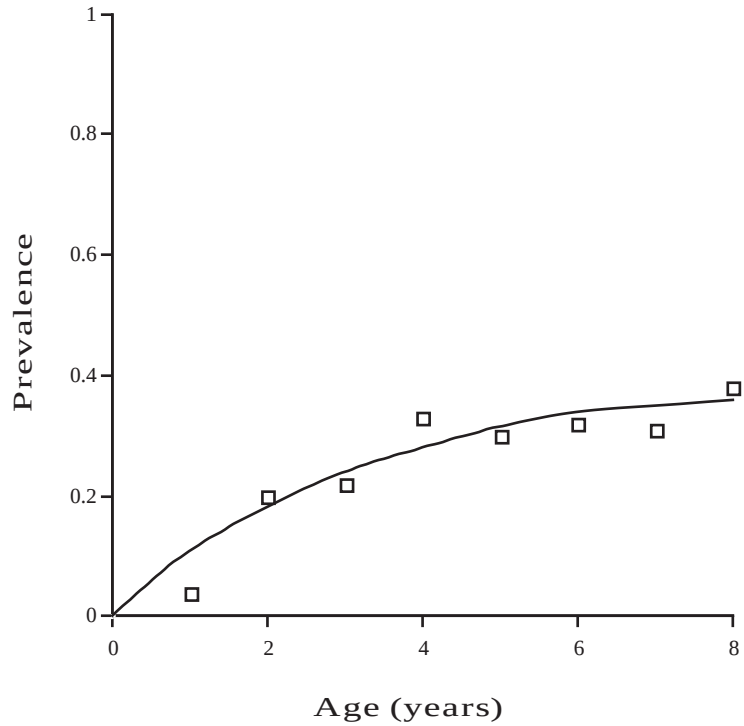

Fig. 4.5. The effect of age on the prevalence of *F. hepatica* in cattle (data from González-Lanza *et al*., 1989).

re-establishment of new infection. Similarly, Baldock and Arthur (1985) demonstrated an increase in age prevalence in male cattle and Hope Cawdery (1984) reported that cows and bulls have higher condemnation rates of livers than younger bullocks or heifers. Boray (1969) suggested that one year after infection cattle are again susceptible to reinfection. In a study in Canada, cows with a mean age of 5 years had a prevalence of up to 68% (depending on the season) with an observed pattern of egg passage closely resembling those of single, primary infections (Bouvry and Rau, 1986). In an endemic area, animals of this age group would normally have been exposed.

The difference between these studies and those of Doyle (1972, 1973) may be, to some extent, explained by the density-dependent constraints on the life span of the parasite. These constraints may include or be independent of immunity. Kendall *et al.* (1978) demonstrated that a sensitizing infection generated protection against challenge 35 or 54 weeks later, regardless of whether there had been treatment with fasciolicides between experimental infections, which provided good evidence for acquired resistance. The variations in the results of field observations and of Clery *et al.* (1996) compared to the experimental studies may also be due to the nature of sensitizing and challenge infections. Trickle infections could result in incomplete stimulation

of protective mechanisms which a single, large challenge infection evokes. Such a situation has been seen with other hepatic parasites such as *Echinococcus granulosus* in sheep. A single sensitizing dose of 50,000 eggs results in resistance to reinfection for at least 9 months (Sweatman *et al.*, 1963). In field conditions, however, the intensity of challenge is much lower and no protective immune response develops (Roberts *et al.*, 1986). The interrupted nature of seasonal challenge in fluke infection may also allow immunity to wane during periods of the year when there is no transmission. Furthermore, if the protective immune response is principally at the level of the gut mucosa, as suggested by McCole *et al.* (1998), rather than at the liver, the established adults would only be eliminated by senescence and, with waning immunity after seasonal challenge, reinfection at the beginning of each period of transmission might occur. Alternatively, acquired resistance in cattle may be a consequence of fibrosis of the liver parenchyma and fibrosis and calcification of the bile ducts (Anderson *et al.*, 1978; Doy and Hughes, 1984; Roberts and Suhardono, 1996). With low intensity of challenge or interrupted transmission patterns, the liver may become susceptible to reinfection. Whatever the nature of protective immunity or resistance, older cattle cannot be ignored either from the point of view of clinical and subclinical disease or as a potential source of pasture contamination.

Bos indicus cattle appear to be more resistant than *B. taurus* to infection with *F. gigantica* (Bitakamire, 1973; Castelino and Preston, 1979). Furthermore, there is also evidence that there is variation between breeds of *B. indicus*. Castelino and Preston (1979) reported an increase in the percentage of cattle infected with *F. gigantica* with age. However, when the intensity of infection is analysed, the prevalence of heavy infections appears to peak at 2–3 years of age and decline in older animals. Although this is insufficient evidence to suggest that complete acquired immunity to *F. gigantica* occurs in cattle, it is consistent with a partial acquired resistance. Other studies (Sukhapesna *et al.*, 1990) have also reported an increase with age in the prevalence of *F. gigantica* in buffalo.

Economic Effects of Fasciolosis in Livestock

The effects of clinical fasciolosis can range from sudden death, when there is a massive challenge infection, to chronic underperformance; subclinical infections, which often go unnoticed, produce marked economic effects.

Effects on live-weight gain and wool production

Ovine fasciolosis can result in significant blood loss representing a loss of metabolizable energy. This, together with impaired appetite and impaired nitrogen retention, can have an adverse effect on weight gain (reviewed by Hope Cawdery, 1984). Sinclair (1962) reported a 70% reduction in weight gain in sheep with a mean burden of 200 flukes. Coop and Sykes (1977) demonstrated that depression of live-weight gain in groups of sheep with a mean of 87, 157 and 233 fluke was 26%, 22% and 33% respectively. Hawkins

and Morris (1978) developed models relating live-weight changes in sheep with fluke burden. Weekly growth rates of wool and live-weight gain decreased with increasing fluke burdens (Fig. 4.6). Burdens of 346 flukes or greater resulted in weight loss and lamb mortality; lower burdens of 46 flukes resulted in 13.6% decrease in wool production and a 5.1% decrease in weight gain. Reductions of 40% in wool production, as a result of fasciolosis, have been recorded by other workers (Roseby, 1970; Edwards *et al.*, 1976).

In cattle, modest fluke infections can result in significant reductions in performance. Infections as low as 54 flukes per animal have been shown to reduce weight gain by 8–9% (Ross, 1970a; Hope Cawdery *et al.*, 1977), even though this degree of infection results in no clinical signs of disease. Recovery in performance occurs about 26 weeks after experimental infection. Consequently, there may be little economic benefit achieved by treatment of light infections acquired more than 6 months previously if there is no further challenge (Kendall and Parfitt, 1975; Hope Cawdery *et al.*, 1977). However, therapy at such a stage may be beneficial in lowering the pasture burdens, as has proved successful with sheep (Armour *et al.*, 1973). Larger burdens of fluke, such as occur after experimental infections with 1000 metacercariae, can reduce weight gains by 28% in previously uninfected animals (Hope Cawdery *et al.*, 1977). The greatest reduction in weight gain occurs in the first 16 weeks of infection and there is still a significant decrease in weight gain during the chronic stage of the disease. Even after the animals are cleared of fluke, the initial impaired performance remains until slaughter (Hope Cawdery *et al.*, 1977). Losses in the early stages of the disease can only be avoided by prevention, not treatment. Losses in reinfected animals are also significant, so immune responses do not provide adequate protection.

Significant effects on performance in beef cattle have been reported in animals that have been infected by natural challenges. In the United States, Johnson (1991) reported an 8% increase in weight gain in feed-lot cattle treated with flukicide. In Belgium, Genicot *et al.* (1991) reported an 18% increase in weight gain in flukicide-treated double-muscled Belgian-blue cattle compared with non-treated controls. Most importantly, the increased profit resulting from the improved productivity was 4.2 times the cost of treatment.

Effects on milk yield and fertility

Studies have suggested that, in dairy cattle, infection can also have a deleterious effect on milk quality (Black and Froyd, 1972). Milk yield can drop by 14% (Ross, 1970a), although 8% is recoverable by treatment. The reduction in the milk yield may be dependent on the magnitude of the parasite burden and animals can, to a certain extent, compensate by an increased appetite (Hope Cawdery and Conway, 1972). Sinclair (1972) attributed to reduced milk production the lower growth rates observed in lambs whose dams were infected. Oakley *et al.* (1979) and Hope Cawdery (1984) suggested lower fertility rates in infected or inadequately treated cattle, while fewer lambs are born to infected ewes (Hope Cawdery, 1976). Crossland *et al.* (1977) demonstrated a 9% increase in fecundity in ewes grazing plots where there was snail

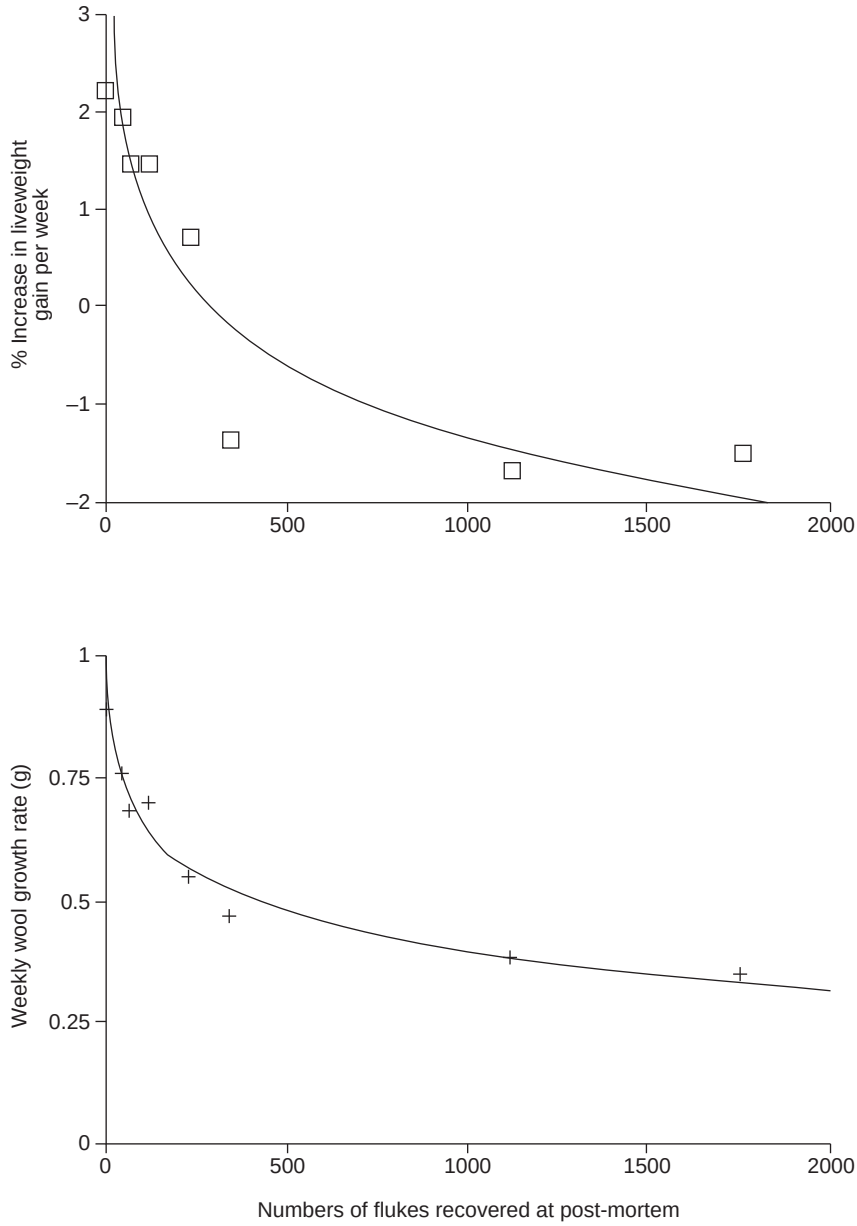

Fig. 4.6. The effects of fluke burden on live-weight gain and weekly wool growth in sheep (data from Hawkins and Morris, 1978).

control by molluscicide. This effect on breeding may be particularly important in the many sheep-rearing areas in Europe as ewes are likely to be exposed to the greatest challenge from this parasite at the same time of year as the breeding season.

In endemic areas the prevalence of infection is often very high (Tables 4.1 and 4.2). Even if the majority of the animals in these surveys have only modest burdens, the economic effects of this parasite on a global scale represent billions of dollars in lowered productivity.

Control Options for Fasciolosis

Eradication of parasitic infections is rarely a practical option and control needs to be aimed at the reduction of disease to allow economic livestock production. Specific aims of the control programme may be to prevent the build-up of parasites in the environment and to avoid areas of heavily contaminated pasture (Brunsdon, 1980). Reduction of pasture contamination may be accomplished through the use of anthelmintics, management regimes, molluscicides and biological competition as components of an integrated control programme. In addition, the use of resistant animals to reduce the impact of infection may have potential, especially where treatment costs are relatively high (Roberts and Suhardono, 1996).

The type of control programme that can be recommended will depend on local husbandry and climatic conditions together with socio-economic factors relating to the livestock owner. These factors will vary widely between temperate and tropical climates as well as between farmers in industrial compared to lesser developed countries. A range of models that predict the likely impact of fasciolosis in particular years, based on climatic data, condemnation rates of livers and the local epidemiology of infection, have been produced, although their use in many specific ecosystems will require further development. Control strategies in any given year can be modified, if necessary, based on the prediction given by these models. The potential environmental impact or consumer acceptance of any control measure must also be taken into account.

Treatment

Anthelmintics

Host treatment with anthelmintics is the principal method employed to control fasciolosis in livestock. There is a range of anthelmintics including benzimidazoles, salicylanides, nitrophenols and halogenated hydrocarbons (Table 4.5; see also Chaper 7 of this volume). These differ in availability, price, efficacy and safety. Most of the products which are listed have efficacy against both *F. hepatica* and *F. gigantica*. However, triclabendazole has reduced efficacy in buffalo as a result of achieving lower serum concentrations of the drug than occur in cattle (Sanyal, 1995). Oral dose-rates of 12 mg kg^{-1}, highly effective in cattle, only reduce infection in buffalo by 19–23%, whereas intraruminal administration of double or triple the normal dose was shown to have a much improved effect (Sanyal and Gupta, 1996).

Table 4.5. Anthelmintics effective against *Fasciola hepatica*.

Anthelmintic	Stage of fluke	Dose	Reference
Halogenated hydrocarbons			
Carbon tetrachloride[1]	>8 weeks old	1–2 ml per 50 kg	Kendall and Parfitt (1962)
Hexachlorophene[2]	Adult flukes	10–15 mg kg^{-1}	Kendall and Parfitt (1962)
	Juvenile flukes	40 mg kg^{-1}	Dorsman (1962)
Tetrachlorodifluoroethane[3]	Adult flukes	0.33 g kg^{-1}	Boray and Pearson (1960)
Hexachlorethane	Adult flukes	220–400 mg kg^{-1} in 3–4 doses in cattle; 20–30 g per sheep	Soulsby (1982)
Nitrophenols			
Oxyclozanide	Adult fluke	10 mg kg^{-1} for cattle; 15 mg kg^{-1} for sheep	Boray (1982); Torgerson (1995)
	Juvenile fluke	45 mg kg^{-1} for sheep	
Nitroxynil	Adult fluke	10 mg kg^{-1} for sheep and cattle	Boray (1982); Torgerson (1995)
	Juvenile fluke	15 mg kg^{-1}	
Salicylanides			
Closantel[4]	70.3–76.3% effective against 6-week-old fluke	10 mg kg^{-1}	Maes *et al.* (1988)
	92.8–96.5% effective against 8-week-old fluke		Maes *et al.* (1990)
Rafoxanide	98–99% effective against fluke ≥6 weeks; 50–90% effective against 4–5-week old fluke	7.5 mg kg^{-1} for cattle and sheep	Soulsby (1982)
Benzimidazoles			
Triclabendazole	Juvenile and adult fluke	10 mg kg^{-1} for sheep; 12 mg kg^{-1} for cattle	Smeal and Hall (1983); Turner *et al.* (1984) Misra *et al.* (1987); Stansfield *et al.* (1987)
Albendazole	Adult fluke	7.5 mg kg^{-1} for sheep; 10 mg kg^{-1} for cattle	Malone and Craig (1990), Torgerson (1995)
Netobium	Adult fluke	20 mg kg^{-1}	Torgerson (1995)
Others			
Clorsulon	Adult fluke	2 mg kg^{-1}	Torgerson (1995)
Diamphenethide[5]	<6 weeks old	100 mg kg^{-1} for sheep	Armour and Corba (1972); Kingsbury and Rowlands (1972); Rew *et al.* (1978)

[1] Low safety margin, especially in cattle. Can produce serious tissue reactions when injected.
[2] Higher dose rates not tolerated well in heavy infections.
[3] Use only documented in sheep.
[4] Additional effect on egg development with reduced production for up to 13 weeks.
[5] Of limited efficacy against adult flukes.

Local remedies to control fluke may also be available, though with unproven efficacy. Extracts of artichoke leaves or of an unclassified plant 'jaya-shipata' in Peru have been used to control *F. hepatica* infection in sheep, where they may reduce the number of adult parasites (Arévalo and Bazalar, 1989).

Emerging evidence of drug resistance against some products (Boray, 1990) may limit treatment options. Resistance of *F. hepatica* to triclabendazole has been recorded in Australia (Overend and Bowen, 1995) and Ireland (Mulcahy and Dalton, 1998) and fluke have shown a degree of resistance to salicylanides (Miller *et al.*, 1994; see also Chaper 7 of this volume).

Treatment strategies

In industrialized countries, because of the significant economic effects of the parasite and the widespread availability of effective anthelmintics, there is likely to be a positive cost–benefit in all endemic areas except where parasite burdens or prevalence of infection are very low. In the lesser developed countries, anthelmintics may be prohibitively expensive and other considerations for treatment may be important. Furthermore, anthelmintic prophylaxis will depend on the principal livestock reservoirs, animal husbandry systems practised, the length and extent of seasonal transmission, and the ability of the intermediate stages to survive local climatological conditions of excessive drought, heat or cold. Implementation of a logical treatment regime thus requires a thorough understanding of the epidemiology of infection under local conditions (see Chapter 5 of this volume).

Strategic anthelmintic control

Regular treatments at 12–13 week intervals with flukicides effective against both mature and immature fluke will reduce the intensity of infection in a flock or herd over time. This should be effective regardless of the climate provided there are no significant wildlife reservoirs of infection or infected irrigation or water courses that could recontaminate the pasture. Once the prevalence of fluke has been reduced to a low proportion, it may be possible to reduce treatment intervals or dispense with treatment completely for a number of years, though reinfection appears to occur eventually. In trials, this approach has reduced the prevalence of *F. hepatica* in sheep by up to 75% on some Irish farms (Taylor *et al.*, 1994) and reduced the prevalence from 49% to 1% on individual Scottish farms (Fawcett, 1990). In France, four annual treatments in the first year followed by three treatments in the second year reduced the prevalence of infection in sheep from 93% to 5% (Maes *et al.*, 1993). Whitelaw and Fawcett (1981) demonstrated that strategic dosing of sheep five times a year over a three-year period reduced the prevalence from over 75% to 1%. However, total eradication was not achieved, possibly due to the presence of infected deer in the area. In New Zealand, similar results have been achieved with treatment of all cattle and sheep on farms with triclabendazole every 8–11 weeks (Boray *et al.*, 1985). As a result of the significant economic effect of the juvenile parasite, more frequent treatments

may be necessary in the early part of a control programme if there is a large fluke challenge. In such circumstances treatment regimes can be modified to give animals additional treatments during the season of transmission. The parasite has a high biotic potential and reservoirs of infection can result in rapid reinfestation of the pasture given suitable climatic conditions, so that it is important to continue to monitor flocks or herds even after the infection has been reduced.

Modifications in light of local environmental or husbandry factors

Some general, strategic control strategies using anthelmintics for different climatic zones are summarized in Table 4.6, which can be modified in the light of local knowledge of the infection including climate, husbandry practices and fluke forecasts. For example, in North America a single annual treatment in cattle has been recommended based on the seasonal transmission dynamics of *F. hepatica* (Kaplan, 1994). In the Gulf Coast states of the United States, local recommendations are based on the fact that transmission ceases during the hot, dry summer. In addition, as a result of high summer temperatures which are often combined with drought, flukes normally only survive within fluke-infested cattle. As few infected snails survive the summer because of the combined stresses of aestivation and infection, a single treatment in early autumn with an anthelmintic against adult fluke, just before (non-infected) snails emerge from summer aestivation, should virtually destroy the entire population of viable flukes. Such a programme should reduce the fluke population to acceptable levels, though this may be inadequate in some high-risk years in or on pasture where previous control has been poor. In these circumstances, in order to minimize economic losses, additional treatments with a flukicide that kills both juvenile and adult flukes may be necessary in the spring.

The situation in the north-western United States is similar to northern Europe where most transmission of infection to cattle occurs in the autumn. A single treatment, in the winter, effective against all stages, will minimize pasture contamination in the spring. This may be adequate in areas where few intermediate stages survive the low winter temperatures but in regions with mild winters significant numbers of overwintering metacercariae or fluke-infested snails may survive. In this case, additional treatments, using flukicides active against juveniles, may be necessary during the transmission season, particularly in the early years of a control scheme. Martignoni *et al.* (1995) suggested that twice-yearly treatment provides a more effective long-term control of fluke infection in cattle than once-yearly treatment.

Modifications for sheep enterprises

As a general rule, effective control in sheep often requires more frequent treatments than in cattle in any given area (Table 4.6). This is partly because sheep are often at pasture for 12 months of the year and because they are more susceptible to the pathogenic effects of the parasite.

Table 4.6. Possible seasonal occurrence of fasciolosis in various climatic zones and suggestions for strategic anthelmintic treatments.

Climatic zone	Development of parasite stages on pasture	Generations of fluke per year	Infection of host	Disease outbreaks	Recommended treatments for cattle[1]	Recommended treatments for sheep
Cool temperate, cool continental	Summer	0.5–1	Late summer to autumn	Autumn and winter	Early winter and early spring	Early spring and 2–3 times from late summer to winter
Warm temperate, warm continental	Late spring to early autumn. Seasonal drought or high temperatures may limit development in summer	1–2	Late spring to mid-winter	Late summer to spring	Early spring and twice in autumn and early winter[2]	Early spring and 3 times from late summer to winter[2]
Sub-tropics	Early spring to late autumn. Seasonal drought or high temperatures may limit development in summer	2	Early spring to mid-winter	All year round	Late autumn and spring[2]	4–5 times per year[2]
Tropics	All year round. Seasonal drought may limit development	3–4[3]	All year round	All year round	3–4 times per year[2]	4–5 times per year[2]

[1] An additional treatment during the periods of cercarial development may be required if calves are grazed.
[2] Fewer treatments will be required if there are regular seasonal droughts.
[3] The maximum possible number of generations per year for *F. hepatica* is four under optimal conditions of temperature and moisture, less for *F. gigantica*. In practice, local conditions are unlikely to support such rapid development of the parasite all year.

Subclinical infection

One problem in devising practical treatment strategies is estimating the degree of subclinical infection at which it becomes economic to treat. Thus, although it may be considered prudent to treat all infections, treatments based on the degree of infection have been suggested. Malone and Craig (1990) reported 25% prevalence rates as being important in a herd and stated that treatment has more effect in least efficient herds, where there may be an improvement of reproductive indices after treatment. Where herd prevalence is less than 25% and the mean egg-count less than 0.5 eggs per gram of faeces, then the herd burden is considered low and there is a low probability of economic loss; where prevalence is 25–75% and the mean egg-count 1–5 eggs per gram of faeces, infection is moderate and economic loss is possible; where prevalence is more than 75% and mean egg-counts are greater than 5 eggs per gram of faeces then the burden of disease is high with concomitant economic loss. However, these suggestions are based on local conditions and, in view of the significant economic losses attributed to modest burdens of fluke, such an approach must be viewed with caution.

Evaluating indirect losses due to fluke infection requires a large amount of production data and this is often lacking, especially in lesser developed countries, a situation further complicated by the presence of mixed parasitic infections in most field situations (Dargie, 1987). Treatment may also be made less effective if diagnosis is confirmed by the identification of eggs in the faeces as the severest form of the disease can occur before the infection is patent.

Models and forecasting systems

Whatever the strategy of anthelmintic prophylaxis, modifications are possible if a fluke forecast or model of infection is available. Ollerenshaw and Rowlands (1959) used a simple *Mt* index-model as an aid to predicting the risk of fasciolosis in different parts of the United Kingdom. The index is calculated for each month from May to October using the difference between monthly rainfall and potential evapotranspiration weighted by the number of days of rainfall each month. Ross (1970b) used a simpler index of accumulated wet-days (potential evapotranspiration being a complex figure to calculate) over the period from June to September to predict disease risk. More recently a computerized system has been developed by McIlroy *et al.* (1990) based on the time-series analysis of a multivariate model that includes both climatic data and the prevalence of liver condemnations in cattle and sheep from previous years. This model can account for 93% of the variation in the prevalence of fasciolosis over an 18-year period. Other models, using population dynamics theory, are also being investigated but are at an early stage of development (Wilson *et al.*, 1982). Using these models, forecasts can be made which optimize strategic preventive measures for the control of disease over the following year. The frequency and strategy of the use of modern flukicidal drugs can, therefore, be used in accordance with the expected prevalence of fasciolosis (see Chapter 5 of this volume).

Snail control

Molluscicides

Molluscicides have been used both successfully and cost effectively to control snail populations (Urquhart *et al*., 1970; Crossland, 1976). However, this approach has not achieved widespread acceptance. Risk of environmental contamination may be unacceptable, particularly when molluscicide kills utility species such as fish and crabs. Furthermore, certain parts of the pasture can be difficult to access with a sufficient amount of the chemical. As a result of the snails' high biotic potential, pasture can quickly become reinfested, making repeated applications necessary, thus adding to expense and increasing risks of contamination. The most important compounds which can be applied to control snails are niclosamide, sodium pentachlorophenate and *N*-tritylmorpholine (Boray, 1982). Niclosamide is highly toxic to snails and eggs at 0.1–0.2 ppm and has a low toxicity for mammals. It is applied at 1–3 ppm for aquatic snails, higher concentrations being necessary if water has a high dissolved salt concentration (Anon., 1970; Meyer Lassen *et al*., 1994). For controlling amphibious snails, a ground application of 0.2 g m^{-2} on snail habitat may be used. Sodium pentachlorophenate is also effective when applied at 0.4–10 g m^{-2} for amphibious snails and 2–5 ppm in areas containing free water. Like niclosamide, it is highly toxic to fish. *N*-tritylmorpholine is effective against snails but not their eggs at concentrations of 0.15–0.5 ppm. Slow release formulations, when combined with snail attractants, may reduce some of the potential risks to the environment (Pfister *et al*., 1994; see also Chapter 2 of this volume).

Environmentally friendly approaches to control of fasciolosis

The disadvantages of anthelmintic chemotherapy and chemical control of snail populations include residues in both the host species and the environment and the development of anthelmintic resistance. To a certain extent the risks of these can be lessened by exploiting forecasting methods to minimize the use of such methods. Nevertheless, there is an increasing interest in developing more environmentally friendly approaches to fasciolosis control. These take the form of natural molluscicides, vaccination, management and biological control and the use of resistant livestock.

Natural molluscicides

There has been an increasing interest in the exploitation of plants with natural molluscicidal activity. Some *Eucalyptus* spp. may have a molluscicidal effect *in situ* (Hammond *et al*., 1994). An aqueous extract of *E. camaldulensis* has an LC_{50} of 3050 mg l^{-1} against *Biomphalaria glabrata*, and a methanolic extract an LC_{50} of 72 mg l^{-1} (Zhou *et al*., 1993). The latex of *Euphorbiales* spp. has been shown to kill some snail species (Singh and Agarwal, 1988, 1992). However, there appears to be no work quantifying the molluscicidal effect of these plants in the field.

Vaccination

At present there are no commercially available vaccines against fasciolosis, although glutathione *S*-transferase and proteinases secreted by *F. hepatica* are possible candidate antigens (Sexton *et al*., 1990; Wijffels *et al*., 1994; Dalton *et al*., 1996; see also Chapter 15 of this volume). It may not be possible – or necessary – for a vaccine to be 100% effective to control fluke in the field but vaccines will need to be cost effective in comparison with anthelmintic treatments. A vaccine which increased herd immunity sufficiently to significantly reduce fluke transmission might effectively control the parasite over a period of time and result in a positive cost–benefit return. Mathematical models backed up by field trials need to be developed for the various host species to define the target efficacy of suitable vaccines in different environments.

Managemental and biological control

Reducing snail abundance through improved pasture, either by draining or fencing-off wet areas, prevents infestation of grazing areas (Plate 1). However, this approach is expensive and often not cost effective (Wilson *et al*., 1982) and may not be feasible in many situations. Lymnaeid snails are vulnerable to predators such as arthropods, amphibians, reptiles, birds and rodents. Generally snails exist in equilibrium with their predator species, although intensive duck and goose husbandry has been shown to eliminate lymnaeid snails and effectively control fasciolosis (Levine, 1970). Certain sciomyzid fly larvae have been shown to predate lymnaeid snails (Berg, 1953) and this has been put forward as a method for the biological control of snails. In Ireland, *Ilione albiseta* has maximum fecundity at temperatures prevailing in the summer and studies are being undertaken to determine if this insect could be exploited in the control of fasciolosis (Gormally, 1988). Other systems of control of snail abundance have involved the competition of non-host species of snail: Ximenes *et al.* (1993) virtually eradicated *Fasciola* infection from some pastures in France using a mixture of molluscicide and competitor molluscs, particularly *Zonitoides nitidus*; Perera de Puga *et al.* (1991) showed reduced *Fossaria* (L.) *cubensis* abundance following competition by *Helisoma duryi* in Cuba; *Marisa cornuarietis* eliminated *L. auricularia/natalensis* from a lake in Tanzania (Nguma *et al*., 1982).

Candidates to reduce fluke infection within snails include the oligochaete *Chaetogaster limnaei*, an external parasite of the snail and second intermediate host to some trematode species, which has been shown to ingest and digest miracidia and emerging cercariae (Khalil, 1961). The nematode *Muellerius capillaris* also antagonizes the trematode in co-infections in snails (Hourdin *et al*., 1993). *Echinostoma audyi* has been shown to displace *F. gigantica* from host snails (Hoa *et al*., 1979).

In an extensive review of antagonism between trematodes within snails, Lim and Heyneman (1972) suggested that locally available parasites might be used to control snail numbers. For such methods to work the definitive host of the controlling parasite must be present in sufficient numbers and be easily maintained and infected in order to produce enough parasites to overwhelm the snails. More importantly, as local parasite dynamics are very variable, Lim

and Heyneman (1972) indicated that a specific antagonistic system would need to be set up for each area of control, a single system not being universally applicable.

Resistant livestock

The use of resistant species to minimize the effects of contaminated pasture, as is common for the control of nematode infections, is limited in the case of fasciolosis due to the large host range of *Fasciola*. Despite the conflicting evidence regarding the generation of protective immunity in cattle, this species does appear to be more resistant than sheep to the pathological effects of the parasite. In some areas cattle production may be possible where sheep production is not. Nevertheless, since individual sheep and sheep breeds demonstrate a variation in resistance it should be possible to create relatively resistant strains of animals that may be productive in endemic areas. Recently, the presence of a gene affecting resistance in thin-tailed sheep in Indonesia has been described and the selection of such sheep has been suggested as a potentially useful factor to reduce the impact of infection (Roberts and Suhardono, 1996; see also Chapter 15 of this volume).

Conclusions

As long ago as 1883 it was suggested that fasciolosis was preventable and that control ought to be integrated to involve snail control and management methods, including (in the absence of effective treatments) killing infected sheep (Thomas, 1883). Although Thomas's reasoning may have been influenced heavily by the lack of effective fasciolicides, it still has application today and adequate control is likely to involve a range of the methods described. In all cases, however, local knowledge of the disease epidemiology is vital to maximize the efficacy and cost effectiveness of *Fasciola* control.

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5 Predicting Outbreaks of Fasciolosis: from Ollerenshaw to Satellites

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Introduction

Recent literature reports suggest that more targeted, cost-effective control of *Fasciola hepatica* and its tropical counterpart, *F. gigantica*, is possible by application of new methods in climatology, mathematical models, and computer-based environmental monitoring systems to classical concepts on the geographic determinants of disease (Pavlovsky, 1945, 1966; Levine, 1968). These new methods can be used to develop geographic models that allow design of better ways to control fascioliasis, a problem that continues to plague much of the world as a cause of food animal production losses and, increasingly, as a food-borne zoonosis of humans.

Climate Forecasts and the Geography of Fasciolosis

In classic work by Ollerenshaw and Rowlands (1959), *F. hepatica* was the target of the first widely used climate forecast system for predicting acute outbreaks and appropriate control of a parasitic disease. The forecast was developed using 1948–1957 disease prevalence data and climate values for five meteorologic stations in the island of Anglesey and validated by extrapolation to all of England and Wales using 1958–1966 data (Ollerenshaw, 1966). Forecast index results were consistent with records of acute disease outbreaks in sheep and rainfall for England and Wales dating back to 1734 (Ollerenshaw and Smith, 1969) and was validated against patterns observed in the field for snail population numbers, number of infected snails, livestock faecal egg counts, liver condemnations and the annual extent of both acute and chronic disease (Ollerenshaw, 1974).

Using the formula [Mt = N (R − PE + 5)], a monthly risk index based on N (the number of raindays) and the difference between R (precipitation) and PET (evapotranspiration calculated by the Penman method) was derived for the months of May to October, the months in which temperatures exceed the

Fasciolosis (ed. J.P. Dalton)

10°C minimum required for life cycle progression. Potential losses in sheep were estimated for both the major 'summer infection' arising from snails infected during late spring and early summer (losses occur autumn to spring), and a minor 'winter infection' arising from carry-over to the next year of snails infected in late summer and autumn (losses in summer). Potential risk of the summer infection was calculated by adding monthly indices for June, July, August and September and half the Mt values of May and October (the latter adjustment was for the typically lower temperatures of those months). Overwintering infection risk, ordinarily of little importance, was represented by adding Mt values of August, September and October to values for May and June of the next year. Disease losses were considered to be heavy at Mt values of >450; little or no disease was expected if Mt values were <300. Rainfall levels in June, the time of rapid snail reproduction, were considered pivotal in most years.

Attempts have been made to adapt the Mt system to other areas in the Oceanic climate zone of Europe, but it has not been routinely used except in the British Isles and Ireland. Limitations are the complex data needed for the Penman equation, and the portability of the forecast system to other climate zones (Trewartha, 1981). The Penman method for calculating potential evapotranspiration (PE) is data intensive and, although variables can be estimated, it requires records of wind, relative humidity, solar radiation and other factors not routinely available except through well-developed national climate services in advanced countries. Most climate stations record only temperature and rainfall.

A simpler Stormont 'wet-day' system was developed for use in Northern Ireland (Ross, 1970) based on the number of days with over 1 mm of rain in the months of June, July, August and September. Calculation of PE values is not needed, but, since no consideration is given to temperature, later attempts to use the method in Scotland were unsuccessful, especially in northern areas where it may take two seasons to complete development of the *Fasciola* life cycle (Ross, 1975). Goodall *et al.* (1989) later developed an autoregressive, multivariate computer model in Northern Ireland using an integrated database of monthly meteorologic data and an 18-year time series on liver condemnations in sheep; they reported greater accuracy than the Mt and wet-day systems and determined that the previous year's prevalence of fasciolosis was an underestimated factor that accounted for 67% of the variation in the time series. Leimbacher (1981) modified the Mt system for use in France and incorporated the growing degree concept, wherein only 'useful' degrees above the critical 10°C are considered. Complete development to cercariae in infected snails was considered to require an accumulative total of 600 degree-days in the field. In addition to predicting disease outbreaks, an indication of the range and relative importance of fasciolosis in different areas of France could be mapped using long-term climate values.

Other climate models have been developed for use in other world areas, including the United States and Australia. In Australia, daily minimum and maximum temperatures and soil moisture under rainfall or irrigation conditions

were used as the driving force for a comprehensive computer simulation model of ovine fasciolosis designed to simulate and predict the value of alternative control strategies for the sheep industry (Meek, 1977; Meek and Morris, 1981) .

A climate forecast was developed for the south-eastern United States based on the growing degree-day concept and the Thornthwaite water budget. It has been used since 1979 to issue advice in Louisiana on the need for flukicide treatment of cattle each spring and autumn. Using only *daily* maximum and minimum temperature and rainfall data as input, a micro-computer program calculates water budget values and a cumulative index at any interval during the fluke transmission cycle. Soil moisture storage (habitat wetness), habitat flood events and sustained periods of drought and cold are taken into account (Fig. 5.1) according to the following formula:

$$\frac{S \times \text{GDD1}}{C} + \text{GDD2} - \frac{1}{D}$$

where *S* is daily surplus water, GDD1 is degrees over a base value of 10°C and *C* is the number of days in the prior 14 with mean temperatures of <10°C. GDD2 is the same as GDD1, except that values are accumulated additively only if the presence of moisture in the top 2.5 cm and lower 12.7 cm of the soil moisture model indicates that adequate habitat wetness is present for life cycle progression. *D* is the number of days in the last 14 in which soil moisture storage was below the adequate level. *C* and *D* are adjustment factors to account for the effects of sustained periods of cold or drought, respectively. GDD1 values are *multiplied* by S to account for the influence of rainfall events on habitat flooding, release of cercariae from lymnaeid snails and dispersal of infective stages on to surrounding pastures. Snails are considered to aestivate during dry periods. Transmission seasons end as metacercariae are killed by sustained summer drought (Malone *et al*., 1982, 1985, 1987; Malone, 1997).

Results of validation studies over a 10-year period indicate there can be a 100-fold difference between years in fluke burdens in the same herd of cattle grazing the same pastures (Table 5.1). Extrapolation of the system to the entire south-eastern United States fluke zone suggested it is possible to use 30-year average data from selected sites to generate information on the range of the enzootic area, the seasonal pattern of transmission and the 'normal' index of severity for *F. hepatica* at each site (Fig. 5.2). Provisional extrapolation of the system to Texas, Oklahoma, Arkansas and Florida, based on 30-year-average climate data, suggests a pattern of diminishing transmission in drier western sites and later transmission at northern sites.

Climate forecast models developed so far have not been extensively used outside the climate zone where they were developed. Satellite-based world climate change models and the increasing use of automated data collection and internet accessible archives by national climate services promise to allow more comprehensive use of forecasts in the future.

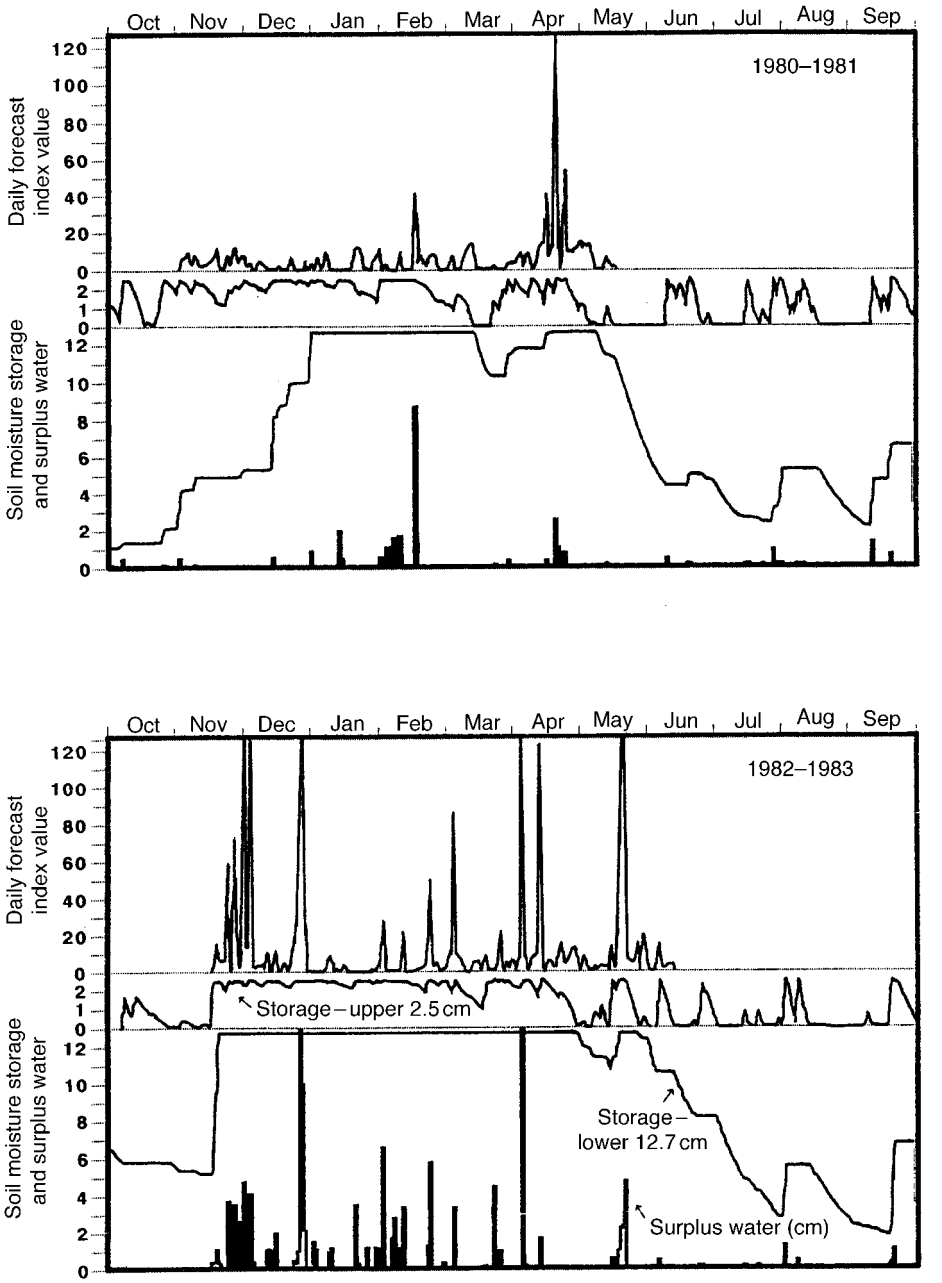

Fig. 5.1. Computer graphical representation of forecast values comparing a low risk year (1980/81) and a very high risk year (1982/83) for *Fasciola hepatica* transmission to cattle at Dean Lee Research Station, Alexandria, Louisiana, USA. (From Malone and Zukowski, 1994.)

Table 5.1. Spring and autumn forecast index values and observed *Fasciola* risk at Dean Lee Research Station, Alexandria, Louisiana.

Year	Spring index	Autumn index	Flukes per calf	Risk
1979	416	1423	2	Low
1980	757	2949	75	High
1981	326	956	5	Low
1982	370	1134	28	Moderate
1983	1304	3671	251	Very high
1984	549	1168	nd	Moderate
1985	2899	3187	325	Very high
1986	2151	2470	146	High
1987	1619	2081	146	High
1988	669	1459	nd	Low–moderate
1989	830	5741	124[a]	Very high
Average[b]	1081	2385	110	

Source: Malone and Zukowski, 1994.
[a] Average fluke number despite subterranean drainage of half of the farm and improved preventive herd treatment in 1983. The neighbouring farm averaged 189 flukes per calf.
[b] 30-year-average reference value computed by the model was 579 for spring and 1978 for autumn.

Geographic Information Systems – Discovering the Multiple Causation of Disease

Geographic Information Systems (GIS) and satellite sensor technology are emerging new tools for epidemiological studies on human and animal diseases, especially vector-borne diseases with strong environmental determinants. By use of statistical and image analysis methods, GIS allows computer-based analysis of multiple layers of mapped data in digital form, including sensor data from earth observation satellites, agroclimatic databases and maps of host populations, vector distribution and disease prevalence. GIS data layers are registered to the identical scale and geographic projection of a reference base map. This allows analysis of all information by location, including descriptive data sets that are 'attached' to specific locations or areas.

Once created, GIS provides a dynamic, easily updated mapping system that can be used to plan and monitor control programmes. By virtue of its potential to 'match' the relative suitability of various environments to the life cycle and transmission dynamics of host–parasite systems, GIS provides a new way to address Pavlovsky's classic concepts of 'landscape epidemiology' and the essential nidality of disease. For the first time, many excellent current and historical local disease investigations can be systematically and accurately placed in a broad geographical and ecological context. Recent applications include schistosomiasis (Cross and Bailey, 1984; Malone *et al*., 1994, 1997), fasciolosis (Malone *et al*., 1992; Zukowski *et al*., 1992, 1993), Rift Valley fever (Linthicum *et al*., 1987), African trypanosomiasis (Rogers and Randolph, 1993), East Coast fever (Lessard *et al*., 1990), Lyme disease (Kitron *et al*., 1992), filariasis (Thompson *et al*., 1996) and malaria (Beck *et al*., 1994).

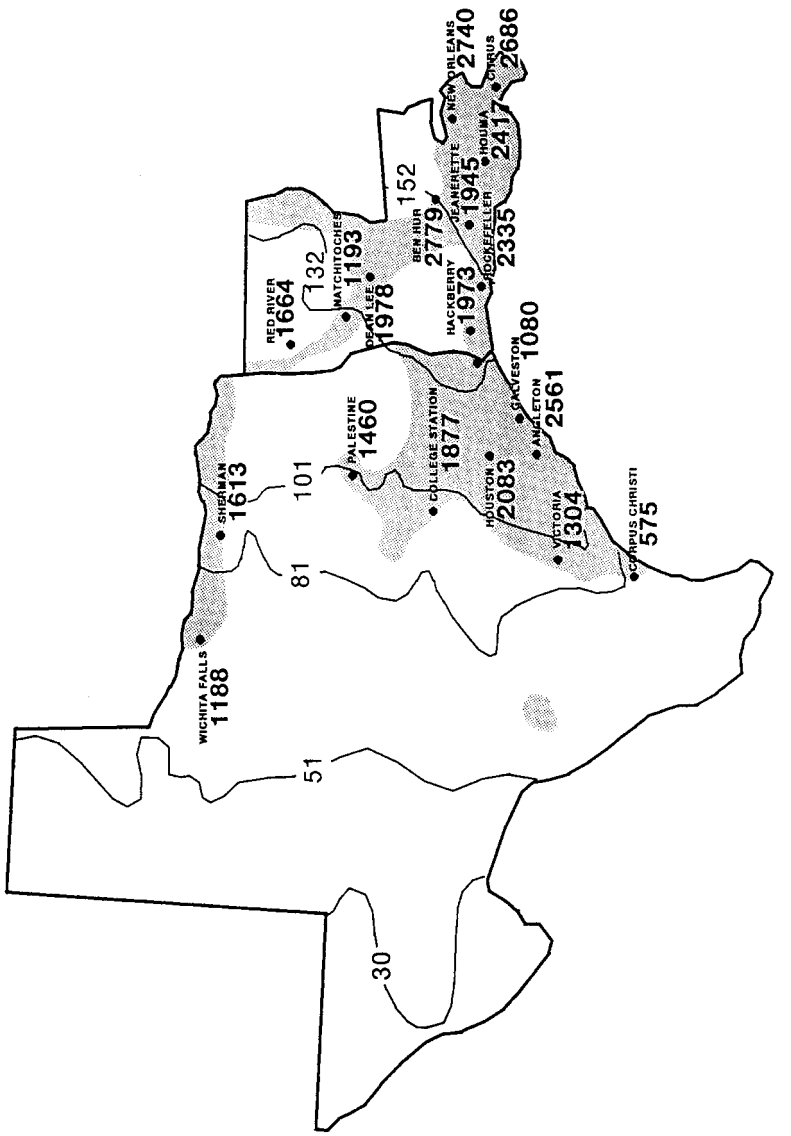

Fig. 5.2. Thirty-year-average fluke forecast values for selected climate stations in fluke-enzootic areas (shaded) of Louisiana and Texas in relation to precipitation isohyets (cm). (From Malone and Zukowski, 1994.)

According to the concepts of landscape epidemiology, a disease has a natural habitat in the same way as a species. The zoonoses in particular have natural habitats in well-defined ecosystems where pathogens, vectors and natural hosts form associations (biocoenoses) within which the pathogen circulates. Fasciolosis is a good candidate for GIS owing to its environmental sensitivity, the tendency to year-to-year geographic stability in snail host habitat distribution, the relative longevity of *Fasciola* in mammalian hosts and the confinement of livestock in identifiable grazing areas.

Soil–hydrology GIS models have been developed for assessing differences in fasciolosis risk between farms in both the chenier plain (coastal marsh) and Red River valley ecosystems of Louisiana using risk indices derived from the percentage of soil types present on pastures, slope, stocking rate and the linear extent of potential habitat associated with mapped hydrologic features on each farm (Malone *et al.*, 1992; Zukowski *et al.*, 1992, 1993). Image overlays of soil-type maps, hydrologic features shown in 7.5′ United States Geologic Survey quadrangle maps and farm boundaries were compared to snail habitat maps and herd fluke egg-shedding prevalence. Farm boundaries were derived from aerial photographs or vegetated pasture areas seen in postharvest Landsat MSS satellite infrared imagery.

Rumen fluke (*Paramphistomum microbothrioides*) faecal egg counts were used as a surrogate for *F. hepatica* prevalence, since both flukes use the same intermediate host *L. bulimoides* (*cubensis*) and metacercariae–herbage mode of transmission and are typically found together in Louisiana. Rumen fluke egg counts are not affected by flukicidal drugs used and were thus not complicated by treatment history. The mean rumen fluke egg-shedding index (mean numbers of eggs per 2 g of faeces in 12–15 random samples per herd $\times$ per cent prevalence) and the *maximum F. hepatica* egg-shedding index observed over a two-year period were placed in rank order and iteratively fitted to soil–hydrology parameters by regression analysis. The egg-shedding index is an indicator of egg abundance.

In the Red River basin, soil types range from sandy loams to hydric clays. The rank of herd egg-shedding indices regressed significantly against a snail habitat risk factor derived from the proportion of soil texture class present, slope and the length of interfaces of pastures with water bodies and other hydrologic features (Fig. 5.3). In the chenier plain region, the ranked egg-shedding indices correlated with the area of Hackberry–Mermentau soils on cheniers (relict beaches), associated Mermentau soils and the length of chenier–marsh interfaces.

These soil–hydrology GIS models reflect the amount of snail host habitat on individual farms and have shown that, as with annual climate risk, there can be a 100-fold variance in fasciolosis risk between similarly managed farms in the same ecologic zone. Climate forecasts can provide a comparison of annual variation in *Fasciola* transmission on a *regional* scale, but no provision is made for potential infection pressure related to the amount of snail host habitat present on specific premises grazed. Results indicate that such wide variation in ‘force of infection’ due to site-specific snail habitat risk must be considered, with regional climate forecasts, in any realistic model aimed at

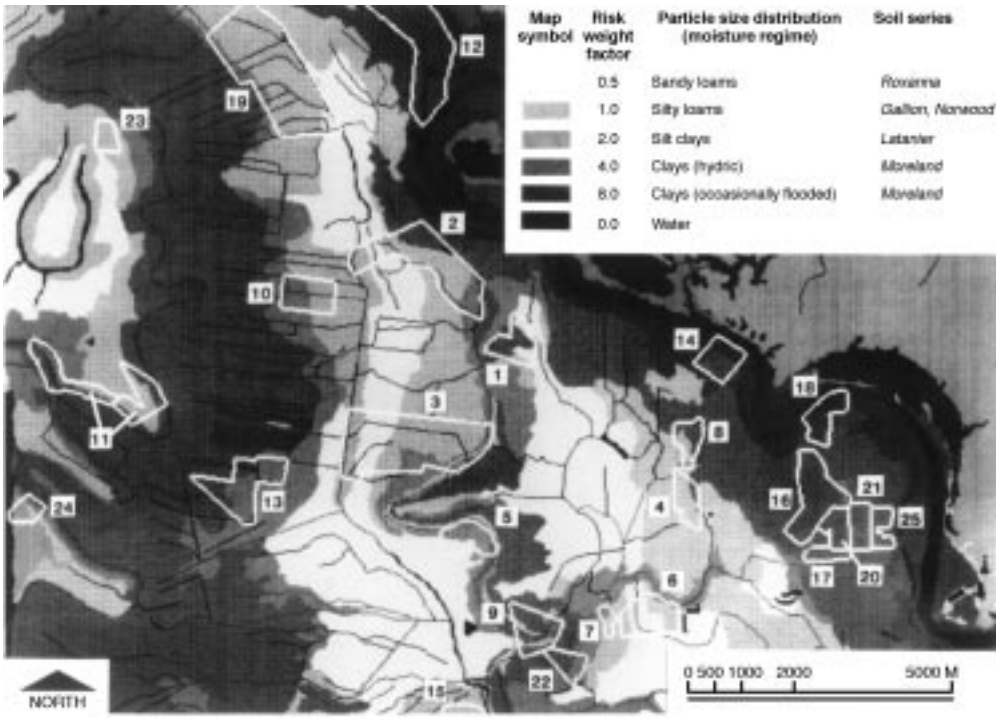

Fig. 5.3. Geographic Information System map showing boundaries of 25 cattle operations in the Red River Valley ecosystem in relation to soil type and major hydrological features. The farm risk model is calculated based on the percentage of each soil texture class on the farm adjusted for slope and length of pasture interface with major hydrological features. Data were registered to 7.5′ quadrangle base maps from the US Geological Survey. (From Malone *et al.*, 1992.)

treatment and control of *Fasciola*. Stocking rate and the amount of snail habitat on pastures were considered to be the two most important components of a comprehensive mathematical model developed for fasciolosis in Australia (Meek, 1977). A general association of certain soils has also been reported for *Fasciola* in Wales (Wright and Swire, 1984) and for schistosomiasis in Japan and Egypt (Nihei *et al.*, 1981; Malone *et al.*, 1997).

Development of a GIS Forecast Model for Fasciolosis in East Africa

Climate risk data can be included in a GIS as separate layers on long-term climate pattern maps (e.g. 30-year-average data), as maps of annual values or even as surrogates of climate. In unique work in Africa, the distribution of

tsetse vectors of trypanosomiasis and tick vectors of East Coast fever (*Theileria*) were successfully characterized by supplementing long-term climate average records with monthly vegetation index values derived from environmental satellite imagery. The latter, from the US National Oceanic and Atmospheric Administration (NOAA), were strongly correlated with long-term average rainfall values and saturation deficit (humidity) and with important biological variables of vector populations, such as population density, mortality rate and size (Rogers and Randolph, 1993). In Egypt, surface temperature data from a thermal channel of the same NOAA satellite system were used to define wet or dry 'moisture domains' in the Nile delta that were correlated with historical schistosomiasis prevalence survey data (Malone *et al*., 1994). By similar methods, a GIS was developed to describe the overlapping distribution of *F. hepatica* and *F. gigantica* in East Africa using a climate forecast system developed for the south-central USA (Malone *et al*., 1987), Atlas GIS software (ESRI, Redlands, CA) and the following Food and Agriculture Organization (FAO) databases and literature resources:

- *CVIEW: IGADD Crop Production System Zones (CPSZ)*. CVIEW is an FAO software product used to map over 500 database variables for 1220 agroecologic/administrative zones in Ethiopia, Eritrea, Sudan, Somalia, Kenya, Uganda and Djibouti. It was designed to view, select and export relevant climate, environmental and crop production data sets to commercial GIS software systems (Van Velthuizen *et al*., 1995).
- *FAOCLIM world climate databases*. FAOCLIM is a CDROM with worldwide monthly climate data and FAO agroclimatic data analysis software programs (FAO, 1995). CVIEW includes interpolated FAOCLIM climate data corresponding to each of the 1220 CPSZ.
- *FAO–ARTEMIS/NASA–GSFC satellite image archive*. Normalized Difference Vegetation Index (NDVI) derived from the AVHRR of the National Oceanic and Atmospheric Administration (NOAA) environmental satellite series for 1981–1991 are included in a CDROM database. Similar NDVI data and estimates of rainfall based on cold cloud duration (CCD) are used for near real-time famine early warning and other applications using a roof-top data receiver at the Rome headquarters of FAO and image display and analysis (IDA, Addapix) software (Griguolo, 1996; Hoefsloot, 1996). Mean monthly NDVI values derived from the entire 1981–1991 database (based on 7 km^2 pixel size spatial resolution at earth surface) were interpolated to provide a mean value for the area covered by each CPSZ and included in the CVIEW database.
- *FAO soil type databases* (1:1,000,000 scale) for the IGADD Sub-region (Nachtergaele, 1996). Map files were extracted from this database on potentially waterlogged soils of neutral or alkaline pH and acid soils of <5.5 pH.
- Prevalence data for *Fasciola* was entered for corresponding CPSZ map units from available literature reports for Kenya, Sudan and Ethiopia (Karib, 1962; Graber *et al*., 1978; Gemechu and Mamo, 1979). Reported prevalence was assigned to all CPSZ included in the survey area described by authors.

Adaptation of the forecast for use of monthly data and incorporation into the GIS

The climate forecast formula, based on *daily* climate values, was adapted for large-scale regional use using *monthly* climate data and average annual mean temperature for each of the 1220 map units in the CPSZ database. Using growing degree-day (GDD) values (average annual mean temperature minus 10°C or 16°C base temperature for *F. hepatica* and *F. gigantica*, respectively) and water budget analysis using the Penman method for calculating PET, forecast indices were calculated for both species according to the following model:

[Index = GDD1 + GDD2]

where

GGD1 = (GDD × Number of surplus raindays) × (Rain − PET)/25), *if* Rain − PET>0

GGD2 = GDD × Days in month, *if* [R − (PET × 0.8)>0]

The first part of the formula counts GDD if monthly surplus water is present due to rainfall events; GDD is *multiplied* by the number of raindays per month that results in surplus surface water during the wet season. This value must be adjusted on a site-specific basis based on annual data or 30-year average rain pattern. In the second part of the formula, subtracting the factor (PET × 0.8) from rainfall was equivalent to *adding* monthly GDD if moisture storage is present in the top 2.5 cm layer of a 15 cm soil depth water budget model. Annual *Fasciola* forecast risk categories were based on cumulative index values during one transmission year: no risk = below 600; low risk = 601–1500; moderate risk = 1500–3000 and high risk = >3000.

Climate data and other relevant environmental data were selected from the CPSZ database and exported to an Excel 5.0 spreadsheet, including monthly rainfall and potential evapotranspiration (PET), average annual mean temperature, NDVI (mean monthly value based on the 1982–1991 database), growing season (length, duration, beginning, end), altitude and irrigation status. Excel software was then used to calculate forecast values and to perform initial statistical analysis. *F. hepatica* risk index was calculated using a GDD base temperature of 10°C (Ollerenshaw, 1966). For the tropical species *F. gigantica*, risk index was calculated similarly but to the reported base temperature of 16°C (Dinnik and Dinnik, 1963). The average annual mean temperature was used since mean temperatures are relatively constant year-round at IGADD sub-region latitudes; this value is successfully used in regional crop forecast models in lieu of daily or monthly temperature data (Van Velthuizen *et al.*, 1995). An average annual mean temperature of over 23°C was considered to be the maximum threshold temperature limit for sustained *F. hepatica* development and the limit of its distribution in the IGADD region. This value corresponded to the 1200 m lower elevation limit described for the distribution of *F. hepatica* in Ethiopia (Bergeron and Laurent, 1970; Graber *et al.*, 1978). The forecast revealed indices of over 6000 in Ethiopian highland areas that receive high annual rainfall. These high

index values reflect the severity of the fasciolosis problem in Ethiopia; indices in fluke zones of the southern gulf coast of the United States seldom exceed values of 3000, even in very high risk years (Malone and Zukowski, 1994).

GIS construction

Excel spreadsheet files containing CPSZ data, climate forecast calculations and prevalence data were exported as DBase IV files [.dbf], each column of which was used by Atlas GIS as values for each of the 1220 CPSZ map units (Fig. 5.4). Results were displayed as maps on the computer monitor as separate layers (or as products of analysis of several layers/values). CPSZ boundaries for the 1220 map units (polygon files), river/water bodies, railroads, roads (line files) and capitals–towns–villages (point files) were exported as longitude and latitude, comma delimited [.bna] files, then imported into Atlas GIS as [.agf] map files. Map files were linked to database [.dbf] attribute data according to name (identical to names of the 1220 map units) and then analysed within Atlas GIS. IGAAD soil database [.bna] files on potentially waterlogged soils of neutral or alkaline pH and soils with pH <5.5 were also imported into Atlas GIS as a separate [.agf] file. *Fasciola* prevalence data were entered as a column in Excel and exported, with data from the CPSZ database and forecast calculations, to Atlas GIS. Regional *F. hepatica* and *F. gigantica* forecast index maps were created and compared to environmental data parameters and to available prevalence survey data and distribution patterns reported in the literature (Bergeron and Laurent, 1970; Graber *et al*., 1978) for each species (i.e. *F. hepatica* above 1200 m elevation, *F. gigantica* below 1800 m, both species at 1200–1800 m).

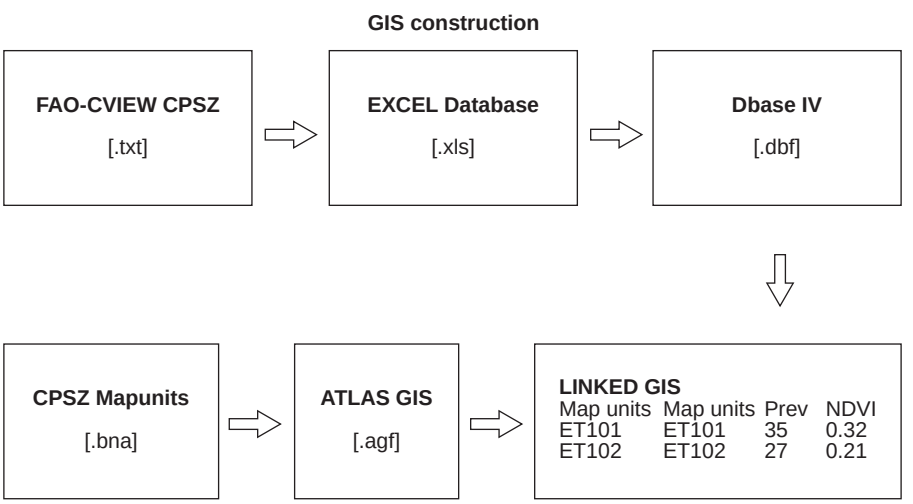

Fig. 5.4. Schematic flow diagram for construction of the East Africa *Fasciola* risk GIS.

GIS model output

Using separate models for *F. gigantica* and *F. hepatica* (Figs 5.5 and 5.6), fasciolosis risk gradients were identified in the IGADD sub-region on the basis of 30-year-average monthly climate data (Malone *et al*., 1998). For both species, the greatest risk occurred in areas of extended high annual rainfall associated with high soil moisture and surplus water, with risk diminishing in areas of shorter 'wet' season and/or lower temperatures. Arid areas were generally unsuitable (except where irrigation, water bodies or floods occur) due to soil moisture deficit and, in the case of *F. hepatica*, high average annual mean temperature >23°C.

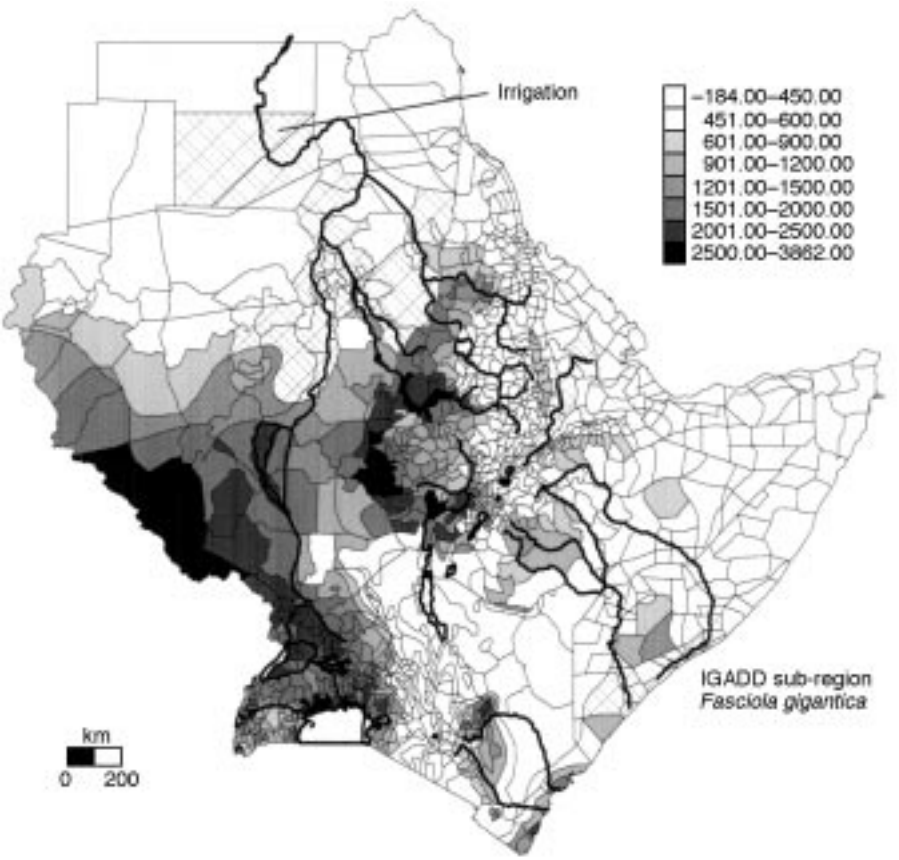

Fig. 5.5. *Fasciola gigantica* distribution and abundance in the IGADD sub-region based on a GIS constructed from FAO–CVIEW agroecologic zone map files, 30-year-average monthly climate databases, a modification of the LSU climate-based parasite forecast system, a base life cycle development temperature of 16°C and known irrigation zones. Irrigated areas and flood zones, also suitable for *F. gigantica*, were not included in the climate forecast analysis. (From Malone *et al*., 1998.)

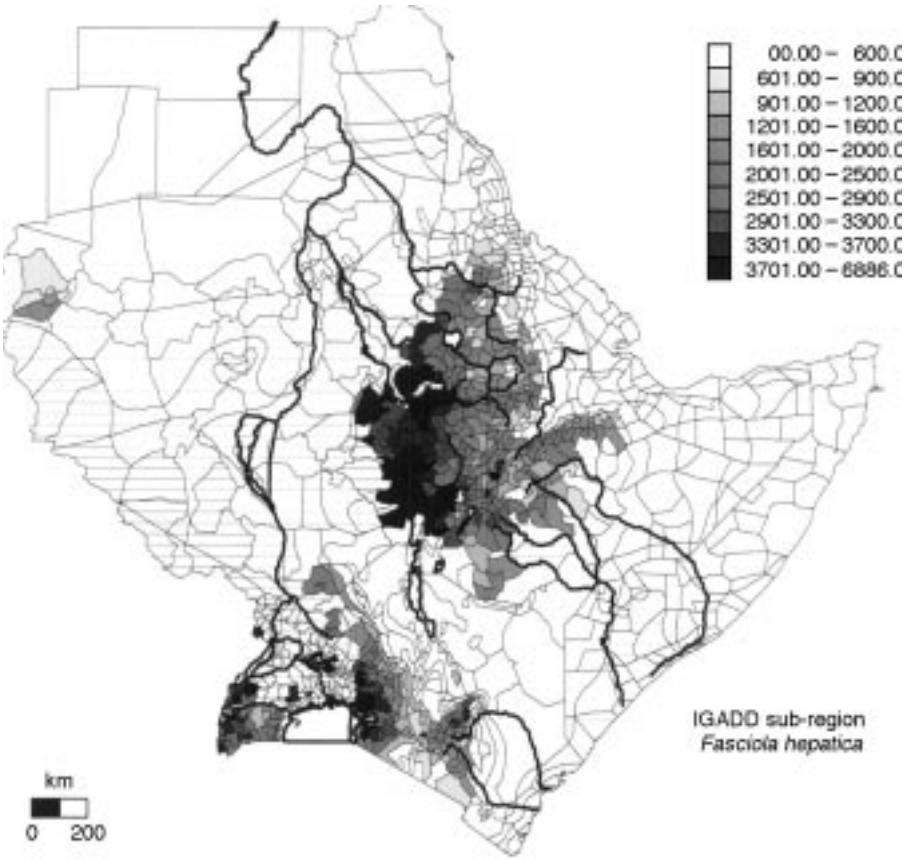

Fig. 5.6. GIS map of *F. hepatica* distribution and abundance gradients for the IGADD sub-region. A base temperature of 10°C for life cycle progression was used. Zones with average annual mean temperatures of >23°C were excluded (shown as areas with horizontal dotted lines). This temperature corresponds to the reported 1200 m elevation lower limit of *F. hepatica* distribution in Ethiopia. Additional areas in Uganda, Kenya and southern Sudan may be unsuitable for *Lymnaea truncatula* snail hosts based on the presence of acid ferralsol soils of <5.5 pH (F. Nachtergaele, personal communications, 1997). (From Malone *et al.*, 1998.)

For *F. gigantica*, regions in the highlands of Ethiopia and Kenya were identified as unsuitable due to inadequate thermal regime (<600 growing degree-days required for completion of life cycle in a single year); this result is consistent with the reported dearth of *F. gigantica* over 1800 m elevation in Ethiopia. *F. hepatica* endemic areas in the highlands of Ethiopia and Kenya were also identified and characterized as to relative risk and geographic distribution. In initial forecast output, 'false positive' areas for *F. hepatica* were seen in the lowlands of Sudan and Uganda. Further analysis suggested that soil acidity regime in western Sudan and Uganda (<5.5 pH) may be unsuitable

for lymnaeid snail hosts of *F. hepatica* and/or that tropical thermal regimes with average annual mean temperatures above 23°C are incompatible with this temperate species.

The combined forecast index (*F. hepatica* + *F. gigantica*) was significantly correlated to literature prevalence data available for 260 of the 1220 agro-ecologic crop production system zones (CPSZ) and to the 1981–1991 FAO–ARTEMIS average monthly NDVI values. The latter suggests that NDVI might be further developed as a surrogate for climate values in the forecast. Taken together, results indicated that the Climate Based Parasite Forecast System, modified for use in a GIS with CVIEW map viewer databases, FAO soil databases and the FAOCLIM worldwide monthly climate database can be used to define: (i) the distribution range of *F. hepatica* and *F. gigantica*; (ii) regional variations in intensity of *Fasciola* spp. transmission; and (iii) seasonal transmission patterns at divergent sites. Such information is essential for design of control programmes and determination of the most cost-effective time(s) for treatment.

Fasciolosis in Ethiopia – validation of the East Africa GIS forecast model and development of strategic control programmes

Ethiopia was identified for validation and further development of the GIS model because of the availability of a more complete local database on *Fasciola* prevalence and a collaborator with knowledge on local environments, host–parasite relationships and livestock management practices. A study was designed: (i) to validate and further develop the model by comparing risk indices and environmental determinants to prevalence data; and (ii) to develop a regional monthly GIS forecast and strategic chemotherapeutic control schemes for four agroclimatic regions in Ethiopia where ruminant fasciolosis is endemic (Yilma and Malone, 1998).

Climate features of Ethiopia

The interannual oscillation of the surface position of the Inter-Tropical Convergence Zone causes a variation in the wind flow patterns over Ethiopia, which results in seasonal rainfall that varies in amount, space and time (EMA, 1988). Mountainous areas have higher rainfall as compared with the surrounding lowlands (Figs 5.7 and 5.8). Generally, in most parts of the country, there is the long and heavy summer rain, often referred to as the big rains or *kiremt* (summer), and the short and moderate rain in spring called the little rains or *belg* (spring). South-western Ethiopia typically receives a long period of rain over 8–10 months of the year. Other regions such as south-eastern Ethiopia have a bimodal pattern which does not coincide with the periods or *kiremt* and *belg*. Ethiopia is a highland country in which tropical temperature conditions are limited to the lowlands in the border peripheries and the Rift Valley. From the borders, the land rises gradually and considerably, culminating in peaks in various parts of the country. Thus temperature, as it is affected by altitude, decreases towards the interior. Ethiopia is, therefore, a country where extremes of temperature and rainfall

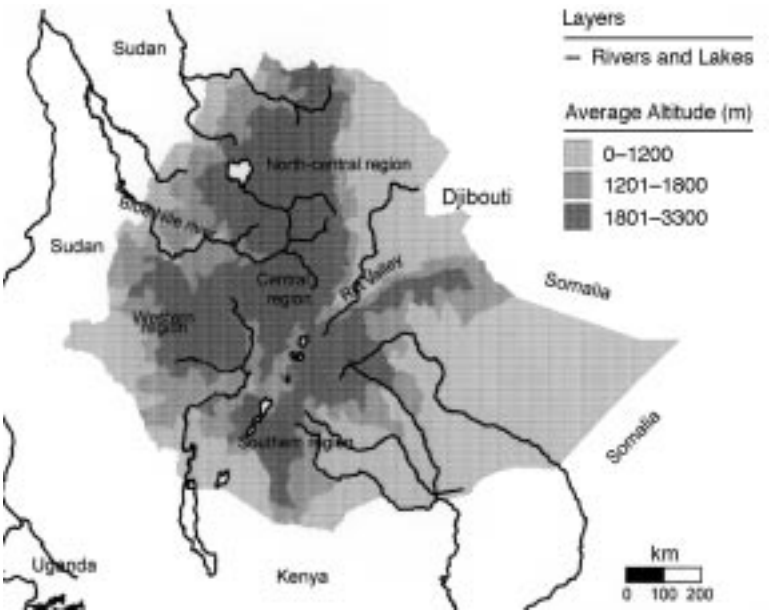

Fig. 5.7. Topographic map of Ethiopia and regions considered in the monthly *Fasciola* forecast. (From Yilma and Malone, 1998.)

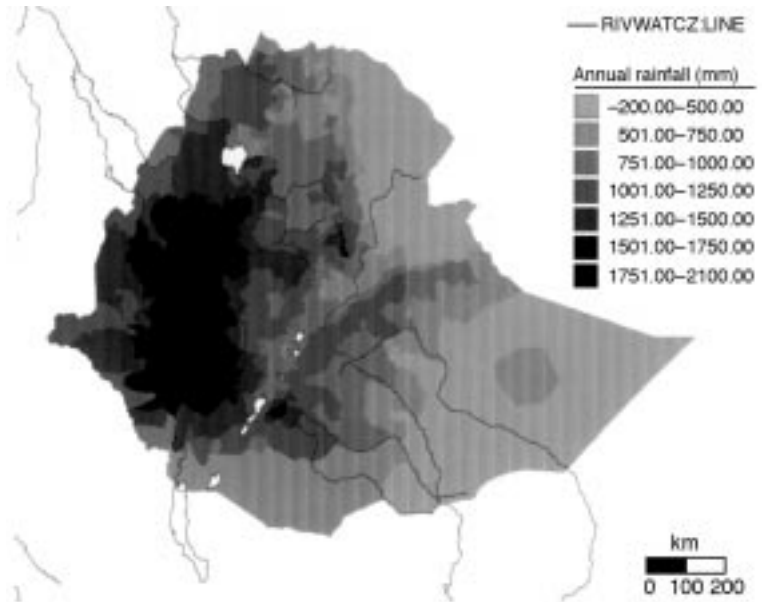

Fig. 5.8. Average annual rainfall in Ethiopia. (From Yilma and Malone, 1998.)

are experienced, altitude being the most important controlling factor. This makes it uniquely possible to study environmental effects on fasciolosis and other diseases in Ethiopia within a relatively small geographic area.

Forecast indices vs. reported prevalence in Ethiopia

A fasciolosis infection prevalence database corresponding to 223 of 460 CPSZ homogeneous agroecologic map units in Ethiopia (Van Velthuizen *et al*., 1995) was developed (Fig. 5.9) using data obtained from available literature, government reports and recent student thesis results (Table 5.2). Survey sites were identified by administrative regions and then assigned to the corresponding CPSZ for analysis. Most of the prevalence data were recently generated from abattoir surveys and field investigations based on coproscopic examinations. Limitations of the data were that most reports did not differentiate the species of *Fasciola* involved in the respective study area, and sampling and examination procedures were not standardized.

Monthly regional *F. hepatica* and *F. gigantica* forecasts were calculated. Long-term (15–30 years) and specific 'dry' (1984) and 'wet' (1987) year climate data were used to assess fluke transmission patterns under varying climatic conditions. Average monthly rainfall, temperature and PET data were extracted from FAOCLIM and used in the computation of regional forecasts. Mean monthly NDVI from the FAO–ARTEMIS image bank for 1984 and 1987 were compared with corresponding forecast indices of the preceding months in order to assess the relationship between the response of vegetation to

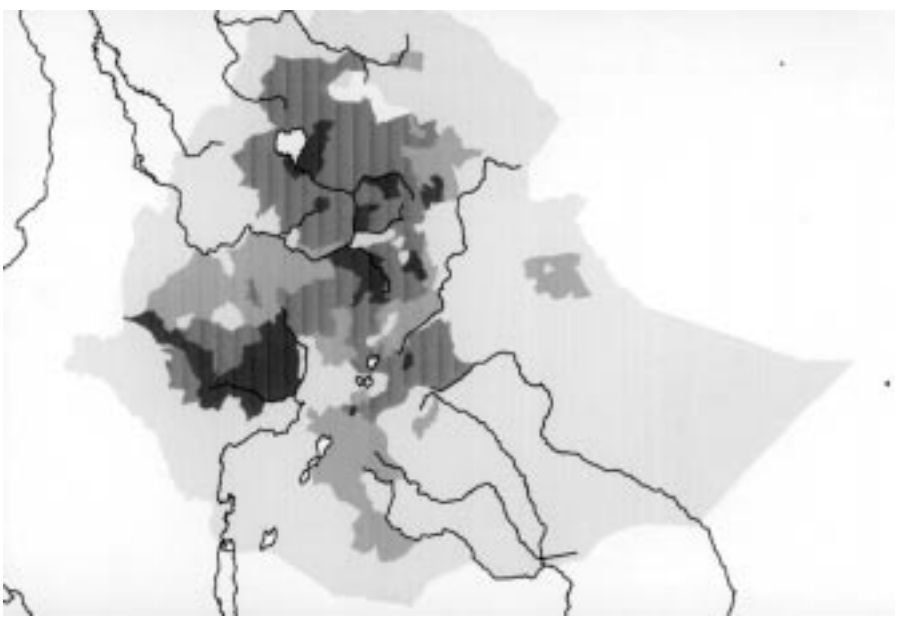

Fig. 5.9. Map of reported prevalence surveys for fasciolosis in Ethiopia. (From Yilma and Malone, 1998.)

Table 5.2. Reported prevalence of *Fasciola* in administrative regions of Ethiopia, corresponding to FAO–CPSZ codes, number of map units surveyed, survey method used and references.

Administrative region	FAO–CPSZ code	No. survey map units	Prevalence Mean±SE (range)	Survey method[a]	References
North-central region					
Gondar	ET1–ET32	20	65.1±2.4 (10–87)	C/A	Mulualem (1966), Gemetchu and Mamo (1978), Roman (1978), Yehenew (1985)
Tigray	ET33–ET69	1	26	A	Takele (1987)
Gojjam	ET70–ET139	18	65.7±2.5 (45–85)	C/A	Gematchu and Mamo (1978), Zerihum (1982), Fekadu (1988), Yohannes (1994), Beyazen (1995)
Wollo	ET102–ET139	32	61.4±2.9 (34–100)	C/A	Gematchu and Mamo (1978), Girmay (1988), Mulugeta (1993)
Central region					
Wellega	ET140–ET175	27	48.3±2.1 (34–78)	CA	Gemetchu and Mamo (1978), Abera (1990), Abebe (1994), Wassie (1995)
Shewa	ET176–ET249	45	61.6±3.0 (11–90)	C/A	Gemetchu and Mamo (1978), Yilma (1983, 1985), Getachew (1984), Kifle (1989), Rhameto (1992), Adem (1994), Yadeta (1994), Mezgebu (1995), Tsegaye (1995)
Arsi	ET250–ET270	18	62.4±3.3 (11–90)	C/A	Gemetchu and Mamo (1978), Graber *et al.* (1978), Wondossen (1990)
Hararghe	ET271–ET331	9	36.2±4.2 (13–43)	A	Solomon (1975), Haymanot (1990), Daniel (1995)
Western region					
Illibabor	ET332–ET345	11	71.4±1.4 (70–86)	C/A	Gemetchu and Mamo (1978), Seyum (1987)
Keffa	ET346–ET369	11	59.4±6.4 (42–86)	C/A	Gematchu and Mamo (1978), Goll and Holmes, Zewdu (1991)
Southern region					
Gamugoffa	ET370–ET392	n.d.	n.d.	n.d.	n.d.
Sidamo	ET393–ET443	30	40.3±1.9 (21–72)	C/A	Gemetchu and Mamo (1978), Gebre-Kiros (1981), Getu (1987), Abduljelal (1992), Hailu (1995)
Bale	ET444–ET460	1	34	C/A	Abduljebar (1994)

Source: Yilma and Malone, 1998.
[a] C = Coproscopic examination; A = Abattoir survey; n.d., no data.

weather conditions and regional *Fasciola* risk. Four agroclimatic regions which represent different rainfall patterns and where ruminant fasciolosis is enzootic were selected for study:

- *South-western region* [Gore (Illibabor Zone), Arjo and Nekemt (Wellega Zone), and Jimma (Kefa Zone)]. This represents the wettest zone in the country, receiving a mean annual rainfall of more than 1500 mm extending over 8–10 months in a year.
- *Southern region* [Kibremengist, Dilla, Hagremariam and Awassa (Sidamo Zone)]. This is a warm region with a mean annual rainfall of about 700–1000 mm occurring in a bimodal fashion.
- *North-central region* [Gondar (Gondar Zone), Bahir Dar and Debre Markos (Gojjam Zone), and Kombolcha (Wollo Zone)]. Both cool and warm weather conditions are experienced in this region together with a moderate annual rainfall of about 1000 mm, divided into spring (*belg*) and summer (*kiremt*) seasons.
- *Central region* [Debre Berhan, Fiche and Addis Ababa (Shoa Zone), and Asella (Arsi Zone)]. A predominantly cool, temperate climate that receives a moderate annual rainfall of about 1100 mm mainly during the summer (*kiremt*) months of June through September.

Jimma (south-west), Awassa (southern), Gondar (north-central) and Addis Ababa (central) were selected as representative sites to develop regional monthly fasciolosis forecasts for 1984 and 1987 'dry' and 'wet' years, respectively.

There was a remarkable spatial correlation between the combined forecast indices for *F. hepatica* and *F. gigantica* and the known distribution of fasciolosis in Ethiopia. *F. hepatica* was the most important species, with a distribution over about three-quarters of the nation. The forecast model showed a variable degree of *F. hepatica* risk occurs except in the north-east and east of the country. High *F. hepatica* risk areas were localized in the western humid zone (Fig. 5.10). *F. gigantica* was predicted to occur in the entire western region of the country with localized foci in the south and east. High risk of *F. gigantica* infection was predicted only at small foci in the East Gojjam zone along the Blue Nile river. The central and north-central highlands were free of *F. gigantica* risk (Fig. 5.11). Combining the annual forecast of both species, fasciolosis occurred in most of the productive and marginally productive highland plateau, an area inhabited by more than 85% of the human and livestock populations of the country (EMA, 1988).

Results are in agreement with literature reports (Erich, 1983) that record the distribution of fasciolosis in Ethiopia and infection prevalence rates varying from 11% in the Rift Valley to 100% in the central highlands (Table 5.2). The infection prevalence data set (Fig. 5.9) revealed that with the exception of a few foci in the north, east and south arid escarpments, fasciolosis is widespread particularly north and west of the great Rift Valley that divides the country into two parts of unequal size. A comparison of forecast indices and average NDVI (Fig. 5.12) versus ranked prevalence data indicated a significant relationship ($P<0.05$) exists between them. In addition,

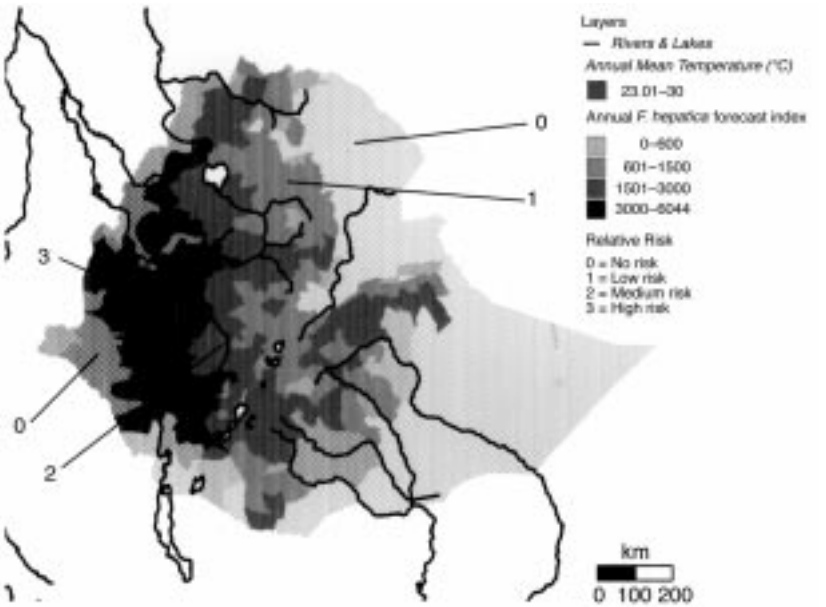

Fig. 5.10. Annual GIS model forecast of *Fasciola hepatica* risk in Ethiopia. The GIS forecast model was constructed based on FAO–CPSZ databases and moisture–thermal regime controlling the distribution of the disease. A base temperature of 10°C was used in the calculation of growing degree-days. Annual mean temperature ranges were overlaid; areas with >23°C average annual mean temperature were excluded as unsuitable for *F. hepatica*. (From Yilma and Malone, 1998.)

annual rainfall (P<0.01), readily available soil moisture and average terrain slope were significantly correlated (P<0.05) with infection prevalence. A statistically significant (P<0.05) linear relationship of surplus water and fasciolosis infection prevalence above 1900 m elevation was also observed. Conversely, a significant inverse correlation (P<0.05) was revealed between infection prevalence and PET below 1950 m of elevation. The finding that surplus water significantly influenced infection prevalence due to *F. hepatica* at altitudes above 1900 m is also consistent with previous reports (Bergeon and Laurent, 1970; Graber *et al.*, 1978). These authors reported that at extreme high (>1800 m) and low (<1200 m) elevations, pure *F. hepatica* and *F. gigantica*, respectively, mark the prevalence scenario while in intermediate altitude zones (1200–1800 m) mixed infections are encountered with an apparent domination of either of the species towards their favoured altitude gradients. A perhaps important model observation was the occurrence of potential *F. gigantica* risk up to 2600 m of elevation, although an effective transmission cycle in a single year can only be maintained at elevations below 1700 m. The effects of terrain slope on infection prevalence were presumably indirect and may reflect the habitat's waterlogging potential and presence of surplus water in grazing fields. The forecast model also

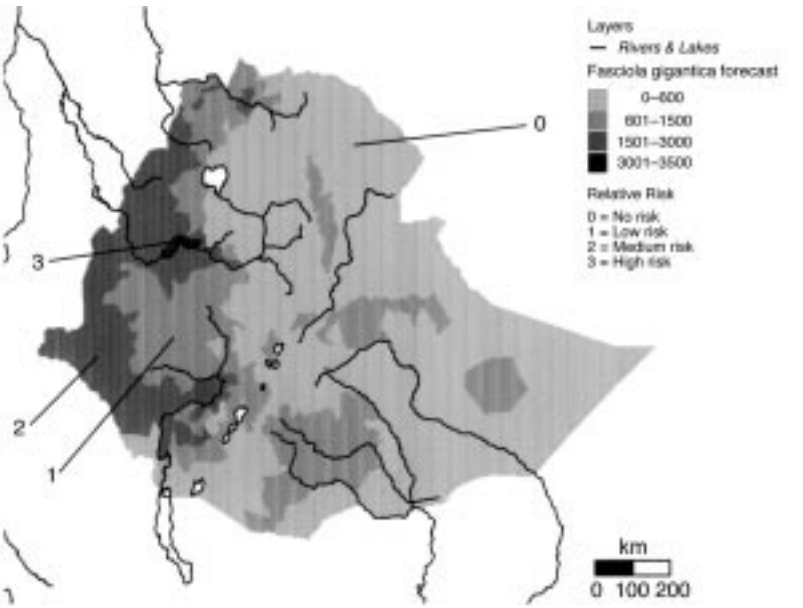

Fig. 5.11. Annual forecast risk of *F. gigantica* in Ethiopia. The GIS forecast model was constructed based on FAO–CPSZ environmental databases, moisture and thermal regimes controlling distribution and abundance of *F. gigantica*; a base temperature of 16°C was used in the calculation of growing degree-days. (From Yilma and Malone, 1988.)

confirmed that prevailing climate in the central and north-central highlands is unsuitable for *F. gigantica*, which is distributed along the western coast of the country covering approximately one-quarter of the nation.

Model forecasts of seasonal transmission pattern

Distinct regional differences in seasonal cercariae shedding and, consequently, fluke transmission patterns were projected by the forecast. In the south-western humid region, conditions suitable for up to 6 months of transmission per year may occur (May to November). Gore and Jimma were considered high *F. hepatica* risk sites where transmission of the tropical species, *F. gigantica*, can also occur in August and September. In Arjo and Nekemt, a complete *F. gigantica* infection cycle may require more than one season. In southern Ethiopia, *F. hepatica* transmission was predicted to be bimodal, resembling the regional rain pattern. In the north-central region and in the central highlands region, *F. hepatica* transmission was confined to the heavy summer rain months. In all four study regions, *F. hepatica* was predicted to be the dominant liver fluke species, with highest annual risk of infection occurring in the humid western regions.

Results indicated that thermal data and soil moisture determine the patterns of seasonal infection variation in each region due to effects on intra-molluscan larva development, free-living phases of the life cycle and activity of the snail vectors. In central highlands elevations above 2800 m (Fiche and

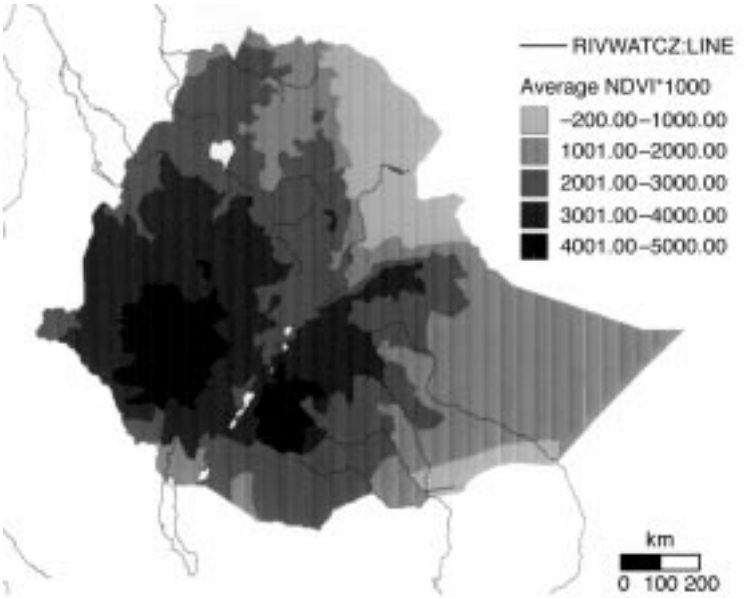

Fig. 5.12. Average normalized difference vegetation index (NDVI) map for Ethiopia using composite imagery data from the FAO–ARTEMIS NOAA AVHRR NDVI Image Bank, Africa 1981–1991. (From Yilma and Malone, 1998.)

Debre Berhan), the prediction that completion of an infection cycle of *F. hepatica* requires more than one season is in agreement with work by Scott and Goll (1977) who disclosed that a reduction in infection transmission in certain months in the year was related to a decline in active snail populations. Erich (1983) and Lemma *et al.* (1985), by contrast, reported that fasciolosis may be caused by aestivating snails remaining after the long rains and becoming active again in the small rains of February and March. Njau *et al.* (1989) disagreed with the latter conclusion and attributed the discrepancy to a reflection of metacercarial survival potential rather than metabolic activities related to the snail intermediate host. In Ethiopia, hay from endemic highland areas is usually carried to arid and lowland areas, particularly during the dry season when suitable grazing pastures are scarce. Njau *et al.* (1989) showed that hay from endemic areas can often transmit fasciolosis up to 3 months after harvest. Hay thus may serve as a major vehicle for disseminating fasciolosis to areas distant from endemic foci in the highlands. This partly explains the relatively high prevalence of the disease in some warmer and drier zones of the country. Local crowding of animals along banks of streams and ponds during the dry season when nutritional conditions are generally compromised may provide an important dynamic in patterns of infection transmission. Epidemiological characterization of the disease at regional levels thus requires knowledge of the husbandry practices in addition to the specific local thermal and moisture conditions necessary for transmission of *Fasciola*.

Annual variation in forecast risk in wet and dry years

In western Ethiopia, a 4–6 month *F. hepatica* cercariae shedding period from May through November was predicted, with no appreciable difference in risk indices and pasture metacercariae contamination between 'dry' and 'wet' years (Fig. 5.13). In the southern region, cercariae shedding was possible in the 'wet' year (1987) in April–May and then in October–November, with the exception of Awassa and Dilla sites, where summer pasture contamination was projected to occur due to extended rains between seasons; in the 'dry' year (1984), however, predicted cercariae shedding was restricted to August and October. In north-central Ethiopia, high forecast indices were found during the summer months and cercariae shedding was possible from August to October; during the 'dry' year, conditions suitable for late summer fluke transmission were absent. In the temperate highlands of the central zone, a similar trend was observed at Addis Ababa and Assela where cercariae shedding may start as early as May and extend to September (Fig. 5.14); during the 'dry' year, cercariae shedding was predicted between July and September. In the central highlands sites of Fiche and Debre Berhan (elevation above 2800 m) completion of an infection cycle required more than one year, a unique epidemiological feature of *F. hepatica* transmission in such areas. In all study regions, a highly significant correlation (P<0.005) was found between monthly NDVI and risk indices in both 'dry' and 'wet' years.

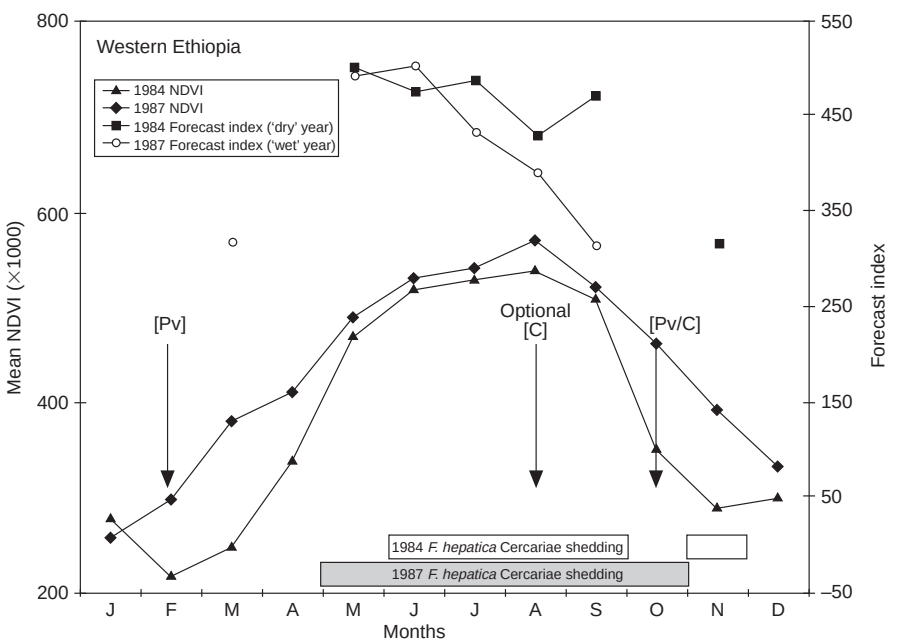

Fig. 5.13. Regional monthly *F. hepatica* forecast, start and end of cercariae shedding and strategic curative/preventive treatment schemes for western Ethiopia. (From Yilma and Malone, 1998.)

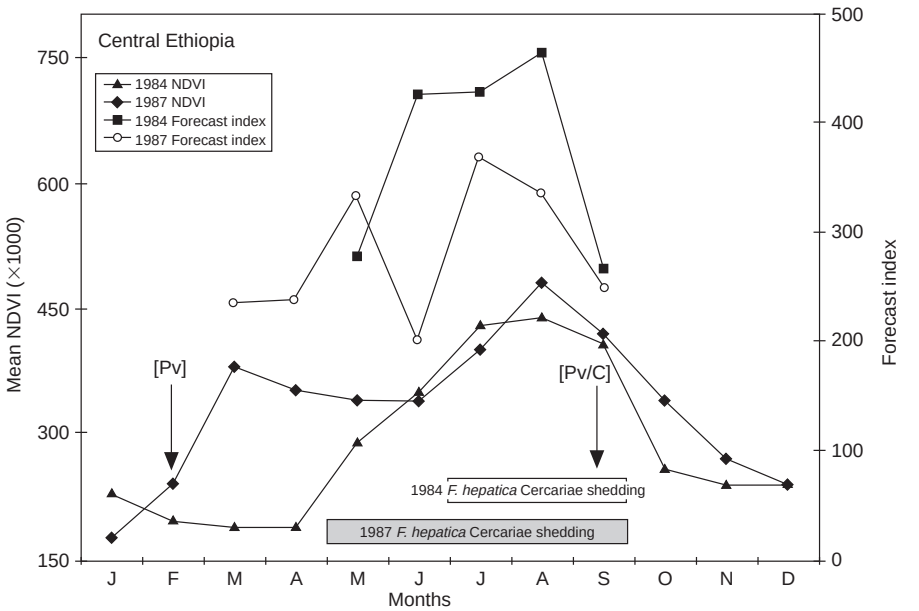

Fig. 5.14. Regional monthly *F. hepatica* forecast, cercariae shedding period and strategic curative/preventive treatment schemes for central Ethiopia. (From Yilma and Malone, 1998.)

Some risk of *F. gigantica* infection was found in each of the four agroclimatic regions with the exception of the central highlands. The altitude range suitable for the occurrence of *F. gigantica* infection varied between 1455 m (Negele, southern region) and 2563 m (Arjo, western region). Completion of one infection cycle of *F. gigantica* in a single year, however, was only possible at sites with elevations of less than 1700 m (Jimma and Awassa). In all study sites where risk of *F. gigantica* infection was predicted, there were no differences in transmission patterns between 'dry' and 'wet' years.

Results of comparisons of wet and dry climate years to 15–30 year 'normal' indicate that major interannual variation in transmission risk may occur and that annual forecasts of fasciolosis risk may be of value for devising cost-effective control of production losses. Although monthly climate values may be used to generate forecasts of annual variation in disease risk, experience with FAO crop models shows that dekadal (10-day-average) data is more suitable (FAO, 1995). If available, daily data could be used in the original forecast model if greater precision is needed.

Development of strategic treatment recommendations

Recommended strategic treatment schedules (Yilma and Malone, 1998) were based on regional *Fasciola* transmission patterns observed and FAO (1994) recommendations for strategic application of anthelmintics. Two treatments

(preventive or preventive–curative) are recommended per year for all four of the studied regions, with an optional curative treatment for the western and southern regions (Table 5.3).

The recommended scheme will effectively control the disease caused by both fluke species. The first treatment is prophylactic and is administered towards the end of the dry season when development of free-living stages and intra-lymnaeid phases of *Fasciola* are retarded and reproduction and activity of snails is minimum. The optional summer treatment is recommended in high risk years and when outbreaks of disease occur. The last obligatory treatment in the autumn removes the bile duct forms prior to the commencement of the dry season. If only one treatment can be given, the September–October strategic treatment is the best choice in all study areas.

Potential extrapolation of the Ethiopia model to other regions

If successful, this application of GIS in East Africa may serve as a model for developing strategic control recommendations in other areas of the world where *F. hepatica* or *F. gigantica* occur and 30-year average climate databases are available. Crop models are well known which describe the suitability of different environments for cultivation and recommended times for planting, fertilizer and pest control (Velthuizen *et al*., 1995). Results from studies on *Fasciola* in East Africa, using FAO databases, suggest that the same agro-climatic databases and satellite sensor data used for crop productivity models may be applicable to strategies for future development of this and other GIS models in animal production and health and for applications in human health.

Very limited *Fasciola* prevalence and snail host distribution data were available for initial development of the East Africa GIS. To provide adequate data for validation and use, cooperating scientists in each region may be identified, as for Ethiopia, to develop further and implement the *Fasciola* forecast GIS. To accomplish this, there is a need to:

- Compile published and unpublished prevalence data and generate new data on local host–parasite–snail systems (e.g. thesis, abattoir records, local meeting reports).
- Generate forecast indices to describe: (i) a 30-year-average 'typical year' in representative agroecological zones; and (ii) the range of interannual variation, using local climate data.

Table 5.3. Recommended strategic treatment schedules.

	Preventive	Curative (optional)	Preventive and curative
Western Ethiopia	February	August	October
Southern Ethiopia	February	July	October
North-central Ethiopia	April	—	October
Central Ethiopia	February	—	September

- Evaluate efficacy of recommended control programmes at selected sites based on seasonal transmission pattern and current year climate forecasts.
- Disseminate model control recommendations to veterinarians and producers, including early warning of high risk years.

The geographic distribution of *F. hepatica* and *F. gigantica* is considered to be mainly determined by the distribution patterns of the snail intermediate hosts (Pantalouris, 1965; Boray, 1982; Over, 1982). *Fasciola* is exclusively transmitted by snails of the family Lymnaeidae, a group that has been plagued by taxonomic controversy and confusing nomenclature in literature reports, much of this due to morphological plasticity associated with strain and environmental factors. According to a revised nomenclature system for the Lymnaeidae by Hubendick (1951), *F. hepatica* is transmitted by the amphibious 'mud' snail *L. truncatula* in the Palaearctic region, including Europe. In North America, L. *cubensis*, *L. columella* and *L. bulimoides* are incriminated and in South America *L. cousini* and *L. viatrix* are proven hosts; all are amphibious snails with habitat preferences similar to *L. truncatula* except *L. columella*, a less important species in terms of transmission that prefers more aquatic conditions such as ponds and floodplains. *L. columella* also occurs in the Caribbean, Central America and South America (Cruz-Reyes and Malek, 1987) and has been introduced to Australia, South Africa and Europe (Boray, 1978). *L. tomentosa*, a semi-amphibious snail species indigenous to Australia (Boray, 1969) assumed intermediate host status in Australia when *F. hepatica* was introduced by European livestock. In New Zealand, fasciolosis was restricted to small areas related to the distribution of introduced *L. tomentosa* but became more widespread following introduction of another species, *L. columella* (Pullan *et al.*, 1972; Harris and Charleston, 1977), a rapidly colonizing, more aquatic, more heat tolerant species.

In the mid-latitude climate zones of the Middle East and central Asia, *F. hepatica* and *F. gigantica* often overlap (Over *et al.*, 1992) in somewhat expected regional patterns. *F. gigantica* has been reported in river floodplains and *F. hepatica* in the piedmont and mountain pastures of south-east Kazakhstan and changes in distribution patterns favouring *F. gigantica* have been associated with increased irrigation in Uzbekistan (Matchanov and Gekhtin, 1991). Intermediate morphological forms of *F. hepatica* and *F. gigantica* that may represent hybrids have been reported (Sarwar, 1957; Watanabe, 1962).

Because of these regional biological variations, adaptation of an environmental risk model developed for *F. hepatica–L. bulimoides* in the Red River Basin of Louisiana may not apply to the *F. hepatica–L. viatrix* system in the central valley of Chile or to the *F. hepatica–L. truncatula* system on the north coast of Morocco (Khalleayoune and El Hari, 1991) without accounting for both environmental differences and possible intrinsic biological differences in the *F. hepatica*–snail host system. There would be major differences where *F. gigantica* occurs. Table 5.4 lists ecologic niche characteristics of the *Fasciola hepatica–L. bulimoides*–cattle system used for developing geographic models of disease risk in Louisiana. It is illustrative to contrast this with data available for

constructing similar geographic models for the *F. gigantica–L. natalensis*–cattle association (Table 5.5), using the Kenya highlands as an example (Van Someren, 1946).

Literature reports indicate that the *F. gigantica–L. natalensis* system is broadly similar to that of *F. hepatica–L. truncatula* but is more aquatic, less tolerant of drought and adapted to higher temperatures (Alicata, 1938; Alicata and Swanson, 1941). Preston and Castelino (1977) reported in Kenya that *L. natalensis* population numbers increased progressively in dam impoundments at a cattle farm between January and September, during the decreasing

Table 5.4. Ecologic niche characteristics of the *Fasciola hepatica–Lymnaea bulimoides*–cattle biocoenose used for developing geographic models of disease risk in Louisiana.

Intramolluscan and free-living phases	
Temperature	No development <10°C; life cycle optimum 18°C
Hydrology of snail habitats	Wet mud and shallow water of <15 cm; depressions in fields, floodplains, seeps, springs Intermittently or seasonally dry, periodically freshened by rainfall or irrigation. Open, sunlit, disturbed (hoof prints, ruts), oxygenated Salinity <5 parts per thousand
Soils	Neutral, well-buffered soils, pH 6–8 High water table favours life cycle Hydric clays>clays>silts>loams>sand
Seasonal dynamics	Crash–boom snail life cycle pattern Snails aestivate until soil moisture recharge (autumn) Slow growth 3–12 mm in winter ± minor reproduction Major reproductive effort, rapid growth early spring Eggs accumulate undeveloped in faecal pats, develop in 10 days at 25°C (150 GDD) when freed in water Egg to cercariae 40 days at 25°C (600 GDD) Cercariae release at rain events – Simulate in laboratory by re-freshened water, pH change, light stimulus Eggs, snails, metacercariae can survive over winter, larvae survive drought in snail Metacercariae death after 2 weeks sustained heat and drought; survival up to 3 months in hay Parasite development suspended in snails during both short-term and long-term drought in habitats
Mammalian phase	Prepatent period 8 weeks Maximum egg shedding in autumn and winter, 4–5 months after peak transmission Herd overdispersion of parasite numbers, egg counts Fluke burdens gradually shed over one year in cattle Transmission to calves *in utero* possible Other hosts: sheep, goat, rabbit, swine, horse, nutria

Source: Malone, 1997.

Table 5.5. Summary of preferred habitat features for *Lymnaea natalensis (caillaudi)* in East Africa (Van Someren, 1946).

1. Type of water body: small, shallow (<10 cm) seepages, pools, streams, and trickles of clean, clear water with slight current; edges of recirculating large open water bodies
2. Stability: permanent in dry season, no cattle disturbance, no violent floods
3. Oxygenation >75%
4. pH 6.5–8.0
5. Hardness of water: >20 ppm $CaCO_3$, lower limit <8 ppm $CaCO_3$
6. Substrate: firm mud bottom
7. Sunlight: limited aquatic weeds, abundant algae

Source: Malone, 1997.

temperatures of the wet season, and peaked at the time of rising temperatures in the late rainy season. Infected snails were found year-round. Dinnik and Dinnik (1963) reported in field studies in Kenya that rediae do not produce cercariae at <16°C but that cercariae develop when temperatures reach 20°C. Schillhorn (1980), however, reported cercariae shedding in the Nigerian savanna in the cool, dry season (13–18°C) and attached less importance to this factor in the total epidemiology of *F. gigantica*. In field studies in South Africa, Appleton (1974) observed that egg production by *L. natalensis* occurred at temperatures of 15–20°C, or 1–2 months later in the cool season than the introduced North American species *L. columella*, which began reproduction at 10–15°C. These reports, combined with the longer prepatent period of *F. gigantica* (12–14 weeks) in the final host and a later maximum egg production (20–22 weeks), suggest adaptation by *F. gigantica* to reduce chances of overwhelming mammalian hosts during the prolonged wet season in tropical wet–dry climates (McCullough, 1965) or the warm season in mid-latitude temperate continental and Mediteranean climates such as Egypt, where extensive year-round irrigation occurs. For *F. hepatica*, shorter life cycle development rates would be expected to be an advantage in northern latitudes where short warm seasons may be limiting on development (Boycott, 1936).

Health Maps: Just in Time Delivery – via Satellite?

In the United States and most other countries control of economic losses due to internal parasites is dependent on convincing livestock producers of the benefits of investing in cost-effective control measures. Most settle on an approach based on their own experience, anecdotal information from other producers and sometimes confusing advice on drug choice and strategic control recommendations for their area by animal health workers in the private sector, academia and the pharmaceutical industry. Treatment is typically given with other anthelmintic products or as combination products effective against both flukes and nematodes (e.g. albendazole, ivermectin–clorsulon). The wide variation in fluke control practices reported for Florida (Simpson and Courtney, 1990) is perhaps typical of internal parasite control practices by producers in

the south-eastern United States where adoption of advocated blanket twice per year, autumn/spring or mid-summer/mid-winter, strategic control programmes for cow–calf operations may cost $7–10 per cow unit per year. In recent years the problem of parasite control has shifted from availability of effective drugs and basic epidemiologic information needed for strategic control to questions on whether livestock producers underuse or overuse products in ways that contribute to inefficient use of resources and potential drug-resistance problems (Murrell, 1994).

The answer may be decision-support systems based on GIS. GIS can provide the environmental context for fasciolosis and a systematic way to evaluate variation in parasite distribution due to *density-independent* population regulation on both a broad scale and a local scale. Results can then be used with mathematical models to suggest and compare control strategies. Mathematical models (Smith, 1984, 1994) that describe intrinsic parasite population development and mortality rates and *density-dependent* regulation of *Fasciola* are relevant, like the parasite population described, in the context of a given environment. Meek and Morris (1981) developed a computer simulation model for evaluating alternative control strategies for ovine fasciolosis in Australia. The model included components to account for climate variation, snail habitat extent, herbage and metacercariae intake by sheep and the effect of fluke burdens on productivity and economic return. The model could be applied to other sites, but only after modifications that represented the new circumstances, notably snail habitat extent and metacercariae intake by sheep, the two variables that most influenced economic return. Decision analysis using the model revealed that the number of treatments required per annum for cost-effective control increased with increasing snail habitat area and stocking rate. This suggests that, in addition to climate, snail habitat related 'force of infection' differences exist between premises that are often not included in mathematical transmission models.

Studies described above suggest it is possible to define: (i) the intrinsic preferences and limits of tolerance of the *Fasciola–Lymnaea*–cattle system; and (ii) the relevant environmental features that determine distribution of its niche in both regional–national scales and local agricultural scales using GIS methodology. Satellite sensor data, which can be used as a surrogate of thermal–hydrology influences on parasite life cycles, is increasingly available in 'near real time' on global scales through the EOS and Global 1 km program of NASA (Eidenshink and Faundeen, 1994). Tapping these new resources, it will be possible to use GIS to provide advice to producers within a week or two of relevant environmental effects on transmission dynamics based on:

- *Climate risk* – regional rainfall and temperature-related differences in transmission potential between years and different climate zones.
- *Pasture risk* – soil-hydrology indicators of differences in snail-habitat extent and 'force of infection' on specific premises.
- *Mathematical population dynamics models* – to define intrinsic life cycle reproduction and mortality rates and to suggest and compare alternative control strategies on given premises.

- *Cost–benefit analysis* – economic and animal productivity factors that indicate whether control is worth while. Control programme success can be periodically confirmed or modified based on herd monitoring by faecal egg counts or immunologic tests in the context of economic loss threshholds.

Computer software and hardware systems needed for GIS and satellite data image analysis are now available at the microcomputer level at reasonable costs. Spatial map databases, digital climate databases and satellite sensor data needed for construction of environmental risk GIS models are increasingly available from government agencies such as the USDA Soil Conservation Service, the National Climate Center, NASA and from academic units such as geography departments that have developed databases for general use. Precedents on use of GIS models are being developed for use in control programmes for several diseases, including the national schistosomiasis control programme in Egypt (Malone *et al.*, 1997). Such evolving new GIS efforts will facilitate government or industry directed control and eradication programmes for animal diseases as well. Using current satellite imagery and GIS climate–soil–hydrology computer models, it should soon be possible to describe and map, in near 'real time', enzootic disease severity on national scales by extrapolation of limited data on the biology of the relevant snail host, the species of *Fasciola* present, local transmission dynamics and climate patterns.

In the future, GIS itself may provide an alternative way to define the range and optimum conditions for parasite species by mapping survey data and then iteratively fitting them to associated climatic and edaphic conditions. For example, an upper limit of 23°C for *F. hepatica* was suggested by the correlation found in Ethiopia with elevation less than 1200 m. While the life cycle may progress with short-term high temperature, continuous or long-term temperatures of above 23°C over several years may provide critical limits in natural habitats. This result is consistent with the reported 18°C optimum for this temperate species (Armour, 1975) and suggests the need for studies on the limiting effect of long-term or continuous higher temperatures on the distribution of *F. hepatica*. Similar conclusions were derived from apparant inadequate thermal regimes for the tropical species *F. gigantica* at cool, high elevation sites in Ethiopia. GIS models may provide the impetus for detailed laboratory and field studies on intrinsic habitat preferences and tolerance limits of *Fasciola–Lymnaea* systems to explain the distribution of both *F. hepatica* and *F. gigantica* and to define host–parasite niche characteristics. Indeed, attempts to construct meaningful GIS models of disease may stimulate rebirth of interest in basic biological studies begun years ago by classical parasitologists and lend new relevance to information on the interaction of host–parasite systems and the environment.

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6 Pathology, Pathophysiology and Clinical Aspects

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Pathology

The pathology and pathogenesis of fasciolosis were described in detail during the 1960s by several workers. Similar descriptions have appeared from several of the sheep-producing countries suggesting that the disease is similar worldwide. *Fasciola hepatica* can naturally infect sheep, cattle, goats, pigs and humans. It is also a pathogen of wildlife and zoo animals. In addition, experimental animals such as mice, rats, guinea pigs and rabbits have been used to explore aspects of life history and pathology. Because the sheep is the definitive host and the pathology of ovine fasciolosis due to *F. hepatica* has been most thoroughly described, it has been chosen here for description. Comparisons with pathology in other species will be drawn. In general, development in the experimental models is quicker than in sheep so the disease process is accelerated. Further, the smaller the liver of the host, the more the relative damage caused by a fluke and the less the functional reserve of the liver tissue. The disease occurs in two phases: the parenchymal phase during migration of flukes through the liver parenchyma and the biliary phase which coincides with their residence in the bile ducts. Some hosts, such as the sheep, rabbit, rat and mouse, are permissive and the biliary stage of the disease is common. In others such as cattle and humans, few flukes survive beyond the migratory phase and biliary disease is relatively rare.

Fasciolosis can be a spectacular disease. The appearance of a heavily infected liver from an animal with acute terminal fasciolosis is not easily forgotten. Nor are sudden deaths which can decimate flocks almost overnight. However, fasciolosis is a more complex disease than it may appear. The complexity arises from several sources. Maturation of flukes involves development and growth over 12–16 weeks during which time the fluke travels between and within organs. Because an individual fluke may pass through the same part of the liver twice (or more) during these

Fasciolosis (ed. J.P. Dalton)

peregrinations, fresh and resolving lesions caused by the sequential insults may be found in the same section of tissue. As the migrating fluke grows, the size of its track through the liver increases as does the damage and the inflammatory response. The level of infection also affects the pathology. Heavy burdens cause more severe pathology and earlier termination by death of the sheep. Smaller infections generally have a more protracted course. Under some environmental and management conditions large simultaneous infections of metacercariae occur. In others, 'trickle' infections, where fluke are acquired over several weeks, are more typical. Both juvenile and older flukes can simultaneously inflict lesions while those caused earlier by older flukes are resolving. As a result examination of an individual liver in an endemic area could reveal all of the features of pathology described below. Because infections of adult flukes in the bile ducts can persist for years, they may be present many years after the source of infection has gone. Both artificial and natural infections have been used to build a picture of the disease.

Prehepatic stages

Newly excysted juveniles penetrate the intestinal mucosa and can be found in the abdominal cavity by 72 h (Kendall and Parfitt, 1962). Flukes start to arrive at the liver at 90 h (Sinclair, 1967). The paucity of information describing these processes in sheep probably relates to the 'needle in the haystack' phenomenon. The most complete description of penetration of the gut is from mice (a smaller 'haystack') (see Dawes and Hughes, 1964, 1970) and probably reflects the events that occur in sheep. After burrowing into the mucosa the juvenile fluke dissolves tissue in the submucosa and muscle layers of the small intestine and passes through the serosa into the abdominal cavity. Penetration is not associated with clinical disease perhaps because only relatively few cells are disrupted. Flukes may carry on to penetrate organs such as the diaphragm and lung. In sheep there appears to be little disease associated with penetration of organs apart from the liver. Lung lesions are found in many animals (Boray, 1969) and pneumonia, fibrous pleuritis and pleural fluid are commonly reported. The pathology is more significant in heavy infections.

Hepatic stages

Flukes have a strong predilection for the tissues of the liver. Recovery rates of flukes from livers vary from about 50% in sheep given 200 metacercariae down to about 30% when given up to 10,000 metacercariae (Boray, 1969). The liver is such an attractive destination that, despite the barriers, flukes can find their way to the livers of fetuses in ewes and cows and set up prenatal infections (Sinclair, 1967).

Black disease is a form of infectious hepatitis caused by *Clostridium novyi*. It is rare now because of the high efficacy of vaccination but may be seen concurrently with fasciolosis. Migration of the flukes through the liver

provides conditions conducive to multiplication of the bacterium and a necrotic hepatitis is usually found at post-mortem. Animals with black disease die suddenly and although flukes may be present they often precipitate disease rather than cause it.

The life history of penetration, migration and localization into the bile ducts has been described in Chapter 1. The salient features of the pathology have been summarized in Table 6.1 using information primarily from Ross *et al.* (1967), Sinclair (1967), Dow *et al.* (1968) and Boray (1969). Readers are referred to these works for more detail. Particular aspects of histopathology are illustrated in Plate 2. When considering this summary it should be remembered that considerable variation exists between animals. The information has been arranged chronologically so that the course of the disease may be appreciated. It is common for one or more lobes of the liver to be infected more intensely than others: usually the lobe closest to the small intestine (i.e. the left, or ventral, lobe) is the most heavily infected. A compensatory hypertrophy of the less-affected lobes occurs and relative liver weight increases. Biliary obstruction by the flukes in the bile ducts appears to be rare.

Cattle

The course of infection follows a broadly similar pattern in cattle (Dow *et al.*, 1967). Calves are susceptible to disease but in excess of 1000 metacercariae are usually required to cause clinical fasciolosis. In this case the disease is similar to sheep and is characterized by weight loss, anaemia, hypoproteinaemia and (after infection with 10,000 metacercariae) death (Boray, 1969). Resistance develops with age so that adult cattle are quite resistant to infection although some benefits to weight gain have been reported following flukicide treatment.

There is considerable variation in both infection rates and the severity of disease between individual animals. In the migratory phase some of the pathology differs only in minor ways. For example, arteritis is a feature of infection and fibrosis is marked. Flukes are concentrated in the ventral portion of the liver and tracks are less distinct than in sheep. Many flukes become trapped in the parenchyma and following high level infections only around 5% of the inoculum reach the bile ducts (Ross, 1965). Surviving flukes confront a hostile inflammatory reaction. The bile ducts thicken due to epithelial hypertrophy and subsequent fibrosis of the walls of the duct. Calcium deposits start to form in the duct walls after 16 to 20 weeks of infection. As a result the ducts enlarge up to 3 cm in diameter and become prominent on the surface of the liver (see Plate 2). 'Stove pipe' or 'clay pipe' liver are typical and apt descriptions. The lumina of the ducts are variously dilated and stenosed and the epithelium shows ulceration and haemorrhage. Few flukes reach the bile duct and few eggs are passed. Most flukes are lost by 30–50 weeks after infection.

Humans

The pathology of human fasciolosis has been summarized by Chen and Mott (1990). Humans are not a natural host and few flukes develop sufficiently to

Table 6.1. Development of *F. hepatica* pathology and clinical signs of fasciolosis in sheep.

Week pi	Flukes	Gross pathology	Histopathology	Bile ducts	Clinical signs	Blood	Eggs
1–3	1–2.5 mm penetrate capsule and form tracks in parenchyma	Congested left lobe, raised white tracks most common on parietal surface, red tracks in parenchyma	Damage limited to track regions. Subcapsular haemorrhage, degenerate hepatic cells and eosinophils in blood-filled tracks. Eosinophils and macrophages in adjacent parenchyma	Some eosinophils	Few clinical effects	Normal	—
4–5	4–5 mm migrating in parenchyma	Tracks yellow and raised, surrounded by haemorrhage centred on left lobe. Fibrinous perihepatitis, possible peritonitis	Old tracks with contracting core, some fibrosis, cuff of infiltrate with giant cells, macrophages and lymphocytes predominating. Eosinophils appear in portal regions adjacent to tracks. May be regions of infarction. Fresh haemorrhagic tracks	Eosinophilic infiltration in ductules near tracks	Few clinical signs	+/− eosinophilia, +/− hyperglobulinaemia	—
6–8	6–10 mm localize in left lobe, start to enter ductules	Tracks are blood red (2–3 mm), haemorrhagic surface plaques present on visceral surface of left lobe (predominantly). Fibrinous adhesions to and congestion of local organs. Enlarged hepatic l.n., congestion of liver	Haemorrhage and hepatocyte debris in fresh tracks, now larger with larger flukes. Hepatic hyperplasia and fibrosis as healing occurs in older tracks. Infiltrate of lymphocytes, plasma cells. Eosinophils restricted to portal areas. Iron pigment accumulates, peripheral fibrosis, venous thrombosis	A few flukes present, duct hyperplasia	ACUTE TYPE I FASCIOLOSIS Sudden death in infections >5000 mc. Ascites, abdominal haemorrhage, icterus, pallor of membranes, weakness	Typically, anaemia, hypoalbuminaemia, eosinophilia	—
8–10	10–12 mm majority enter bile ducts	Subcapsular haemorrhage on visceral surface. Tracks form grooves on the surface. Fibrinous/gelatinous liver surface	Necrotic cores with giant cells, granulation tissue and lymphocytes forming concentric rings	Flukes in dilated ducts. Duct and ductule hyperplasia and fibrosis. Mucous cell hyperplasia. Some inflammatory infiltration	ACUTE TYPE II FASCIOLOSIS 1000 flukes cause death but animals may briefly show pallor, loss of condition and ascites	As above	+/− in faeces

12–40	12–30 mm resident in bile ducts	Enlarged liver of lighter than normal colour, diffuse cirrhosis. Enlarged bile ducts with thickened but pliable walls contain brown detritus and flukes. Ducts project above the rough visceral liver surface, thinned in places, overlaid by healed tracks. Gall bladder, hepatic l.n. enlarged	Fibrous tracks surrounded by loss of liver tissue including fibroblasts, lymphocytes macrophages. Occasionally parenchymal granulomata containing fluke eggs	Hyperplastic cholangitis. Mild fibrosis of ductules. Extreme cell hyperplasia and wall thickening of main bile ducts. Necrosis of duct mucosa caused by presence of flukes. Eggs and tarry bile also present. Haemorrhagic epithelium	SUBACUTE FASCIOLOSIS: >800 flukes, often acquired over time. Flukes are mostly in bile ducts by 20 weeks. Sheep are lethargic, anaemic and may die. Weight loss	Anaemia (normochromic, normocytic) hypoalbuminaemia, elevated liver enzyme activities. Eosinophilia	Many in faeces and bile ducts
					CHRONIC FASCIOLOSIS: >200 flukes. Gradual development of bottle jaw and ascites (ventral oedema), emaciation	Anaemia, hypoalbuminaemia, elevated liver enzyme activities. Eosinophilia	

Notes:
Data are based on reports by Ross *et al.* (1967), Sinclair (1967), Dow *et al.* (1968) and Boray (1969).
This is a guide to pathology. Animals may simultaneously carry infections at different stages and show a complex of pathologies. The timing and severity will vary between animals. For example, in heavy infections the development of flukes may be delayed.
Heavier infections generally result in death at earlier times.
Eggs refers to fluke eggs in faeces.
pi: post-infection; mc: metacercaria; l.n.: lymph nodes.

reach the bile duct. The severity of infection may vary from being long term (up to 9 years) and asymptomatic to a severe and debilitating disease. There are fewer opportunities for post-mortem examination or experimentation with human subjects but from the data available it appears that the disease parallels that in animals and the development rate of the parasite is similar to that in sheep. A major symptom is abdominal pain, mild to excruciating, localizing to the right hypochondrium as infection progresses. A second symptom is fever which may reach 42°C in severe cases. The clinical syndromes are as follows. The 'hepatic' phase is equivalent both in pathology and clinically to 'acute fasciolosis' in sheep. Occasionally parenchymal calcification accompanies the fibrotic lesions. The 'biliary phase' is like the 'chronic' disease of sheep. In chronic cases liver abscesses (1–30 mm), often containing eosinophils, are a common finding. Ectopic infections, a result of rare aberrant migrations, can be found in several possible sites, e.g. subcutaneous, lymph node, lung. In such cases, tissues may contain evidence of flukes, eggs, tracks, fibrous repair and inflammatory infiltrates including eosinophils. Peripheral eosinophilia is also a common finding.

Rabbits

Rabbits have been used as experimental models of fasciolosis and can tolerate infections of about 75 metacercariae. The pathology and course of infection has been described by Urquhart (1956). Although migration follows a similar course as in sheep, the life cycle is shortened in rabbits: juveniles reach the body cavity within 48 h and flukes enter the bile duct as early as 5 weeks after infection. Liver pathology is relatively severe. Organized adhesions between liver, stomach, intestine and diaphragm are common and are often accompanied by fibrinous peritonitis. The margins of the liver can contain infarcts and cirrhosis is severe. Egg granulomata and haemorrhagic ulcers are also features of infection. Dilated bile ducts show hyperplasia, fibrosis and ulceration.

Rats

Thorpe (1965b) has described the pathology of fasciolosis in rats. Juveniles reach the body cavity within 24–48 h and flukes enter the bile duct at between 4 and 6 weeks. Although the migratory phase is abbreviated, the pathology is similar to that of sheep. Flukes reside in the common bile duct. It appears that the intrahepatic ducts are too small to accommodate flukes and, in the absence of a gall bladder, they seek the enlarged common duct which can reach 20 times its normal size (Isseroff *et al.*, 1977). The location of flukes in a duct remote from liver tissue raises questions about how closely fasciolosis in rats mimics chronic disease in sheep. Ulceration and haemorrhage of duct epithelium is also lacking. Infections of 20–30 metacercariae are tolerated by rats.

Mice

Although flukes appear to undergo a similar migration in mice as in sheep, the small size of the mouse liver both speeds up and exacerbates the pathology.

Juveniles reach the body cavity in under 24 h and first start to appear in the bile duct after 24 days (Dawes, 1961b, 1963b). A single metacercaria may be sufficient to kill a mouse, which indicates how large individual tracks, especially of older flukes, can be in relation to the liver. Sudden death of inbred mice accompanied by extensive haemorrhage into the body cavity is not uncommon after 24 days. Hyperplasia of the duct epithelium precedes fluke invasion of the duct (Dawes, 1963a). After about 4 weeks of migration in the liver, flukes enter the bile duct. Flukes within ducts cause ulcers but haemorrhage into the duct does not occur. Feeding physiology of flukes in the bile duct of mice also appears to differ from the situation in sheep. As a model host, the mouse may not be ideal.

Clinical Aspects

The disease classifications together with clinical signs and fluke numbers are described in Table 6.1. The severity of disease varies depending on the level of infection, the nutritional plane of the animals and also varies between animals in a group.

Acute fasciolosis may cause sudden death of stock, especially sheep and goats. Often there is no warning, but there may be abdominal pain and ascites. Because it generally occurs as a result of a large intake of larvae over a short period its occurrence may be predicted by a study of climatic conditions. Deaths two months after the start of grazing on swampy pasture is a typical scenario. Subacute disease is a haemorrhagic anaemia which is slightly more protracted than the acute disease. Losses of up to 25% of a flock are possible in outbreaks of acute fasciolosis. Acute haemonchosis also causes a rapidly fatal anaemia. Calves may suffer from acute fasciolosis in very heavy infestations.

Chronic disease is accompanied by weight loss, pallor of mucous membranes, ventral oedema and wool break. Sheep die with obvious signs including the presence of typical eggs in faeces. A history of grazing fluke-prone areas is usually found. In cattle the chronic disease is uncommon and often manifests as a small production loss. Reductions of 13 kg carcass weight over 124 days for cattle have been reported (Marley *et al.*, 1996). Reductions in milk production are equivocal. Fasciolosis may be involved in a complex with bovine ostertagiosis and be associated with parasitic gastroenteritis.

Effects on Blood Components

Profound changes in the composition of the blood occur in infected hosts, as outlined below.

Anaemia

Anaemia is probably the single most important factor contributing to host morbidity and mortality in fluke infections. Its aetiology has long been debated but it is now widely accepted that it is a haemorrhagic anaemia.

There is still some mystery why the anaemia is normochromic and normocytic. Erythropoiesis occurs, there is a mild reticulocytosis but no iron deficiency until late in the infection. Blood loss is from direct blood feeding by the flukes: blood has been recovered from regurgitated caecal contents, and from haemorrhage into the parenchyma, the bile duct and the abdominal cavity as a result of activity of the flukes. It has been estimated that blood is lost at the rate of 0.2–0.5 ml per day per fluke (Dawes and Hughes, 1964; Jennings, 1976). As well as the protein components of plasma and the blood cells, which are reabsorbed after digestion in the intestine, considerable amounts of iron are lost, much of which is not reabsorbed (see e.g. Dargie and Mulligan, 1970). Under these circumstances, the rate of erythropoiesis is increased but is limited in the later stages of the infection by the availability of dietary iron and protein, which is influenced by the quality of the diet and intensity of anorexia (Sinclair, 1965; Berry and Dargie, 1976; Dargie, 1981).

Anaemia is not generally a feature of the parenchymal stage of the disease except in mice, where hepatic haemorrhage is common (Dawes, 1963c; Eriksen, 1980b), unless the infection is very heavy, in which case significant mortality coincides with hepatic haemorrhages at the 'critical' stage of the infection when the flukes are passing into the bile ducts.

Plasma proteins

The major protein components of the plasma are albumin and immunoglobulins, along with about 30 other minor proteins. The liver is the only site of synthesis of serum albumin, whereas immunoglobulins are synthesized by leucocytes at a variety of sites in the body. Hypoalbuminaemia and hyperglobulinaemia commonly occur in liver fluke infections in all host species.

During the parenchymal stage of the infection, liver damage caused by the migrating flukes compromises liver function, which in sheep and calves is reflected in a decline in plasma albumin concentrations, attributed partly to reduced rate of synthesis and partly to an expansion of the plasma volume (see Anderson *et al.*, 1977; Dargie, 1981; Symons, 1989). In rabbits plasma albumin is not reported to decline during this stage of the infection (Dargie and Mulligan, 1971). In rats albumin declines during this stage only in very heavy infections (Thorpe, 1965a).

During the biliary stage of the infection loss of blood to the intestines is so extensive that the synthetic capacity of the liver is insufficient to replace the lost albumin. Even though the liver parenchymal tissue has largely healed and regained normal function by this time, in field infections incoming metacercariae may further damage the liver, compromising function further. Thus a progressive loss of plasma albumin occurs in all infected host species, starting from around the time the flukes commence blood feeding. In calves the loss of albumin declines only late in the infection (Anderson *et al.*, 1977), due possibly to the ability of calves to resolve the infection at the migrating stage. In infected sheep on a low protein diet the rate of albumin synthesis was significantly higher than in pair-fed controls, yet both the absolute rate of catabolism of albumin and the fraction of the intravascular albumin pool

catabolized per day increased (from about 0.05 to above 0.15) late in the infection; at the same time the plasma half-life of albumin decreased from 500–600 h to below 300 (Dargie, 1981). Similar changes have been observed in infected rabbits (Dargie *et al.*, 1968). The loss of albumin is influenced by diet as sheep fed a high protein diet lost albumin more slowly than those on a low protein diet. In poorly fed animals, in particular, the diversion of amino acids to albumin and haemoglobin synthesis would compromise the availability of amino acids for protein synthesis in muscle and the body generally, as reflected in loss of body weight.

Increased immunoglobulin synthesis commences within several weeks of infection and elevated immunoglobulins, including IgM, IgG1, IgG2a and IgE, persist throughout the infection (e.g. Holmes *et al.*, 1968; Sinclair, 1968, 1970; Berry and Dargie, 1976; Sykes *et al.*, 1980; Dargie, 1981; Hughes *et al.*, 1981; Poitou *et al.*, 1992; Jemli *et al.*, 1993). In sheep the fractional catabolic rate of immunoglobulins rises during the infection (Holmes *et al.*, 1968), consistent with their loss to the intestines along with albumin and blood cells. The rise appears less dramatic than that for albumin because of the normally higher turnover rate of immunoglobulins (around 250 h for IgG (Nielsen, 1976)).

Hepatic enzymes in blood

Levels of hepatic enzymes released into the blood as a result of damage to liver tissue have been used to monitor the progress of the infection in a variety of hosts under experimental conditions and as a sensitive diagnostic aid in field infections. Activities in serum of the hepatocyte enzymes glutamate dehydrogenase and glutamate-oxaloacetate aminotransferase increase during early infection, reaching a peak towards the end of the parenchymal stage (e.g. Thorpe, 1965a; Ross *et al.*, 1966; Sinclair, 1967, 1975; Hughes *et al.*, 1973; Schuster and Lammler, 1973; Anderson *et al.*, 1977, 1981; Rowlands and Clampitt, 1979; Rajasekariah and Howell, 1980; Sykes *et al.*, 1980; Sandeman and Howell, 1981; Jemli *et al.*, 1993; Ferre *et al.*, 1994, 1996). The appearance in the blood of γ-glutamyl transferase, an enzyme present in the bile duct epithelium, is an indicator of damage to the bile ducts. The peak of enzyme activity follows the peak of the hepatocyte enzymes (e.g. Anderson *et al.*, 1977, 1981; Rowlands and Clampitt, 1979; Sykes *et al.*, 1980; Sandeman and Howell, 1981; Bulgin *et al.*, 1984; Jemli *et al.*, 1993; Ferre *et al.*, 1994, 1996; Marley *et al.*, 1996). In the biliary stage, in the absence of concurrent parenchymal damage, enzyme levels fall but remain higher than in uninfected controls.

Leucocyte populations

The outstanding change that occurs during *F. hepatica* infection in all host species is a dramatic peripheral blood eosinophilia which appears soon after infection, increases rapidly during the parenchymal stage and persists at a high level after the flukes enter the bile ducts (e.g. Ross *et al.*, 1966; Schuster

and Lammler, 1973; Sinclair, 1973, 1975; Poitou *et al.*, 1992, 1993; Jemli *et al.*, 1993). Other white blood cell populations that have often been observed to increase are those of lymphocytes and neutrophils, and occasionally monocytes and basophils.

Bilirubinaemia

Although the mature flukes occupy the bile ducts and might be expected to interfere with bile production and flow, the occurrence of bilirubinaemia has been reported only occasionally, e.g. in rats during the parenchymal stage (González *et al.*, 1991) and in sheep during both the parenchymal and biliary stages (Prache and Galtier, 1990; Ferre *et al.*, 1995); most studies in rats or sheep have reported no significant change in serum bilirubin concentration.

Other serum components

Ascorbic acid in the plasma declines steadily throughout the infection in sheep (Gameel, 1982a; Kouider and Kolb, 1994). A deficiency in plasma or tissue ascorbic acid indicates the possibility of oxidative stress in the tissues as well as adversely affecting the metabolism of iron and processes of tissue repair.

A study in sheep infected with 200 metacercariae reported no changes in mineral components, or in organic components such as urea, creatinine, triglycerides, cholesterol in the serum obtained over 14 weeks after infection (Jemli *et al.*, 1993).

Hepatic Pathogenesis

Liver trauma

Abrasion caused by spines and the prehensile action of the suckers appear to account for the majority of the damage caused in the liver. Death of the host is a consequence of the haemorrhage induced by this damage. However, the precise causes of pathology are still unknown. Most of our knowledge is based on histopathology of flukes *in situ* and examination of caecal contents following regurgitation. These observations are like snapshots of dynamic events and are open to many interpretations.

The early life of *F. hepatica* in mice has been described in a series of papers by Dawes (1961a, b, 1963b, c). Penetration of the intestinal mucosa causes little damage (Dawes, 1963c). Debris is left in the wake of the fluke but little else is described. Much has been made of abrasion of the spiny tegument of the fluke on the liver parenchyma and the bile duct epithelium (Dawes, 1963a). In infections of mice (Dawes, 1963c), cattle (Dow *et al.*, 1967) and sheep (Sinclair, 1967) desquamation and ulceration were observed in regions adjacent to the spiny bodies and in some cases indentation of spines in the tissue was observed. It is difficult to prove how important spines are in causing damage. The gravity of their effects has been widely accepted because powerful but unsubstantiated comments such as ‘epithelial

damage caused by the spines of the fluke' (Dawes 1963a) have not been confirmed or disputed. Would a spineless fluke be less irritating?

The oral sucker is the route by which liver flukes gain most of their nutrition. It appears to cause considerable damage to liver tissue and macerated hepatic cells have been observed inside the sucker and pharynx (Dawes and Hughes, 1964). The oral sucker extends during migration and feeding and even from the earliest stages is capable of disrupting cells. The muscular pharynx assists in this process. Food as cell debris enters the caeca where it is digested and what is not absorbed is regurgitated. Observation of postures of fixed flukes within excavated tracks in the parenchyma also suggest that the oral suckers are the major organ involved in tissue disruption. In the experiments of Sukhdeo *et al*. (1988) tissues of rabbits harbouring duct-stage flukes were rapidly frozen in order to minimize the movement of flukes prior to fixing. Following observation of serial sections they found that in the large majority of cases areas of chronic ulceration and haemorrhage were associated with areas of the bile duct adjacent to oral suckers.

The ventral sucker is a holdfast organ which the fluke uses for attachment while feeding. Dawes (1963c) observed broken cells in the ventral sucker of fluke in mice and assumed that they contributed to the damage. On the other hand Sukhdeo *et al.* (1988) described papillae in bile ducts of rabbits and concluded that they were the sites of 'permanent' attachment which allowed the fluke to graze on an ulcerated region of the epithelium.

Enlargement of the bile duct wall and lumen due to hyperplasia of the epithelial and subepithelial cells, particularly fibroblasts, and deposition of collagen occur long before the maturing flukes enter the bile ducts (Dawes, 1963a). Bile duct enlargement can be induced by ectopically implanted flukes, showing that the changes are induced chemically by factors produced or induced by the flukes (Isseroff *et al.*, 1977). Elevated concentrations of the amino acid proline appear to be an important factor in this process: proline is essential for collagen synthesis by fibroblasts and is released in large quantities by liver flukes. Infusion of proline into rats mimicked in part the bile duct enlargement induced by ectopically implanted flukes (Modavi and Isseroff, 1984).

Proteases

It has long been held that proteases contribute to the tissue damage suffered in fasciolosis. In addition, proteolytic cleavage of immunoglobulins may have a role in immune evasion. Howell (1966) described a collagenase which may cause tissue liquefaction. Dawes (1963a) pondered why the cavity caused by migrating flukes, especially those in the latter stages of migration, was much larger than the fluke itself. He suggested that tissue lysis was due to the action of leucocyte (i.e. host) enzymes although the possibility that proteases in the regurgitant were responsible for tissue lysis was raised (Dawes 1963c).

Despite research effort in the last 30 years there remains no convincing evidence that fluke proteases – or other tissue-degrading enzymes – are responsible for aspects of the pathogenesis of fasciolosis. Several proteases have been recovered from fluke homogenates, regurgitant or culture fluids and

the genes encoding some have been cloned. Studies have revealed a battery of proteases and many unique enzymes can be recovered from several life cycle stages of flukes. For example, electrophoretic analysis has revealed 11 thiol proteases in immature and mature flukes although one of these, at least, appears to be a product of only immature flukes (Dalton and Heffernan, 1989). Amongst others, cathepsin B (Chapman and Mitchell, 1982) and cathepsin L-like (Carmona *et al.*, 1993) proteases have also been described. Several roles for proteases have been suggested. These include: ameliorating the immune response to the fluke, formation of fibrin clots, penetration of the tissues of the host, excavation of liver tissue and ulceration of the bile duct.

Excretory/secretory products from immature and adult flukes which have characteristics of cathepsin B proteins cleave IgG and IgM from several species (Chapman and Mitchell, 1982). The cleavage, which has been performed *in vitro*, is sensitive to protease inhibitors. These enzymes also cleave haemoglobin. Rats vaccinated with crude preparations of culture medium in which parasites were maintained are partially protected from infection by flukes (Rajasekariah and Howell, 1979). However this hardly implicates proteases as protective given that other components of the medium could be responsible. Several stages of fluke, including newly excysted juveniles, produce a cathepsin L-like enzyme which is capable of preventing antibody-mediated attachment of eosinophils to the flukes (Carmona *et al.*, 1993). Antibodies to the protease reversed this inhibition (Smith *et al.*, 1994). Except for the vaccine trial in rats all of the above observations were from *in vitro* experiments and there is no current evidence that the enzymes perform the suggested functions *in vivo*.

An unusual activity of a cathepsin L-like proteinase is in the proteolytic cleavage of fibrinogen to form a novel type of clot (Dowd *et al.*, 1995). Fibrinous adhesions are a feature of the migratory phase of fasciolosis (Boray, 1969). The question remains: are clots part of a normal inflammatory response or are they caused by parasite factors? If there were a specific role for this protease in creating fibrin-clotting ability it should be at peak activity in immature flukes, which is yet to be demonstrated.

Several proteases have been recovered from regurgitant. One would expect that these enzymes – as well as culture-derived proteases – have roles in feeding and nutrition. Most are secreted as proenzymes and carry out extracellular digestion. The multiplicity of enzymes found can be explained by the variety of the diet available to the fluke as it includes liver, bile duct epithelium and blood. Developmental regulation of proteases could also be expected as the environment and diet of the developing fluke changes. Flukes which have been used as the source of enzymes have been recovered from a variety of hosts including rats, mice and sheep. It might be expected that a different suite of enzymes would be synthesized depending on which tissue and from which host the flukes were recovered. Alternatively, the proteases liberated by the parasite may have a broad specificity. Immunoreactivity to several cathepsin-like proteins has been identified in epithelial cells lining the gut wall of the fluke, specifically in intracellular vesicles (Smith *et al.*, 1994; Creaney *et al.*, 1996). In addition, a

dipeptidylpeptidase present in several stages of the fluke would function at later stages of digestion to form dipeptides suitable for absorption (Carmona *et al.*, 1994). The presence of an endogenous fluke protease inhibitor which is located in membrane vesicles of the gut and in fluke parenchyma (Bozas *et al.*, 1995) suggests that flukes can modulate proteolytic activity.

Although there is ample evidence for feeding roles for proteases, there is no direct evidence to link fluke proteases with tissue penetration. The regurgitant is expelled *in vitro*, but whether it is expelled in a similar fashion *in vivo*, remains active and interacts with host tissue is unproven. Because of the prevailing pH, several acid thiol proteases are probably inactive in host tissues but the basic ones potentially have a role in extracorporeal tissue digestion (Dalton and Heffernan, 1989). Although proteases secreted *in vitro* by juvenile flukes have collagenolytic activity and also degrade fibronectin and laminin (Berasain *et al.*, 1997), there is no evidence to suggest these activities are more than nutritive. Because the penetration of the gut, the liver capsule and the bile duct are specific events one would expect the putative enzymes to be stage-specific and to be present in host tissues during invasion. A cathepsin L-like protease has been immunolocalized to the gut of flukes (Smith *et al.*, 1993) but its presence in the path of migrating flukes *in vivo* has not been reported. The difficulty of these studies is considerable given the plethora of enzyme epitopes, the size of the flukes and the inaccessibility of the host tissues. More progress has been made with *Schistosoma mansoni* where a fluke serine protease which is synthesized in the acetabular cells has been found in the migratory path of the cercariae through human tissues (Fishelson *et al.*, 1992). Furthermore skin penetration by cercariae can be prevented with protease inhibitors (Cohen *et al.*, 1991). An indirect proof of the role of proteases in the pathogenesis of fasciolosis would be to demonstrate protection, specifically prevention of establishment or invasion, by immunization with proteases. In a trial using a molecular mass 28 kDa regurgitant thiol cathepsin in sheep, no protection against establishment of *F. hepatica* was observed, although fluke egg production was reduced in vaccinated sheep (Wijffels *et al.*, 1994). This suggests that in sheep this protease had no role in tissue invasion. Levels of antibodies to the protein did not correlate with the reductions in egg production and the effect may be attributed to impaired digestive activity. In cattle, however, liver pathology was reduced and a 40–50% reduction in fluke burdens was achieved by vaccination with preparations of purified cathepsin L1; established flukes were smaller and egg embryonation was also inhibited (Dalton *et al.*, 1996). Therefore in cattle cathepsin L1 is probably an important factor in the pathogenesis of the infection.

Inflammatory responses

Accompanying the mechanical liver damage caused by the migrating juvenile flukes is a cellular inflammatory reaction mounted by the host (Urquhart, 1956; Dawes, 1963c; Thorpe, 1965b; Ross *et al.*, 1966, 1967; Dow *et al.*, 1967, 1968). The tracks fill with cell debris, erythrocytes, lymphocytes, neutrophils,

eosinophils and macrophages and damage to the hepatic cells surrounding the tracks is evident. Older parts of the tracks gradually fill with macrophages and fibroblasts, forming fibrotic granulation or scar tissue. In heavy or prolonged infections fibrosis of the liver becomes severe. Fibrosis is more marked in cattle than other hosts and may restrict the movement of the flukes. Once the flukes have entered the bile ducts, the parenchymal tissue recovers and inflammation is restricted to the epithelia of the bile ducts and areas of the parenchyma immediately adjacent.

Early studies in sheep by Sinclair (1968, 1970) showed that the inflammatory response is important in partially protecting the host against the tissue damage caused by the flukes. Treatment of infected sheep with dexamethasone, an anti-inflammatory corticosteroid that kills lymphocytes, allowed a more rapid development of the flukes and increased the physical damage to the liver. In the treated sheep there was extensive haemorrhage, little hepatic fibrosis and no thickening of the bile duct walls. The sheep exhibited clinical signs of illness (not seen in the infected controls) such as anorexia, pallor and weakness, they lost weight and rapidly developed anaemia. Similarly, splenectomized sheep exhibited a higher fluke burden, with larger flukes, more extensive liver damage and more severe clinical disease. In each case, hypoalbuminaemia developed earlier than in untreated sheep.

A similar situation occurs in mice, where infected athymic (nude) mice almost invariably die during the parenchymal stage with extensive liver damage characterized by absence of cellular infiltration in the fluke tracks, considerable intrahepatic bleeding and necrosis, little thickening of the bile duct epithelium and little evidence of tissue repair processes (Eriksen, 1980a, b). Studies with rabbits rendered immunodeficient by treatment with antilymphocytic serum showed reduced cellular responses in the liver which were associated with increased hepatic damage and death of the hosts in the third week post-infection (Dodd and O'Nualláin, 1969). In genetically immunodeficient calves lacking a normal thymus gland, there was little gross hepatic pathology or cellular reaction in the liver to the presence of flukes (Flagstad *et al.*, 1972; Flagstad and Eriksen, 1974). The animals failed to gain weight, unlike the controls, but it is not possible to attribute this directly to the lack of response in the liver to the flukes because of the presence of secondary bacterial infections. In rats, flukes recovered from athymic hosts were larger than those from heterozygous littermates (Doy and Hughes, 1982); although there has been no detailed study of hepatic pathology in infected athymic rats, one report (Hanisch *et al.*, 1992) noted that gross pathology was less severe. An increase in circulating acute phase proteins, indicative of inflammation, was demonstrated 3–4 weeks post-infection, coincident with maximum liver damage; as with sheep, treatment of rats with anti-inflammatory agents led to increased fluke burdens (Baeza and Poitou, 1994).

Immunopathology

Although the inflammatory response has an important role in protecting the host against the severest consequences of liver damage by the flukes, perhaps

by retarding the growth of the parasite slightly and contributing to hepatic healing processes, there is accumulated evidence, in rats, that the response also contributes to hepatic dysfunction. Using athymic rats or treatment with dexamethasone, a number of studies (Hanisch *et al.*, 1991, 1992; Lenton *et al.*, 1995; Topfer *et al.*, 1995) have shown that many aspects of biochemical dysfunction (discussed below), including bioenergetic abnormalities, accumulation of non-esterified fatty acids, depletion of P450 and phospholipids, do not occur in hosts whose T-cell function was absent or had been eliminated.

There is evidence also that the infected rat liver is under oxidative stress during the parenchymal stage of the infection. Oxidative stress would be one of the consequences of the activity of inflammatory cells such as neutrophils, macrophages and eosinophils in producing oxygen-derived free radicals, nitric oxide and their products. A useful indicator of oxidative stress is the concentration of reduced glutathione (GSH) in cells. Several (Maffei Facino *et al.*, 1990, 1993), but not all (Galtier *et al.*, 1991b), studies in rats have reported that the concentration of hepatic GSH declines. A decline in cytosolic hepatic GSH has also been reported in infected lambs (Galtier *et al.*, 1986b). As well, in rats the concentrations of products of lipid peroxidation, malondialdehyde and conjugated dienes are elevated. Administration of exogenous GSH to infected rats restored hepatic glutathione levels almost to normal and also normalized the levels of malondialdehyde (Maffei Facino *et al.*, 1993). Glutathione treatment also partially prevented the decline in P450 concentration and P450-dependent enzyme activities as well as the decline in phospholipid content (discussed below), all of which could be attributed at least in part to oxidative damage to the membranes of hepatocytes. Treatment of infected rats with UDP–glucose (Maffei Facino *et al.*, 1990) also increased hepatocyte GSH levels, reduced malondialdehyde accumulation and partially restored P450 concentration and P450-dependent enzyme activities, although the mechanism is less clear: one hypothesis is that UDP–glucose could provide additional substrate for glucose 6-phosphate dehydrogenase, thus increasing intracellular NADPH levels and permitting regeneration of GSH via glutathione reductase activity.

These studies highlight the importance of a balance between the inflammatory response on the one hand contributing at least some protection to the host against the damaging activities of the flukes and, on the other hand, causing functional damage to the liver. Although there is little information on the immunopathological damage to hepatic function for hosts other than rats, it seems not unlikely that the situation in other hosts could be similar. Therefore it could be important when designing vaccines aimed at preventing liver fluke infection to ensure that the vaccine does not exacerbate liver pathology by provoking inflammation.

Pathophysiology

The liver plays a central role in the physiology of the body, being responsible for a large proportion of the body's amino acid metabolism, for carbohydrate and lipid balance, urea synthesis, detoxification metabolism, ketogenesis, albumin and glutathione synthesis as well as aspects of homeostasis. It is not

surprising therefore that many systemic changes are induced by liver fluke infections which ultimately cause reduced productivity in livestock. Although the magnitude and significance of many of the systemic and tissue-specific effects described below clearly depend on the intensity of the infection, most of the changes have been recorded even for subclinical infections involving relatively small numbers of flukes (e.g. two or more mature flukes for rats, around 20 mature flukes for sheep).

Weight gain, food intake and nitrogen balance

Forty to sixty adult flukes compromise the weight gain and wool growth of young sheep; the effect is evident within a few weeks of infection but is most prominent during the biliary stage (Hawkins and Morris, 1978). Sheep harbouring more than about 250–350 mature flukes progressively lose weight, starting a few weeks after infection. Studies of weight gain and productivity in cattle with subclinical infections (up to 200 mature flukes) have shown rather variable outcomes. In a recent study with cattle harbouring up to 90 mature flukes, weight gain was not compromised during the parenchymal phase but significant effects on weight gain and feed conversion efficiency were observed during the biliary stage of the infection (Marley *et al.*, 1996).

Reduced weight gain in sheep and cattle has two apparent causes: reduced feed conversion and anorexia (inappetence). Fluke burdens <200 in sheep do not appear to induce significant anorexia (e.g. Sinclair, 1975; Berry and Dargie, 1976; Hawkins and Morris, 1978; Dargie *et al.*, 1979; Sykes *et al.*, 1980; Ferre *et al.*, 1994), indicating that reduced weight gain in these infections is due to compromised conversion of feed. In sheep with higher fluke burdens, anorexia is a consistent and important feature of chronic fasciolosis (for reviews see Dargie, 1987; Symons, 1989). What causes this reduced appetite is not known. In single-dose experimental infections of sheep, which included pair-fed controls, anorexia coincided with entry into bile ducts around weeks 6–7 post-infection (Berry and Dargie, 1976; Dargie *et al.*, 1979; Sykes *et al.*, 1980; Ferre *et al.*, 1994). Animals on poorer diets exhibited more severe disease than those on a higher level of nutrition (e.g. Berry and Dargie, 1976). Further, Dargie *et al.* (1979) showed that in heavy infections (1000 metacercariae) in sheep nitrogen retention was lower after week 8 post-infection, which could account for the differences in body weight, and that the loss of nitrogen was a result of increased urinary excretion rather than decreased intestinal absorption. The authors postulated that catabolism of muscle proteins might also contribute to the emaciated state of the animals late in the infection. Both anorexia and the quality of the diet of infected sheep contributed to hypoalbuminaemia during the infection (see below). In contrast to heavy single-dose infections, in subclinical multiple-dose infections of sheep Sykes *et al.* (1980) found no differences in nitrogen balance from pair-fed controls and no changes in plasma urea. It seems likely that the availability of amino acids for protein synthesis would be an important factor in determining the extent to which the liver can compensate for the damage done by the flukes.

Hepatic function

Liver has remarkable functional redundancy and, unlike most other organs in mammals, is able to regenerate functional tissue after physical or chemical injury. It has been estimated that 60–70% of liver tissue can be lost before significant dysfunction becomes apparent (Jubb and Kennedy, 1970), though this probably depends on what aspect of liver function is examined. Although only a few aspects of liver function have been directly studied in fluke-infected hosts, significant disturbances have been detected, even when only small areas of the liver are overtly directly damaged, as discussed below.

Mitochondrial bioenergetic metabolism

The synthesis of ATP is an essential function of all viable cells and is a prerequisite for almost all other cellular functions. Van den Bossche *et al.* (1980, 1983) reported that mitochondria prepared from infected rat livers were uncoupled, i.e. that mitochondrial electron transport was not coupled to ATP synthesis. These reports were confirmed by Rule *et al.* (1989) who showed that at 2, 4 and 6 weeks post-infection the respiration of isolated mitochondria did not respond to chemical uncoupling agents or to oligomycin, an inhibitor of ATP synthesis in coupled mitochondria. At 2 and 6 weeks post-infection the rate of respiration without added ADP was high, indicating that the mitochondria were already largely uncoupled. The uncoupling persisted until at least 21 weeks post-infection and required a fluke burden of greater than two flukes in the liver. An additional effect was evident at 4 weeks post-infection – the time in rats when maximum liver damage occurs (Thorpe, 1965a, b) – when the respiration of isolated mitochondria was severely attenuated, indicating significant damage to the mitochondrial electron transport system. Mitochondria isolated from rats at 3 weeks post-infection were also unable to synthesize ATP (Hanisch *et al.*, 1992). The ATP concentration in extracts of freeze-clamped infected rat livers at 4 weeks post-infection was 75–85% of the control preparations (Millard *et al.*, unpublished), implying the possibility of a shortage of ATP in the liver *in vivo*.

A similar study of mitochondria isolated from infected sheep livers showed that respiration was attenuated and uncoupled in preparations from 4 to 15 weeks post-infection (Rule *et al.*, 1991). The respiratory aberrations were more severe in the left lobe than in the middle lobe, indicating that greater tissue damage causes greater biochemical damage. Hepatic mitochondrial respiratory aberrations also occurred in preparations from Balb/c mice but developed only at the 'critical' stage of the infection at around 28 days post-infection (Somerville *et al.*, 1995). Interestingly, the effect was not seen in outbred Swiss mice under the same conditions.

Additional studies in rats showed that the mitochondria were permeable to NADH, to which they are normally impermeant, that the mitochondrial F_1F_O-ATP synthase was insensitive to a variety of inhibitors to which it is normally sensitive and that it appeared to be structurally unstable (Lenton *et al.*, 1994). Intact hepatocytes isolated from infected rat livers at 3 weeks post-infection were abnormally permeable and also exhibited respiratory

abnormalities (Hanisch *et al.*, 1992). An explanation for some of these effects in rat liver has been provided by the observation that mitochondria isolated from infected livers contain elevated concentrations of non-esterified ('free') fatty acids, which could account for their uncoupled state (Lenton *et al.*, 1995). As well, the study showed a 40% decline in the concentration of total mitochondrial phospholipids, accompanied by changes in the phospholipid composition of the mitochondrial membranes. These alterations could account at least in part for the increased non-esterified fatty acid concentration in the mitochondria as well as their altered respiratory and permeability properties.

Infected sheep, in contrast, showed no changes in non-esterified fatty acid concentration or phospholipid composition or content in liver mitochondria isolated at 4 weeks post-infection (Lenton *et al.*, 1996). Furthermore, the attenuation of respiration was attributed to direct damage to the electron transport chain. Thus, the nature and aetiology of the damage to mitochondrial respiration are quite different in sheep and rats.

It is not yet clear to what extent ATP synthesis in hepatocytes in the liver as a whole is compromised during the infection in any host, although ATP concentrations in liver extracts from infected rats are lower than in uninfected rats. *In vivo*, the effects could be restricted to the areas of tissue immediately adjacent to the flukes or their tracks. The work outlined below shows that certain aspects of liver function are indeed altered in infected hosts, some of which could be due to reduced ATP synthesis and the detrimental changes in the composition of hepatocyte membranes.

Carbohydrate metabolism

An important function of the liver is regulation of the concentration of blood glucose. This is especially critical in ruminants because glucose is not obtained directly from their diet but rather by hepatic conversion of three-carbon precursors into glucose or glycogen by the process of gluconeogenesis. Under hormonal control, glucose is released from the liver into the blood to maintain stable levels of blood glucose. In ruminants, another important function of the liver is the conversion of two-carbon units derived from the gut into ketone bodies such as acetoacetate or β-hydroxybutyrate which are released into the blood and serve as energy substrates for tissues other than the brain.

A few studies have assessed blood glucose concentrations in infected hosts (rats and sheep) and found them to be in the normal range (Rowlands and Clampitt, 1979; Millard *et al.*, 1997 unpublished results). One study, however, reported a decline, from about 5.2 mM to about 4.3 mM, in sheep from 60 days post-infection, i.e. during the biliary phase (Ferre *et al.*, 1994). Although blood glucose levels may not be compromised under experimental conditions, the evidence outlined below supports the notion that dysfunction in hepatic carbohydrate metabolism may occur in field conditions and when the host is stressed.

The glycogen content of infected rat livers is significantly lower throughout the infection than those of controls, even if pair-fed (Gameel, 1982b; Millard *et al.*, 1997 unpublished results). Lowered glycogen content would imply that less glucose would be available to maintain blood glucose levels in short-term fasting, during exercise or in stressful situations. This has been confirmed in

liver perfusion experiments with rat livers (Hanisch *et al.*, 1991; Millard *et al.*, 1997 unpublished results) where hormone-dependent release of glucose from livers was severely attenuated (20–60% of controls) throughout the infection (up to 21 weeks). Gluconeogenesis was also attenuated by 50% or more up to 12 weeks post-infection (Millard *et al.*, 1997 unpublished results). Thus hepatic carbohydrate metabolism is impaired in infected rats. The only report of hepatic glycogen concentrations in ruminants showed a decline in the left lobe of infected sheep but no change in the right lobe (Lenton *et al.*, 1996). Thus, in low to moderate infections of sheep carbohydrate metabolism may be compromised in the more damaged left lobe but not in the less-affected lobes, which may be able to compensate for this damage.

Protein metabolism

It is not clear whether the processes of protein synthesis and turnover are specifically affected in infected livers as this question has not been directly investigated. Several studies found a decline in microsomal and cytosolic protein content in rats in the early biliary stage (Biro-Sauveur *et al.*, 1994, 1995) but other studies in rats and sheep found no change in hepatic protein (Galtier *et al.*, 1983, 1986b; Jemli *et al.*, 1994). In sheep infected with 1000 metacercariae the relative rate of albumin synthesis declined during the parenchymal stage but increased during the biliary stage in comparison with pair-fed controls; the protein content of the diet influenced the rate of albumin synthesis in the latter stage (Dargie, 1981).

Lipid metabolism

Although no studies have directly addressed the synthesis and turnover of lipids and phospholipids in fluke-infected livers, the concentrations of both the phospholipid and total lipid components of hepatic homogenates decline dramatically in infected rats, commencing in the second and fourth weeks post-infection, respectively (Maffei Facino *et al.*, 1990, 1993; Lenton *et al.*, 1995). Both microsomal and mitochondrial fractions exhibited significant decline in phospholipids (Lenton *et al.*, 1995) and it appears likely that other membrane fractions would also be affected. In both the mitochondrial and microsomal fractions the decline in phospholipids was accompanied by an increase in degradation products or precursors including, in particular, large increases in the concentration of non-esterified fatty acids, suggesting the possibility of elevated phospholipase activity in infected rat livers. Increases in the concentration of malondialdehyde in homogenates and in conjugated dienes in microsomal preparations from rat liver have also been reported (Maffei Facino *et al.*, 1990, 1993), which is evidence for peroxidative damage to hepatic lipids. The consequences of severely decreased phospholipid content to the function of membrane-associated processes in liver cells would be extreme and could explain many of the functional changes observed in rats, such as respiratory aberrations, increased permeability of hepatocytes and reduced cytochrome P450-associated activities (see below). The phospholipid composition of infected sheep liver, however, was normal, even in the more severely affected left lobe (Lenton *et al.*, 1996).

Steroid metabolism

The liver is the principal site of steroid catabolism, which is catalysed by the mixed-function oxidase system. The rate of clearance of exogenous testosterone was reduced in postpubertal rams infected with mature *F. hepatica* (Fleming and Fetterer, 1986). Such impaired *in vivo* steroid hormone metabolism has been implicated as a possible factor in the failure of fluke-infected livestock to maintain pregnancy (Biro-Sauveur *et al.*, 1994). A study in infected rats, at 6 weeks post-infection, demonstrated reduction *in vitro* of most hydroxylations of progesterone and testosterone by hepatic microsomal fractions (Biro-Sauveur *et al.*, 1994); the exceptions were 6β- and 21-hydroxylations of progesterone and 7α-hydroxylation of testosterone, which did not decline. These effects correlated with decreased contents of the P450 isoform CYP2C11 and, to a lesser extent, of CYP2B1/2 and CYP3A1/2, as determined by immunoblotting.

Bile flow and composition

It might be expected that the flow and composition of bile would be altered during liver fluke infection, given the location and activities of the flukes, but only a few studies, in rats, sheep and calves, have addressed this question. In rats the flow of bile declined during the parenchymal stage of the infection; this change was accompanied by a decline in the concentration of bile acids (López *et al.*, 1994). In the biliary stage, however, bile flow was increased and bile acid concentration normal. The rate of excretion of bilirubin was elevated during both the parenchymal and biliary stages. In infected sheep bile flow at 6 weeks post-infection was about 50% of control levels and progressively returned to normal values by 16 weeks (Ferre *et al.*, 1995). There was a slight decline in the concentration of bile acids at 6 and 8 weeks post-infection; biliary bilirubin levels also declined slightly from 6 to 14 weeks, due to a significant decrease in the proportion of conjugated bilirubin. Another study in sheep did not detect bilirubinaemia (Jemli *et al.*, 1993). In infected calves bile flow increased and the rate of bile salt (cholate) secretion was normal, from 10 weeks post-infection, i.e. during the biliary stage (Symonds *et al.*, 1983). The rate of excretion of iron in the bile increased enormously after week 8 as a consequence of leakage of red blood cells into the bile due to the activity of the parasites. During the same time frame the rates of excretion of zinc, copper and manganese were unchanged. In rats and calves the increase in bile flow coincided with the entry of the flukes into the bile ducts and their commencement of blood feeding: it would seem plausible that the increase in bile flow could be due to a combination of hyperplasia of the bile duct epithelium plus a significant contribution of blood to the bile.

A study by Isseroff *et al.* (1972) showed very large changes in amino acid composition of the bile in cattle, rabbits and rats during the biliary stage. The concentrations of almost all amino acids changed significantly. Much of this alteration could be due to amino acids derived from blood leaking into the bile ducts, but they also showed that possible excretion of proline by the flukes could be a major source of elevated proline in the bile.

Detoxification of xenobiotics and pharmacokinetics of drugs

One of the critical functions of the liver, and one that is affected in many diseases, is the detoxification of xenobiotics and endogenous compounds prior to excretion from the body via the bile or urine. It has become clear that infection with liver flukes affects both the metabolism of xenobiotics and the pharmacokinetics of many administered drugs. These changes could influence the efficacy and toxicity of administered therapeutic compounds, the toxicity and clearance of xenobiotics such as pasture and ectoparasite toxins, insecticides or environmental pollutants, as well as compromise legislated post-treatment withdrawal periods for milk and/or meat.

Metabolism of lipophilic xenobiotics generally occurs in two stages, termed Phases I and II. In Phase I lipophilic compounds are converted, usually by oxidation reactions catalysed by the mixed-function oxidase (MFO) system (comprising NADPH–cytochrome P450 reductase, cytochrome P450 and cytochrome b_5), into products that are more water soluble and, generally, less toxic. The MFO system is highly versatile and can adapt to metabolize a diverse range of substrates. This is due largely to the P450 family of haemoproteins and the regulatory processes that control their expression. In mammals there are currently 14 recognized genetic families of P450 (named CYP1, CYP2, etc.), many containing numerous subfamilies (CYP1A, CYP1B, etc.), with a variety of isoforms within the subfamily (CYP1A1, CYP1B1, etc.), each capable of metabolizing different groups of compounds. Several isoforms of P450 are expressed constitutively in the liver (e.g. CYP1A2 and CYP2A1), whereas others are induced selectively by exposure to different xenobiotics. For example, CYP1A1 is induced by polycyclic aromatic hydrocarbons such as benzo(*a*)pyrene whereas CYP2B1 is induced by phenobarbital. Most of the earlier work on P450 relied on the analysis of specific enzymatic activities (e.g. aniline hydroxylase, aminopyrine *N*-demethylase) or spectroscopic shifts to detect changes in P450 function or concentration. Although these studies provided useful information that could often account for any changes in xenobiotic metabolism observed *in vivo*, they could not provide accurate information about changes in P450 isoforms, as enzymatic activities and spectroscopic shifts overlap between isoforms. The more recent development of isoform-specific antibodies and nucleic acid probes is now providing the tools to interpret *in vivo* changes more precisely in terms of P450 isoforms.

The products of P450 oxidation act as substrates for the Phase II enzymes, of which the most important are the UDP–glucuronosyl transferases (UDPGT) and glutathione *S*-transferases (GST), that catalyse conjugation reactions, producing water-soluble products that are readily excreted from the body. Xenobiotic molecules that are water soluble are generally processed by Phase II reactions only. Important factors affecting the disposition and excretion of xenobiotics include their route of entry to the body (e.g. oral, intravenous) and their route of excretion (e.g. via kidneys or bile), the extent to which they are bound by plasma proteins, vascular flow through the liver and kidneys, bile flow and the metabolic integrity of hepatocytes.

GSH is important in detoxification metabolism as it is both a substrate for GST and an intracellular reducing agent capable of binding directly to

endogenous or exogenous electrophilic compounds such as peroxides or free radicals. It is synthesized by the liver and released into the blood to supply other tissues. A decline in GSH concentrations in infected livers has been reported in some studies in rats (Maffei Facino *et al.*, 1990, 1993) and sheep (Galtier *et al.*, 1986b) which could thus affect both GST and non-enzymatic reducing activity in the liver and other body tissues.

Phase I and/or II reactions are also important in the metabolism and/or excretion of a variety of endogenous molecules such as steroids, cholesterol, bile acids, fatty acids, eicosanoids and biogenic amines. In the liver Phase I detoxification reactions occur primarily in the smooth endoplasmic reticulum of hepatocytes, termed the microsomal fraction in *in vitro* subcellular preparations; Phase II reactions take place either in the cytosolic or microsomal fractions of hepatocytes.

A variety of studies have been carried out on drug pharmacokinetics and activities of detoxification enzymes in *F. hepatica*-infected hosts. The results of the major studies *in vivo* in rats, sheep and cattle are shown in Tables 6.2, 6.4 and 6.6. The infections have been classified into 'parenchymal' or 'biliary' on the basis of the time of post-infection, as indicated in the tables, or, for natural infections, other criteria provided in the publications cited. Numerous studies have also been carried out *in vitro* on the activities or content of individual enzymes of xenobiotic metabolism, which are summarized in Tables 6.3, 6.5 and 6.7.

Rats

There appear to be no studies *in vivo* of hepatic drug metabolism in infected rats, but several studies show reduced uptake and biliary excretion of administered compounds (see Table 6.2). Tetracycline is normally excreted into the bile chemically unchanged; after administration it was cleared more slowly from the plasma due to decreased uptake by hepatocytes and decreased secretion into the bile under conditions where bile flow was not changed (Galtier *et al.*, 1985a). Choleresis induced by cefmetazole, which is not metabolized by the liver but excreted rapidly in the bile, was attenuated in infected rats (López *et al.*, 1992). On the other hand, the disposition of rafoxanide, which is extensively metabolized by the liver, was unchanged because most of the administered dose remains in the plasma, bound to albumin, and entry to hepatocytes is normally very slow (Galtier *et al.*, 1985a).

The content of both cytosolic and microsomal proteins declined in rat liver at 6 weeks post-infection (Galtier *et al.*, 1991b; Biro-Sauveur *et al.*, 1994,

Table 6.2. Effects of infection with *F. hepatica* on the pharmacokinetics of drugs in rats.

Drug	<28 days	>28 days	Effect	Reference
Tetracycline	✔	—	Reduced plasma clearance	Galtier *et al.*, 1985a
Cefmetazole	n.d.	✔	Reduced bilary excretion	López *et al.*, 1992
Rafoxanide	—	—	No change	Galtier *et al.*, 1985a

n.d. Not determined; — no significant effect; ✔ significant effect observed.

1995); a selective and disproportionately greater decline in the activities of a variety of enzymes of xenobiotic metabolism was demonstrated in conjunction with this general loss of protein. Spectroscopic studies of Phase I enzymes show a consistent decline in the concentration of cytochrome P450 during both the parenchymal and biliary stages of the infection (see Table 6.3). Loss of P450 is one of the earliest biochemical effects reported in rat liver as it is evident at day 19 post-infection (Maffei Facino *et al.*, 1990). An accompanying decline in the content of cytochrome b_5 during the biliary phase has been observed occasionally. The activity of NADPH–cytochrome *c* reductase appears unchanged at this time, indicating that the loss of P450 is not due to a general decline in the synthesis of microsomal proteins. There is a 60% decline in total phospholipid in hepatic microsomal preparations from rats 21 days post-infection, accompanied by a large increase in non-esterified fatty acids (Lenton *et al.*, 1995); such large changes are evidence of severe damage to the microsomal membranes that could adversely affect insertion and function of the highly hydrophobic P450 molecule. These changes in phospholipids are unlikely, however, to account for the selective decline in certain isoforms of P450 (see below).

In vitro functional studies of P450-based enzymatic activities (*N*-demethylase, hydroxylase, *O*-deethylase, *O*-demethylase) with a variety of xenobiotic substrates show, in most cases, decreased activity during both the parenchymal and biliary stages of the infection (Table 6.3). The unchanged rate of *N*-demethylation of erythromycin was an exception to this trend (Galtier *et al.*, 1994a). It appears that the inducibility of those P450 isoforms that are induced by 3-methylcholanthrene or β-naphthoflavone (e.g. CYP1A1), both of which utilize the Ah receptor induction process, is not affected by *F. hepatica* infection, whereas the induction of isoforms induced by phenobarbitone or Arochlor 1254 (e.g. CYP2B1), which are induced by a different mechanism (Waxman and Azaroff, 1992), is more sensitive to inhibition during the infection (Galtier *et al.*, 1985b).

Many studies have shown that enzymatic activities are not a reliable indicator of the activities of individual isoforms of P450. The first studies to correlate directly the decline in P450 content and activities with specific isoforms of P450 were carried out by Galtier *et al.* (1986a), who showed that different isoforms of the enzyme appear to be affected differently by the infection. Using specific antibodies they demonstrated that there is a decline during both the parenchymal and biliary stages in the concentration of the main constitutive isoform in male rats, CYP2C11, and of CYP2B1, which is induced by phenobarbital, but not of CYP1A1, which is induced by polycyclic aromatic hydrocarbons. The decline in CYP2C11 correlated with loss of aminopyrine *N*-demethylase activity and that of CYP2B1 with loss of benzphetamine *N*-demethylase activity, but the results for CYP1A1 were less clear. CYP1A1 is the principal (but not sole) inducible ethoxycoumarin *O*-deethylase activity. In the 1986 study (Galtier *et al.*, 1986a) there was no effect of infection on this activity either before or after induction by 3-methylcholanthrene; this correlated with unchanged CYP1A1 detected by antibody. But a later study by the same group found that the enzymatic activity in

Table 6.3. Effects of infection with *F. hepatica* on enzymes of xenobiotic metabolism in rats.

Enzyme	Substrate	<28 days	>28 days	Effect	Reference*
Phase I metabolism					
P450	—	✔	✔	Decreased total content	[1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12]
CYP1A1	—	—	—	No change	[3]
CYP2A1/2A2	—	n.d.	✔	Decreased content	[10]
CYP2B1/2B2	—	n.d.	✔	Decreased content	[10]
CYP2B1	—	✔	✔	Decreased content	[3]
CYP2C7	—	n.d.	✔	Decreased content	[10]
CYP2C11	—	✔	✔	Decreased content	[3, 10]
CYP2E1	—	n.d.	✔	No change	[10]
CYP3A1/3A2	—	n.d.	✔	Decreased content	[10]
N-demethylase	Aminopyrine	✔	✔	Decreased activity/binding	[1, 2, 3, 5, 7, 11]
	Benzphetamine	✔	✔	Decreased activity	[3, 7, 11]
	Erythromycin	—	—	No change	[7]
	Ethylmorphine	—	✔	Decreased activity	[7]
Hydroxylase	Aniline	✔	✔	Decreased activity	[1, 2, 5, 9, 11]
	Benzo(*a*)pyrene	✔	✔	Decreased activity	[7, 11]
O-demethylase	*p*-Nutroanisole	✔	✔	Decreased activity	[5, 9]
O-deethylase	Ethoxycoumarin	—	—	No change	[2, 3]
	Ethoxycoumarin	✔	✔	Decreased activity	[7]
	Pentoxyresorufin	n.d.	✔	Decreased activity	[11]
Nitroreductase	Nitroxynil	n.d.	✔	Decreased activity	[9]
NADPH–P450 reductase	—	n.d.	✔	Decreased activity	[9]
NADPH–cytochrome *c* reductase	—	n.d.	—	No change	[2]
Cytochrome b_5	—	—	✔	Decreased total content	[1, 5]
		n.d.	—	No change	[2]

Phase II metabolism					
UDP–glucuronosyl transferase	*p*-Nitrophenol	✔	✔	Decreased total activity; loss of latency	[5, 9, 13]
	p-Nitrophenol	—	—	No change	[4, 11]
	Chloramphenicol	—	—	No change	[4]
	Bilirubin	✔	✔	Decreased total activity	[8]
Glutathione *S*-transferase (GST)	1-Chloro-2,4-dinitrobenzene	✔	✔	Decreased activity	[4, 6, 11]
	1,2-Dichloro-4-nitrobenzene	✔	✔	Decreased activity	[6, 11]
GST-α	—	✔	✔	Decreased total amount; Decreased subunit 1	[6]
GST-μ	—	—	—	No change	[6]
Glutathione reductase	—	—	—	No change	[6]
Glutathione peroxidase	—	—	—	No change	[6]
Arylamine *N*-acetyl transferase	Isoniazid	✔	✔	Decreased activity	[4, 11]
	Sulphamethazine	n.d.	✔	Decreased activity	[11]

n.d. not determined; — no significant effect; ✔ significant effect observed.

* References: [1] Galtier *et al.* (1983); [2] Galtier *et al.* (1985b); [3] Galtier *et al.* (1986a); [4] Galtier *et al.* (1987); [5] Maffei Facino *et al.* (1990); [6] Galtier *et al.* (1991b); [7] Galtier *et al.* (1994a); [8] López *et al.* (1994); [9] Maffei Facino *et al.* (1993); [10] Biro-Sauveur *et al.* (1994); [11] Biro-Sauveur *et al.* (1995); [12] Topfer *et al.* (1995); [13] Maffei Facino *et al.* (1985).

untreated rats declined at both parenchymal and biliary stages of the infection (Galtier *et al.*, 1994a). In the same study the authors noted that the loss of benzo(*a*)pyrene hydroxylase activity could be due to loss of the constitutive CYP1A2 and that the lack of decline in erythromycin *N*-demethylase could be attributed to a lack of effect of the infection on the CYP3A subfamily. The latter hypothesis appears to be negated by the observation of a decline in CYP3A1/2 in a study of hepatic microsomal preparations at 6 weeks post-infection using isoform-specific antibodies (Biro-Sauveur *et al.*, 1994). This study confirmed the decline in CYP2B1/2 and CYP2C11 content and also demonstrated relative losses of CYP2A1/2 and CYP2C7, but not CYP2E1.

The molecular mechanisms underlying these isoform-specific decreases in P450 concentrations are not understood. They may be due to the actions of immunological or inflammatory mediators. Recent experiments using T-cell-deficient (athymic) or dexamethasone-treated rats (Topfer *et al.*, 1995) have shown that the decline in P450 concentration during the parenchymal stage of the infection is a consequence of T-cell-dependent inflammatory reactions in the host. There are examples in other diseases of cytokines and other factors influencing hepatic P450. Oxidative stress in the liver is also a contributory factor to the loss of P450 (Maffei Facino *et al.*, 1989, 1993) but it is not clear how such effects could be isoform-specific: perhaps they reflect different rates of turnover of the different P450 isoforms in the infected liver.

Hepatic Phase II enzymes are also affected by infection of rats with *F. hepatica*. UDPGT activity has been variously reported as either decreased during the parenchymal and biliary stages of the infection (Maffei Facino *et al.*, 1985, 1990; López *et al.*, 1994) or unchanged (Galtier *et al.*, 1987; Biro-Sauveur *et al.*, 1995). The conflict could be due to different substrates, fluke burdens and ages of rats in the studies involved. A decline in conjugation of bilirubin by UDPGT, along with the decreased bile flow, would contribute to the bilirubinaemia reported during the parenchymal stage in rats (López *et al.*, 1994). The activity of GST also declined during both the parenchymal and biliary stages of the infection (Galtier *et al.*, 1987, 1991b; Biro-Sauveur *et al.*, 1995), along with a loss of GST protein as determined by HPLC. The decline in both activity and protein was shown to be due mainly to loss of subunit 1 of the GST-α isoenzyme. The activities of glutathione reductase and glutathione peroxidase did not change under the same conditions, although loss of subunit 1 of GST-α would be expected to contribute to loss of Se-independent glutathione peroxidase activity.

Thus hepatocyte function with respect to uptake and/or secretion of xenobiotics into bile is compromised in infected rats as well as *in vitro* activities and content of Phase I and II enzymes. The effect is selective for particular enzymes or isoforms. Additional *in vivo* studies are now required to test whether hepatic metabolism of xenobiotics is deficient in infected rats.

The enzymes of xenobiotic metabolism have also been examined in extra-hepatic tissues in infected rats at 6 weeks post-infection (Biro-Sauveur *et al.*, 1995). In kidneys, the concentration of P450 increased, along with associated benzphetamine *N*-demethylase activity, but aniline and benzo(*a*)pyrene hydroxylase activities declined. This result is indicative of differential effects on

P450 isoforms as benzphetamine *N*-demethylase activity is normally associated with CYP2B1 whereas benzo(*a*)pyrene hydroxylase is predominantly associated with CYP1A2. Similarly, increased CDNB-supported GST activity was reported, though activity with DCNB as substrate was unchanged. Changes in the enzymes of pulmonary xenobiotic metabolism were also observed: benzphetamine *N*-demethylase and pentoxyresorufin *O*-deethylase activities were reduced but the activities of other enzymes were unchanged. In both tissues the protein content of cytosolic and microsomal fractions was unchanged. Thus, in rats aspects of the detoxification capacity of key organs other than the liver is potentially altered by infection with *F. hepatica*.

Sheep

Many studies have investigated the disposition of drugs and xenobiotics in *F. hepatica*-infected sheep (see Table 6.4). Plasma clearance of many compounds is reduced during the biliary stage of the infection, including that of bromosulphophthalein, indocyanine green, antipyrine, prednisone, pentobarbital, albendazole sulphone, erythromycin and mebendazole. These changes have been attributed to: (i) reduced hepatic Phase I metabolism (for antipyrine, prednisone, pentobarbital, erythromycin, mebendazole), resulting in decreased rates of extraction of the compound from the plasma; and (ii) reduced Phase II metabolism (for bromosulphophthalein, mebendazole and possibly albendazole sulphone), leading to decreased elimination of the conjugated product. In the case of indocyanine green, a compound normally excreted in bile unchanged, a transient reduced plasma clearance was observed only at 4 weeks post-infection and was explained by increased distribution volume in the liver due to liver hypertrophy at this stage of the infection (Tufenkji *et al.*, 1987). Some xenobiotics (methylprednisolone, prednisolone) are cleared from plasma more rapidly in sheep during the biliary stage of the infection, an effect attributed to increased hepatic blood flow (Alvinerie *et al.*, 1993). Other compounds tested, such as lidocaine, oxytetracycline, ampicillin, thiabendazole and rafoxanide, exhibited unchanged pharmacokinetics, because Phase I or II metabolism by the liver does not contribute significantly to the pharmacokinetic distribution of these molecules. The news is not all bad, however: reduced hepatic metabolism of xenobiotics in infected sheep had a host-protective effect against hepatotoxins secreted by concurrent infections with larvae of the sawfly *Arge pullata* (Olaechea *et al.*, 1991).

Most spectroscopic studies of the hepatic Phase I enzymes in sheep (see Table 6.5) have identified a decrease in microsomal content of P450 and cytochrome b_5 during both the parenchymal and biliary stages (Galtier *et al.*, 1986b). There was no change in the activity of microsomal NADPH–cytochrome *c* reductase or, at 4 weeks post-infection, glucose 6-phosphatase (Galtier *et al.*, 1986b; Lenton *et al.*, 1996). Loss of P450 is greatest in the heavily infected regions of the liver, i.e. normally the left lobe, leaving undamaged regions relatively unaffected (Lenton *et al.*, 1996). Unlike rats, there appears not to be a decline in hepatic microsomal or cytosolic protein or phospholipid content accompanying this change (Jemli *et al.*, 1994; Lenton

Table 6.4. Effects of infection with *F. hepatica* on the pharmacokinetics of xenobiotics in sheep.

Drug	<56 days	>56 days	Effect	Reference*
Bromosulphophthalein	—	✔	Reduced plasma clearance	Tufenkji *et al.*, 1987
Lidocaine	—	—	No change	Tufenkji *et al.*, 1987
Indocyanine green	✔	—	Decreased plasma clearance; increased distribution volume	Tufenkji *et al.*, 1987
Antipyrine	✔	✔	Reduced plasma clearance	Tufenkji *et al.*, 1988
	n.d.	✔	Reduced plasma clearance	Burrows *et al.*, 1992
	—	✔	Reduced plasma clearance	Benchaoui and McKellar, 1993
	—	✔	Reduced plasma clearance	Ferre *et al.*, 1996
Prednisone	—	✔	Reduced plasma clearance	Alvinerie *et al.*, 1989
Methylprednisolone	n.d.	✔	Increased plasma clearance	Alvinerie *et al.*, 1989
Ampicillin	—	—	No change	Tufenkji *et al.*, 1991
Pentobarbital	✔	✔	Reduced plasma clearance; increased sleep time	Tufenkji *et al.*, 1991
Albendazole	—	—	No change in sulphoxidation	Galtier *et al.*, 1991a
Albendazole	—	✔	Reduced conversion of sulphoxide to sulphone	Galtier *et al.*, 1991a
Albendazole	✔	✔	Reduced clearance of plasma sulphone	Galtier *et al.*, 1991a
Erythromycin	✔	—	Reduced plasma clearance	Burrows *et al.*, 1992
Oxytetracycline	—	—	No change	Burrows *et al.*, 1992
Prednisolone	—	✔	Increased plasma clearance	Alvinerie *et al.*, 1993
Rafoxanide	—	n.d.	No change	Benchaoui and McKellar, 1993
Mebendazole	—	✔	Increased plasma residence time	Galtier *et al.*, 1994b
Thiabendazole	—	—	No change	Galtier *et al.*, 1994b

n.d. not determined; — no significant effect; ✔ significant effect observed.

Table 6.5. Effects of infection with *F. hepatica* on enzymes of xenobiotic metabolism in sheep.

Enzyme	Substrate	<56 days	>56 days	Effect	Reference
Phase I metabolism					
P450	—	✔	✔	Decreased total content	Galtier *et al.*, 1986b, 1991a, 1993; Jemli *et al.*, 1994; Lenton *et al.*, 1996
		—	—	No change	Ferre *et al.*, 1995
	Mebendazole (MBZ)	n.d.	✔	Decreased NADPH–MBZ reductase activity	Galtier *et al.*, 1994b
N-demethylase	Aminopyrine	—	✔	Decreased activity	Galtier *et al.*, 1986b, 1993; Jemli *et al.*, 1994
	Benzphetamine	—	✔	Slightly decreased activity	Galtier *et al.*, 1986b, 1993; Jemli *et al.*, 1994
	Bromhexine	n.d.	4	Decreased activity	Galtier *et al.*, 1993
	Chlorpheniramine	n.d.	✔	Decreased activity	Galtier *et al.*, 1993
	Chlorpromazine	n.d.	✔	Decreased activity	Galtier *et al.*, 1993
	Ephedrine	n.d.	✔	Decreased activity	Galtier *et al.*, 1993
	Erythromycin	n.d.	✔	Decreased activity	Galtier *et al.*, 1993
	Imipramine	n.d.	✔	Decreased activity	Galtier *et al.*, 1993
	Spiramycin	n.d.	✔	Decreased activity	Galtier *et al.*, 1993
	Ethylmorphine	n.d.	✔	Decreased activity	Jemli *et al.*, 1994
Hydroxylase	Aniline	—	✔	Slightly decreased activity	Galtier *et al.*, 1986b
O-deethylase	Ethoxycoumarin	✔	✔	Decreased activity	Galtier *et al.*, 1986b
Sulphonation	Albendazole sulphoxide	✔	—	Decreased activity	Galtier *et al.*, 1991a
Cytochrome b_5	—	✔	✔	Decreased total content	Galtier *et al.*, 1986b
	—	—	✔	Increased total content	Jemli *et al.*, 1994
NADPH–cytochrome *c* reductase	—	—	—	No significant change	Galtier *et al.*, 1986b

Table 6.5 (*continued*).

Enzyme	Substrate	<56 days	>56 days	Effect	Reference
Phase II metabolism					
UDP–glucuronosyl transferase	*p*-Nitrophenol	—	—	No change	Galtier *et al*., 1986b
	Chloramphenicol	—	—	No change	Galtier *et al*., 1986b
	Bilirubin	—	—	No change	Ferre *et al*., 1995
Glutathione *S*-transferase	1-Chloro-2,4-dinitrobenzene	—	✔	Decreased activity	Galtier *et al*., 1986b
γ-Glutamyl transferase		—	✔	Increased activity	Galtier *et al*., 1986

n.d. not determined; — no significant effect; ✔ significant effect observed.

et al., 1996). Decreased activities of P450-dependent activities with a variety of substrates are evident at both parenchymal and biliary stages of the infection (Table 6.5). No change in activity was observed for the Phase II enzyme UDPGT, whereas GST exhibited decreased activity during the biliary stage. A significant bilirubinaemia has been reported in the parenchymal and early biliary stages in sheep, accompanied by a large increase in the proportion of conjugated bilirubin (Ferre *et al.*, 1995). Thus, conjugation activity appears unaffected *in vivo* and any bilirubinaemia might be a result of the decline in bile secretion evident at this stage. However, a significant bilirubinaemia was observed in sheep during the later biliary stage also (Prache and Galtier, 1990), when the rate of secretion of bile is reported to be normal (Ferre *et al.*, 1995).

There have been no investigations of changes in the specific isoforms/isoenzymes of biotransformation enzymes in infected sheep. A pharmacokinetic study of antipyrine by Tufenkji *et al.* (1988) suggested selectively decreased formation of the 4-hydroxylated product, compared with other products of P450 activity, which could be indirect evidence for differential effects on different isoforms of P450. To date the presence only of members of the CYP2B and CYP3A families has been positively identified in sheep liver (Galtier and Alvinerie, 1996).

Calves

Unlike sheep, the disposition of antipyrine and erythromycin was unchanged during the biliary stage in calves whereas plasma clearance of oxytetracycline increased (Burrows *et al.*, 1992) (Table 6.6). Oxytetracycline is not normally metabolized to any great extent by the liver and is usually excreted primarily in the urine. Its increased elimination was attributed to the severe cholangitis that occurs in the chronic infection in calves which, if it led to increased bile flow, could cause more rapid biliary elimination of the drug.

Phases I and II enzymatic activities are also compromised in infected cattle (see Table 6.7). In common with sheep and rats, spectroscopic studies have shown decreased concentrations of both P450 and cytochrome b_5 in hepatic microsomal preparations, during both the parenchymal and biliary stages (Maffei Facino *et al.*, 1984). NADPH–P450 and NADPH–b_5 reductase activities also decreased at these times, as did P450-based *N*-demethylase, hydroxylase and *O*-demethylase activities. Nitroreductase activity with the anthelmintic nitroxynil as substrate also declined, indicating a potentially

Table 6.6. Effects of infection with *F. hepatica* on the pharmacokinetics of xenobiotics in calves.

Drug	<84 days	>84 days	Effect	Reference
Antipyrine	n.d.	—	No change	Burrows *et al.*, 1992
Erythromycin	n.d.	—	No change	Burrows *et al.*, 1992
Oxytetracycline	n.d.	✔	Increased plasma clearance	Burrows *et al.*, 1992

n.d. not determined; — no significant effect; ✔ significant effect observed.

Table 6.7. Effects of infection with *F. hepatica* on enzymes of xenobiotic metabolism in calves.

Enzyme	Substrate	<84 days	>84 days	Effect	Reference
Phase I metabolism					
P450	—	✔	✔	Total content decreased	Maffei Facino *et al.*, 1984
Cytochrome b_5	—	✔	✔	Total content decreased	Maffei Facino *et al.*, 1984
NADPH–P450 reductase	—	✔	✔	Decreased activity	Maffei Facino *et al.*, 1984
NADPH–b_5 reductase	—	✔	✔	Decreased activity	Maffei Facino *et al.*, 1984
Nitroreductase	Nitroxynil	✔	✔	Decreased activity	Maffei Facino *et al.*, 1984
N-demethylase	Aminopyrine	✔	✔	Decreased activity	Maffei Facino *et al.*, 1984
Hydroxylase	Aniline	✔	✔	Decreased activity	Maffei Facino *et al.*, 1984
O-demethylase	*p*-Nitroanisole	✔	✔	Decreased activity	Maffei Facino *et al.*, 1984
Phase II metabolism					
UDP–glucuronosyl	*p*-Nitrophenol	✔	✔	Decreased activity	Maffei Facino *et al.*, 1985
transferase	Oxyclozanide	✔	✔	Decreased activity	Maffei Facino *et al.*, 1985

n.d. not determined; — no significant effect; ✔ significant effect observed.

reduced capacity of the host to detoxify this compound. This is important because the maximum tolerated dose of nitroxynil is only threefold higher than the therapeutic dose, so there is increased potential for toxicity in infected animals. The only Phase II enzyme examined in infected cattle to date is UDPGT, the activity of which was significantly decreased at both parenchymal and biliary stages (Maffei Facino *et al.*, 1985). The ability of hepatic microsomal preparations to conjugate the anthelmintic oxyclozanide with glucuronic acid was severely reduced; if this reflects the situation *in vivo*, there could be potential toxicity and residue problems in cattle treated with this compound, which is normally excreted in bile in the conjugated form.

Acknowledgements

We wish to thank Professor T. Rothwell and Sally Pope for their generous contributions to Fig. 6.1. C.A. Behm acknowledges the support of the Australian Research Council.

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7 Mechanisms of Fasciolicide Action and Drug Resistance in *Fasciola hepatica*

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Introduction

The purpose of this chapter is to review our understanding of the mechanism of action of fasciolicides used for the treatment of liver fluke infections. Also, to assess the extent to which resistance has developed to existing fasciolicides and strategies that can be adopted to overcome the problem. The fasciolicides in common use today (or in the more recent past) fall into five main chemical groupings:

1. *Halogenated phenols*, e.g. bithionol ('Bitin', 'Actamer'), hexachlorophene (formerly 'Bilevon', now obsolete), niclofolan ('Bilevon'), nitroxynil ('Trodax').
2. *Salicylanilides*, e.g. brotianide ('Dirian'), closantel ('Flukiver', 'Seponver', 'Supaverm', 'Cosicare'), oxyclozanide ('Nilzan', 'Zanil'), rafoxanide ('Flukanide', 'Ranizole').
3. *Benzimidazoles*, e.g. albendazole ('Valbazen'), mebendazole ('Telmin', 'Vermox', 'Supaverm'), triclabendazole ('Fasinex').
4. *Sulphonamides*, e.g. clorsulon ('Curatrem', 'Ivomec F', 'Ivomec Plus').
5. *Phenoxyalkanes*, e.g. diamphenethide ('Coriban').

The chemical structures of these fasciolicides are presented in Fig. 7.1.

The experimental data for each group of fasciolicides will be collected together to determine how close we are to defining their primary mode of action. Wherever possible, concentrations of fasciolicides used in *in vitro* studies have been expressed in terms of μg ml^{-1}, in order to permit direct comparison with the maximum blood levels of drug circulating *in vivo*. Due to the constraints of space, only the most salient points will be made. For a more detailed discussion of fasciolicidal action, the reader is referred to Fairweather (1997).

Before discussing the mode of action of individual fasciolicides, brief consideration will be given to the chemotherapy of *Fasciola* infections.

Fasciolosis (ed. J.P. Dalton)

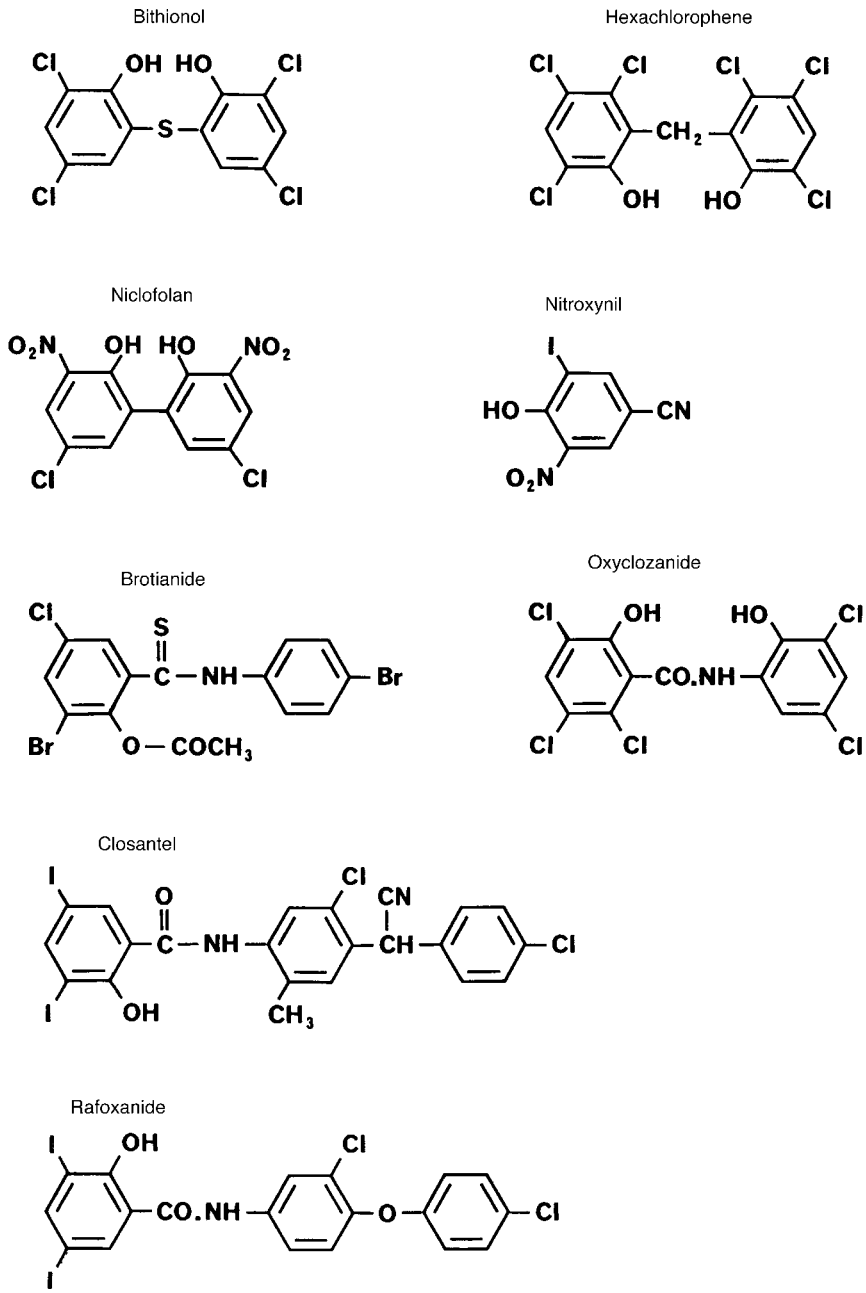
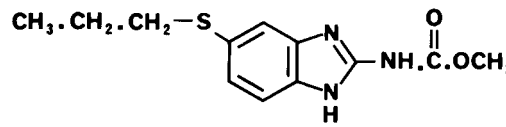
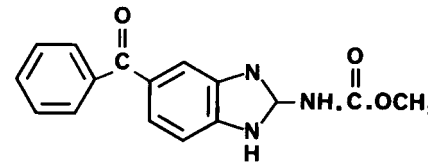


Fig. 7.1 (*and opposite*)**.** Chemical structures of fasciolicides.

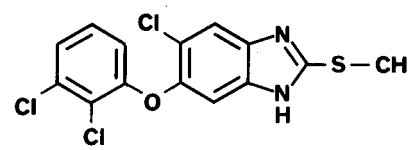
Albendazole



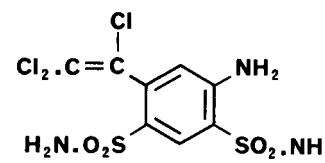
Mebendazole



Triclabendazole



Clorsulon



Diamphenethide

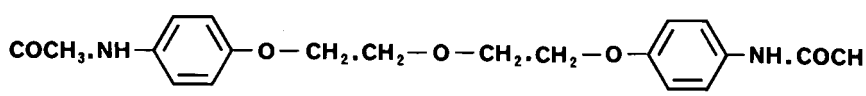


Fig. 7.1 (*continued*).

Chemotherapy

Data pertaining to the efficacy and safety of fasciolicides are presented in Tables 7.1 and 7.2. The use of the most effective drugs with efficacy against both mature and early immature fluke is essential. It is of particular importance in an efficient strategic control programme, with a minimum number of annual treatments and an expected seasonal elimination of pasture

Table 7.1. Comparative efficiency of drugs against *Fasciola*.

Anthelmintic	Route of application	Recommended dose rate (mg kg^{-1})		Maximum tolerated dose in sheep (mg kg^{-1})	Safety index at recommended dose rate in sheep	Minimum age of fluke in weeks, efficiency ≥90%	
		Sheep	Cattle			Sheep	Cattle
Hexachlorophene	Oral	15	20	40	2.6	12	12
Bithionol	Oral	75	30	75	1	>12	>12
Oxyclozanide	Oral	15	13–16	60	4.0	12	>14
Niclofolan	Oral	4	3	12	3.0	12	>12
	sc	NR	0.8			NR	>12
Nitroxynil	sc	10	10	40	4.0	8	10
Brotianide	Oral	5.6	NR	27	4.8	12	NR
Rafoxanide	Oral	7.5	7.5	45	6.0	6	12
	sc	NR	3			NR	12
Closantel	Oral	7.5–10	NR	40	4.0	6–8	NR
	sc	NR	3			NR	>12
Diamphenethide (acemidophene, CIS)	Oral	80–120	100	400	3.3–5.0	1 day to 6 wk	1 day to 7 wk
Albendazole	Oral	4.75	10	30	8	>12	>12
Triclabendazole	Oral	10	12	200	20–40	1	1
Clorsulon	Oral	—	7	100	5		8
	sc	—	2				>12

sc = subcutaneous; NR = not recommended.

Table 7.2. Efficiency spectrum of drugs at recommended dose rates against *Fasciola hepatica* in sheep.

	Age of fluke in weeks													
Drug	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Bithionol, Hexachlorophene, Oxyclozanide, Niclofolan, Albendazole, Clorsulon+Ivermectin (inj)										50–70%		80–99%		
Clorsulon (oral)								90–99%						
Nitroxynil, Closantel							50–90%			91–99%				
Rafoxanide				50–90%			91–99%							
Triclabendazole		90–99%		99–100%										
Diamphenethide	100–91%						80–50%							

contamination for extended periods. The reduction of treatment frequency may prevent development of drug resistance, which will be discussed later. Drugs which only work against adult fluke, such as albendazole, oxyclozanide and clorsulon at the low dose rate combined with ivermectin, are unsuitable for effective chemoprophylaxis. Strategies for the treatment and prophylaxis of *Fasciola* infections based on epidemiological data have been discussed by Boray (1997) and Malone (1997).

Suppression of *F. hepatica* infections has been attempted using treatments every 6 weeks with rafoxanide from spring to autumn for 2 years resulting in 90% reduction of infection and reduction of the infection rate in snails (Armour *et al.*, 1973). Good results were achieved with increased dose rates of rafoxanide given five times between June and January/February in a heavily infected area in Scotland and the infection level remained very low for some time after the programme. It was predicted that virtual eradication of fasciolosis could be achieved using the regimen for 3 to 4 years (Whitelaw and Fawcett, 1977, 1981).

In a field experiment in New Zealand, sheep suffering from heavy acute, subacute and chronic infections were treated with triclabendazole at 10 mg kg^{-1}, with an efficacy of 99.8%. Subsequently, all sheep, cattle and horses were treated on the property every 8 to 11 weeks for a period of 14 months. No patent infection could be detected and the contamination of the pastures was reduced to a negligible level for a period of 12 months after the last treatment (Boray, 1986). It was concluded that effective treatment during the prepatent period for an extended duration could eliminate *Fasciola* infection or reduce contamination to a very low level, requiring less frequent treatments for a considerable time.

Frequent treatments of sheep were carried out in the field with triclabendazole by Fawcett (1990) in a strategic pattern, between June and January, for a period of 5 years and reduced the prevalence of infection from 49% to 1%. Eight-week treatments with triclabendazole between April and October did not reduce infection in the first year but achieved a 70–75% reduction when the treatments commenced in February and were carried out four times a year for 2 years (Taylor *et al.*, 1994).

A small flock of sheep, initially suffering from clinical subacute fasciolosis, was treated every 8 weeks for a period of 3 years with 10 mg kg^{-1} triclabendazole in a pasture contaminated only by the surviving flukes reaching early maturity. The infection was reduced to a negligible level but the surviving fluke population developed a degree of resistance to triclabendazole (Boray, 1990, 1997).

In comparative efficacy trials it has been shown that triclabendazole, rafoxanide and closantel exert an appreciable retardation of the development of immature flukes in treated sheep. The level of retardation was directly related to the level of efficacy of the drugs against early immature flukes and considerably extended the prepatent period of the surviving fluke populations (Boray, 1997). This phenomenon has a great influence on the success of strategic control programmes. Since triclabendazole is highly effective against flukes aged 1 week or older, the drug is most suitable for reducing the

pasture contamination for extended periods. Retarded development of flukes after treatment with closantel has been reported (Maes *et al*., 1985, 1990) and a lower degree of retardation also observed in cattle after treatment with clorsulon (Malone *et al*., 1984; Yazwinski *et al*., 1985).

Retardation of immature flukes which survive treatment appears to be applicable to all anthelmintics and the degree of retardation depends on the efficacy of the drugs against the immature stages. This phenomenon has a great advantage in strategic control by reducing early pasture contamination with eggs. Unfortunately, recent studies to be discussed later have demonstrated that resistance may develop to most anthelmintics, mainly affecting the flukes aged 6 weeks or younger (Boray, 1990). It appears that during the early immature stages selection for resistance will occur rapidly if eradication of *Fasciola* spp. is attempted with frequent treatments and this method of control is not desirable. Less frequent strategic treatments with a possible yearly rotation of anthelmintics or anthelmintic combinations which are effective against both immature and adult flukes will provide the best method of successful control of fasciolosis.

Mode of Action Data

Group 1: halogenated phenols

These drugs contain a phenolic group and bear a structural similarity to 2,4-dinitrophenol (2,4-DNP), a known uncoupler of oxidative phosphorylation in mammalian systems. This fact has been used to support the idea that they act in a similar way in fluke tissues (Van Miert and Groeneveld, 1969; Corbett and Goose, 1971; Yorke and Turton, 1974; Campbell and Montague, 1981). However, as is evident from Table 7.3, experimental studies were carried out in systems (such as isolated tapeworm or mammalian mitochondria) that are not related to the fluke or at concentrations far higher than those which occur *in vivo*. Consequently, the results may have little bearing on the *in vivo* activity of the drugs. Moreover, oxidative phosphorylation makes a minor contribution to energy production by flukes, accounting for only 10% of the total carbohydrate metabolized (Lloyd and Barrett, 1983). It can generate a greater percentage of ATP production – up to 20% in aerobic conditions in the adult fluke *in vitro* (Tielens *et al*., 1984). Therefore, most of ATP synthesis in the mitochondria will take place by anaerobic processes, even under aerobic conditions.

Phenolic fasciolicides have also been shown to inhibit certain key enzymes in the pathway from phosphoenolpyruvate to propionate:malate dehydrogenase (hexachlorophene; Lwin and Probert, 1975) and succinic dehydrogenase (bithionol, hexachlorophene and niclofolan; Panitz and Knapp, 1970). Again, the concentrations used were far higher than those attained *in vivo* following drug treatment and so it is difficult to assess the true physiological significance of these findings (see Table 7.3).

Perhaps of greater importance is the fact that the phenols induce a rapid (<3 h) spastic paralysis of the fluke at concentrations comparable to effective

Table 7.3. A comparison of studies on the mode of action of halogenated phenols against the liver fluke, *Fasciola hepatica* (studies *in vitro* unless stated otherwise; concentrations expressed as $\mu g\ ml^{-1}$).

Parameter tested	Niclofolan	Bithionol	Hexachlorophene	Nitroxynil
Maximum blood level *in vivo*	4.5[1] (dose 3 mg kg^{-1} in sheep and cattle)			80[2] (dose: 10 mg kg^{-1} in sheep)
Minimum concentration for paralysis within 3 h[3]	1.0 (spastic paralysis)	1.0 (spastic paralysis)	1.0 (spastic paralysis)	50 (spastic paralysis)
Stimulation of oxygen uptake by *Fasciola hepatica*[4]			0.41–4.07	2.90–29.0
Induction of ATPase activity of rat liver mitochondria[5]	0.35–34.51		0.41–40.69	0.29–29.0
Minimum concentration for uncoupling of rat liver mitochondria[4,7]			0.24–0.33[4] 1.22–4.07[7]	7.83–10.15[4] 8.70–29.0[7]
Minimum concentration for maximum uncoupling of *Hymenolepis diminuta* mitochondria[6]				130.5
Inhibition of malate dehydrogenase activity in *F. hepatica*[8]			406.9	None at 290.0
Inhibition of succinic dehydrogenase activity in *F. hepatica*[9]	20.71–153.57	20.30–203.0		
Disruption of spermatogenesis *in vivo*[10]	Within 4 h (dose: 5.4 mg kg^{-1} in rabbit)		Within 4 h (dose: 4.0 mg kg^{-1} in rabbit)	Within 4 h (dose: 20 mg kg^{-1} in rabbit)
Decrease in egg production *in vivo*[11,12]		After 3 or more single doses of 10 mg kg^{-1} in rat[11]		dose: 10 mg kg^{-1} in sheep[12]

References: 1. Flucke *et al.* (1969); 2. Parnell (1970); 3. Fairweather *et al.* (1984); 4. Corbett and Goose (1971); 5. Van Miert and Groeneveld (1969); 6. Yorke and Turton (1974); 7. Campbell and Montague (1981); 8. Lwin and Probert (1975); 9. Panitz and Knapp (1970); 10. Stammers (1975b); 11. Dawes (1966); 12. Stammers (1976).

blood levels *in vivo* (see Table 7.1). It is believed that the action is not due to uncoupling *per se*, but rather to changes in the permeability of the muscle membrane to certain ions, particularly K^+: for a more detailed explanation of the rationale behind this view, see Holmes and Fairweather (1985) and Fairweather (1997). However, the precise nature of this neuromuscular action remains to be defined.

Few studies have been carried out on morphological changes in the fluke induced by phenolic compounds. Vacuolation of the cuticle (viz. tegument) leading to widespread stripping of the tegument has been observed in histological sections following treatment with bithionol *in vivo*; the dorsal surface was more seriously affected than the ventral surface (Dawes, 1966). A posterior–anterior spread of surface damage has been described for bithionol and for hexachlorophene (Gusel'nikova, 1974).

Disruption of the reproductive system – in particular the testes – has been described for each of the phenols *in vivo*. With nitroxynil, niclofolan and hexachlorophene, disruption is rapid, occurring within 4 h (Stammers, 1975b). For both nitroxynil and bithionol, the testis was more severely affected than other reproductive organs, such as the ovary and vitellaria, although in each case egg production declined (Dawes, 1966; Stammers, 1976). The cause of the latter effect differs for the two drugs, being due to disruption of oogenesis for nitroxynil (Stammers, 1976) and disruption of vitellogenesis for bithionol (Dawes, 1966). The precise mechanism behind the disruption of spermatogenesis and other reproductive activities is not known, although for nitroxynil it has been linked to its potential uncoupling action leading to less energy being available for cell division (Stammers, 1975a). However, for these fasciolicides it may simply be an illustration of the axiom advanced by Dawes (1968) that in a stress situation 'flukes which are threatened by drug action on the host will tend first to sacrifice non-vital organs and to conserve vital organs', presumably in an attempt to save energy and therefore aid survival. So, for these compounds, changes to the reproductive organs may be an indirect or secondary effect of some other primary drug action.

Group 2: salicylanilides

Metabolic and motility data for the salicylanilides rafoxanide, oxyclozanide and closantel are summarized in Table 7.4. For these fasciolicides there is more direct evidence for an uncoupling of oxidative phosphorylation in the fluke. With oxyclozanide, for example, some metabolic changes induced *in vitro* are characteristic of an uncoupler – stimulation of oxygen consumption, increases in respiratory intermediates, increase in oxaloacetate:malate ratio and a rise in succinate production (Edwards *et al.*, 1981a; see also Table 7.4). However, there was no indication of any decline in ATP levels in the fluke and the authors concluded that the primary action may be a neurotoxic one (Edwards *et al.*, 1981a). Oxyclozanide causes a rapid (within 0.5 h) spastic paralysis of the fluke at a concentration comparable to maximum blood level attained *in vivo* following drug treatment (Fairweather *et al.*, 1984). This point will be returned to later.

Table 7.4. A comparison of studies on the effects of salicylanilides against motility and energy metabolism of *Fasciola hepatica* (concentrations expressed as $\mu g\ ml^{-1}$).

Parameter tested	Rafoxanide	Oxyclozanide	Closantel
Maximum blood level *in vivo*	18.6 (dose: 7.5 mg kg^{-1} in sheep)	10–15[2] (dose: 10 mg kg^{-1} in cattle)	45–55[3] (dose: 10 mg kg^{-1} in sheep and cattle) 35.7[4] (dose: 7.5 mg kg^{-1} in sheep)
Minimum concentration for paralysis *in vitro* within 3 h[5,6]	1.0[5] (spastic paralysis)	1.0[5] (spastic paralysis)	1.0[6] (spastic paralysis)
Stimulation of oxygen uptake by *Fasciola hepatica in vitro*[7,8]		20–40% at 0.40–4.02[7] 23% at 0.5[8]	
Induction of ATPase activity of rat liver mitochondria *in vitro*[9]		318% at 4.02[9]	
Minimum concentration for uncoupling of rat liver mitochondria *in vitro*[7,10]	0.63–1.88[10]	1.21–1.61[7] 0.40–1.20[10]	
Stimulation of rat liver mitochondrial respiration *in vitro*[9]		222% at 40.2	
Minimum concentration for maximum uncoupling of *Hymenolepis diminuta* mitochondria *in vitro*[11]	43.82	96.36	
Increased glucose uptake[12,13]	*In vitro*: not after 3 h at 400[12]		*In vivo* (dose: 5 mg kg^{-1}) *In vitro*: not after 6 h at 1.32[13]
Decreased glycogen content[1,8,12–14]	*In vivo*: 54% after 24 h (dose: 7.5 mg kg^{-1})[1]; significant decrease within 4 h (dose: 9 mg kg^{-1})[14]; *in vitro*: not after 48 h at 400[12]	*In vitro*: not after 7 h at 0.1–0.5[8]	*In vivo* (dose: 5 mg kg^{-1}) *In vitro*: 20% after 6 h at 1.32[13]

Changes in levels of respiratory intermediates[1,8,13–15]	*In vivo*: decreased pyruvate (63%) after 24 h; other intermediates increase within 4 h but return to normal by 24 h (dose: 7.5 mg kg^{-1})[1] *In vivo*: increased oxaloacetate and decreased malate after 24 h; other intermediates increase but return to normal by 24 h (dose: 9 mg kg^{-1})[14]	*In vitro*: increased pyruvate and oxaloacetate, also glucose 6-phosphate (G6P) and phosphoenolpyruvate (PEP) after 3–5.5 h at 0.25[8]	*In vivo*: decreased G6P (42%) and malate (73%) after 12 h (dose: 5 mg kg^{-1})[13] *In vitro*: increased pyruvate (473%), decreased G6P (35%) and malate (66%) after 3 h at 1.32[13] *In vitro*: decreased G6P (27%) after 4 h at 3.3 (39%) after 1 h at 33.0[15]
Increase in oxaloacetate/malate ratio[8,13,14]	*In vivo* (dose: 9 mg kg^{-1})[14]	*In vitro*: 67% after 5.5 h at 0.25[8]	*In vitro*: 513% after 12 h at 1.32[13]
Changes in levels of respiratory end products[1,8,12,13]	*In vivo*: increased succinate (71%) after 24 h but total unchanged (dose: 7.5 mg kg^{-1})[1] *In vitro*: increased succinate and decreased propionate; total decreased (11%) after 9 h at 400[12]	*In vitro*: increased succinate after 3–5.5 h at 0.25[8]	*In vivo*: increased succinate and acetate (dose: 5 mg kg^{-1})[13] *In vitro*: increased succinate (491%) and lactate (55%) after 3 h at 1.32[13]
Reduction in ATP and total nucleotide levels[1,8,12–17]	*In vivo*: 28–40% and 14–15%, respectively, after 24 h (dose: 7.5 mg kg^{-1})[1,16]; 18% decrease in ATP levels after 4 h and 61% decrease after 24 h (dose: 9 mg kg^{-1})[14] *In vitro*: 27–29% decrease in ATP levels after 3–9 h at 400[12,16]	*In vitro*: not after 5.5 h at 0.25[8]	*In vivo*: 53% and 28%, respectively, after 12 h (dose: 5 mg kg^{-1})[13]; 41% decrease in ATP levels after 4 h (dose: 5 mg kg^{-1})[17] *In vitro*: 20% decrease in ATP levels after 3 h at 1.32; 25% decrease in ATP levels after 20 h at 3.3 and 20% after 4 h at 33[15]

Table 7.4 (*continued*).

Parameter tested	Rafoxanide	Oxyclozanide	Closantel
Stimulation of ATPase activity of fluke mitochondria *in vitro*[17]			0.33 (50% inhibition at 66.31)
Increase in cytochemically demonstrable mitochondrial ATPase activity[19]			*In vivo* (time and dose unknown)
Inhibition of malate dehydrogenase activity in *F. hepatica in vitro*[18]	none at 626.0	80–100% at 401.5	

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2. D.A.D. McIntosh, Macclesfield, 1982 (personal communication)
3. Closantel Information Booklet (Janssen Animal Health Ltd) (1986)
4. Mohammed-Ali and Bogan (1987)
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6. Skuce (1987)
7. Corbett and Goose (1971)
8. Edwards *et al.* (1981a)
9. Veenendaal and De Waal (1974)
10. Campbell and Montague (1981)
11. Yorke and Turton (1974)
12. Cornish and Bryant (1976)
13. Kane *et al.* (1980)
14. Prichard (1978)
15. Rohrer *et al.* (1986)
16. Bryant *et al.* (1976)
17. Van den Bossche *et al.* (1979)
18. Lwin and Probert (1975)
19. Verheyen *et al.* (1979)

Comparable data have been obtained for rafoxanide from both *in vitro* and *in vivo* studies: viz. changes in respiratory intermediates, increase in oxaloacetate:malate ratio, changes in end products and decreased ATP synthesis (Bryant *et al.*, 1976; Cornish and Bryant, 1976; Cornish *et al.*, 1977; Prichard, 1978; see also Table 7.4). The major changes are essentially long term in nature and coincide with (or follow the onset of) removal of the flukes *in vivo* (Cornish *et al.*, 1977; Prichard, 1978). There are some differences between *in vivo* and *in vitro* observations: e.g. depletion of glycogen reserves *in vivo* but not *in vitro* (Cornish and Bryant, 1976; Cornish *et al.*, 1977). This has been attributed to the presence of a ready glucose supply in the culture medium and the passive diffusion of glucose into the fluke obviating the need to draw on glycogen reserves *in vitro*. In contrast, *in vivo*, detachment of flukes and reduced activity lead to cessation of feeding and a greater necessity to call on glycogen deposits (Behm and Bryant, 1979). The effects of rafoxanide on ATP and other nucleotide levels, while important in their own right, also impact on respiratory metabolism because of the modulatory actions of nucleotides on respiratory enzymes and consequently the flux through the metabolic pathways (Behm and Bryant, 1979).

There is much evidence, too, from both *in vivo* and *in vitro* studies, that closantel acts as an uncoupler in the fluke. An increased carbon flow along energy-producing pathways is indicated by increased glucose uptake, decreased glycogen content, increased end-product formation (especially succinate), changes in respiratory intermediates, increase in oxaloacetate:malate ratio, decreased ATP synthesis and changes in mitochondrial ATPase activity (Van den Bossche *et al.*, 1979; Kane *et al.*, 1980; Rohrer *et al.*, 1986; see also Table 7.4). The *in vitro* studies on closantel are a welcome rarity among *in vitro* studies involving fasciolicides in that they were carried out at concentrations well below the maximum blood level attained *in vivo* following drug treatment (for details see Table 7.4). An alternative suggestion has been put forward for the action of closantel, namely, that the initial effect is not on oxidative phosphorylation, but rather on glycolysis, specifically glucose 6-phosphate accumulation, since decreases in the level of the latter *precede* any drop in ATP levels. It was also suggested that the reductions in ATP levels may be correlated with the 'death' of the fluke (Rohrer *et al.*, 1986). However, the fall in glucose 6-phosphate accumulation may be due to acidification of the tegument: a rapid (within 10–20 min) decrease in pH and lowering of membrane potential occurs at a closantel concentration of 0.66 µg ml^{-1} (Pax and Bennett, 1989).

The three major salicylanilides cause a rapid spastic paralysis of *F. hepatica* at very low levels (Fairweather *et al.*, 1984; Skuce, 1987; Table 7.4). The effect is very similar to that induced by uncouplers such as CCCP and FCCP (Holmes and Fairweather, 1985). However, the raised muscle tone may not be due directly to uncoupling, but may simply reflect an increase in calcium ions in the muscle cells. This may result from the release of Ca^{2+} from internal stores, from an ionophore effect across the plasma membrane or organelle membrane, or from a membrane-perturbing effect that alters membrane permeability to ions (for an additional discussion of these ideas see

Fairweather *et al*., 1984; Holmes and Fairweather, 1985; Fairweather, 1997). The rapidity of the neuromuscular actions of the salicylanilides may make them more significant than any disruption of energy metabolism because the knock-on effect of paralysis, leading to detachment *in vivo*, is a cessation of feeding. Consequently, the fluke enters a state of starvation and this will impose a severe metabolic stress on it. The fluke has to draw on its energy reserves in an attempt to survive, and this may account, at least in part, for some of the biochemical changes observed and the altered fluxes along respiratory pathways; hence, they may be secondary to a more direct neuromuscular effect.

Morphological studies on the changes induced by salicylanilides are restricted to closantel. The SEM data on the *in vivo* effects are confusing: a posterior–anterior spread of damage has been observed in flukes from closantel-treated sheep (and attributed to a posterior accumulation of drug in the fluke's gut) (Verheyen *et al.*, 1980), whereas loss of tegument was observed over large areas of the anterior and posterior regions of flukes from closantel-treated rats, with the intervening midbody region remaining normal (Skuce and Fairweather, 1990). Internally within the tegument a swelling of the basal infolds has been observed in studies of both the *in vivo* and *in vitro* effects of closantel (Verheyen *et al*., 1980; Skuce, 1987; Skuce and Fairweather, 1990). Further swelling of the infolds leading to their detachment from the basal plasma membrane will account for the loss of tegument observed with SEM. The swelling may have a metabolic basis, resulting from restriction of the energy supply to the ATPase-driven ion pumps associated with the tegumental membranes (Skuce and Fairweather, 1990). A number of morphological changes evident in the tegumental syncytium and underlying cell bodies are compatible with an uncoupling type of metabolic inhibition, viz. deformed mitochondria, vesiculation and reduction of the Golgi complex, dilation of the GER cisternae and reduction in numbers of secretory bodies (Verheyen *et al*., 1980; Skuce, 1987; Skuce and Fairweather, 1990). Similar changes take place in the gut, together with increased autophagy, shedding of gut lamellae and eventually 'complete desquamation of the necrotized epithelium' (Verheyen *et al*., 1980) which indicate a state of stress and/or starvation. Increased autophagic activity is also evident in the vitelline cells, and a marked reduction of glycogen deposits occurs in them and in the parenchymal cells (Skuce and Fairweather, 1990). The latter observation suggests that carbohydrate reserves have been diverted into the glycolytic pathway to maintain energy production in the face of uncoupling of oxidative phosphorylation. Uncoupling leads to an increased carbon flux along energy-producing pathways, in an attempt by the fluke to produce more energy. Furthermore, the hydrolysis of ATP is promoted and electron transfer will continue at an uncontrolled rate until the respiratory substrate is exhausted. Consequently, many of the morphological data support the concept of a metabolic action for closantel, though they may also indicate the consequences of a stress (starvation) response induced by an as yet undefined neuromuscular action. There may even be a synergistic association between the two effects, each magnifying the extent of the other. For more

detail on the timing of the morphological changes induced by closantel see Fairweather (1997).

Apart from the vitelline changes induced by closantel, the only other study showing the effects of salicylanilides on the reproductive system was that describing the disruption of spermatogenesis by rafoxanide, an effect that was evident within 4 h *in vivo* (Stammers, 1975b).

Group 3: benzimidazoles

Comparative data for triclabendazole, albendazole and mebendazole is presented in Table 7.5. Triclabendazole (TCBZ) has an unusual structure for a benzimidazole in that it contains a chlorinated benzene ring but has no carbamate group (Bennett and Köhler, 1987; Lipkowitz and McCracken, 1991). It has an unusual activity, too, in that unlike other broad-spectrum benzimidazoles which show only marginal activity against the liver fluke, its efficacy appears to be restricted to *F. hepatica*, *F. gigantica* and *Fascioloides magna*. Triclabendazole lacks activity against nematodes and cestodes and against other trematodes, including *Dicrocoelium dendriticum*, *Schistosoma mansoni* and *Paramphistomum* spp. (Wolff *et al*., 1983; Guralp and Tinar, 1984; Coles, 1986). It is a significant fasciolicide because it displays high efficacy against both adult and juvenile flukes (Boray *et al*., 1983; Smeal and Hall, 1983; Turner *et al*., 1984). Whether this unusual activity of TCBZ is achieved via a mechanism different from that of other benzimidazoles will be discussed below.

The effects of benzimidazoles on fluke motility are essentially long term in nature and require concentrations far higher than those which are effective *in vivo*, so they probably have little relevance to their mode of action. There are interesting differences between certain benzimidazoles and their active metabolites. For example, albendazole produces a prolonged stimulation of motility before movement finally declines, while albendazole sulphoxide induces a gradual suppression of activity (Fairweather *et al*., 1984). A number of studies have shown that both TCBZ and its active sulphoxide metabolite (TCBZ-SX) induce a gradual suppression of activity (Fairweather *et al*., 1984; Coles, 1986; Bennett and Köhler, 1987). Immature flukes are more sensitive to TCBZ than adults (Bennett and Köhler, 1987). The effects on motility are paralleled by a gradual hyperpolarization of the tegumental membrane potential, although this was shown not to be due to an inhibition of ATPase-driven ion pumps (Bennett and Köhler, 1987). Surface membrane changes as observed by SEM occur fairly quickly (within 3 h) following TCBZ-SX treatment (albeit at higher concentrations) and it is difficult to envisage how the ion pumps would remain unaffected by prolonged exposure to the drug given the severe disruption evident (Stitt and Fairweather, 1993a).

With regard to potential disruption of respiratory pathways, there is some evidence for an uncoupling action of mebendazole. *In vitro*, end-product formation (especially succinate) is increased by 12 h and remains so through to 36–48 h, although glucose uptake and glycogen mobilization are not affected. Levels of a number of respiratory intermediates are decreased

Table 7.5. A comparison of studies on the mode of action of benzimidazoles against the liver fluke, *Fasciola hepatica* (concentrations expressed as μg ml^{-1}).

Parameter tested	Triclabendazole	Triclabendazole sulphoxide	Albendazole	Albendazole sulphoxide	Mebendazole
Maximum blood level *in vivo*[1–6]	27[1] (dose: 10 mg kg^{-1} in sheep)	13.3[2] (dose: 10 mg kg^{-1} in sheep)	0.05[3] (dose: 10 mg kg^{-1} in sheep)	3.2[3] (dose: 10 mg kg^{-1} in sheep) 0.35[4] (dose: 7.5 mg kg^{-1} in cattle)	0.006–0.117[5] (dose: 40 mg day^{-1} in man) 0.4[6] (dose: 40 mg kg^{-1} in rat) 0.224[7] (dose: 100 mg kg^{-1} in sheep)
High activity (>87.5%) against flukes from 1 to 12 weeks in age[8–10]	Dose: 5–10 mg kg^{-1}				
Activity against flukes as young as one-day-old in sheep[10]	Dose: 15 mg kg^{-1}				
Total immobilization of fluke *in vitro*[11]					
(i) adult	(i) 24 h at 9.0–18.0	(i) —			
(ii) 3-week-old juvenile	(ii) 24 h at 3.6; 6 h at 9.0	(ii) 24 h at 3.6			
Total immobilization of adult fluke *in vitro*[12]	24 h at 2.5				
Total immobilization of adult fluke *in vitro*[13]	>30 h at 100	>18 h at 100	>21 h at 100	>36 h at 100	12 h at 500
Lowering of resting tegumental membrane potential in 3-week-old juvenile flukes *in vitro*[11]	4 h at 3.6 24 h at 0.36–1.08	24 h at 0.36–3.6			

Inhibition of Na^+-, K^+-, Mg^{2+}- and Ca^{2+}-ATPases in adult fluke *in vitro*[11]	No inhibition at 18.0	No inhibition at 18.0		
Uncoupling of rat liver mitochondria *in vitro*[14]			0.165–6.6	
Respiratory control index effective concentration (RCI I_{50}) for uncoupling of rat liver mitochondrial oxidative phosphorylation *in vitro*[15]	6.48	3.38 (sulphone metabolite 0.39)		
Changes in glucose uptake[16]				*In vitro*: no change after 3 h at 400
Change in glycogen content[16,17]				*In vitro*: no change after 48 h at 400[16] *In vivo*: 44.8% decrease after 30 h (dose: 100 mg kg^{-1})[17]
Changes in levels of respiratory intermediates[16,17]				*In vitro*: decreases in various intermediates (e.g. G6P, malate) after 48 h at 400[16]

Table 7.5 (*continued*).

Parameter tested	Triclabendazole	Triclabendazole sulphoxide	Albendazole	Albendazole sulphoxide	Mebendazole
Changes in levels of respiratory intermediates[16,17]					*In vivo*: decreases in G6P (30%) and malate (17%) after 30 h (dose: 100 mg kg^{-1})[17]
Changes in levels of respiratory end products[11,16–18]	*In vitro*: increased acetate (157%) and propionate (164%) after 24 h at 3.6[11]		*In vitro*: no change after 24 h at 1.65[18]	*In vitro*: no change after 24 h at 1.81[18]	*In vitro*: increased succinate (14%) and decreased lactate (7%) after 36–48 h at 400[16] *In vivo*: increased lactate (83%) after 30 h (dose: 100 mg kg^{-1})[17]
Reduction in ATP and total nucleotide levels[16,17,19]					*In vitro*: 33–60% and 19–37%, respectively, after 36–48 h at 400[16,19] *In vivo*: 28% and 13%, respectively, after 30 h (dose: 100 mg kg^{-1})[17]
Inhibition of succinate decarboxylase system in *F. hepatica in vitro*[20]					36–43% at 38.39
Inhibition of colchicine binding to tubulin from adult flukes[11,21]	(i) *In vitro*: no inhibition at 3.6[21] (ii) *In vitro*: 86% inhibition at 3.6[11]		*In vitro*: inhibition at 1.65 *In vivo*: no inhibition after 12, 24 h (dose: 20 mg kg^{-1})[21]	*In vitro*: 25–75% after 5–15 min at 1.81[21]	*In vitro*: inhibition at 2.95[21]

Inhibition of protease enzyme secretion *in vitro*[11]		
(i) adult	(i) 12 h at 3.6–9.0	
(ii) 3-week-old juvenile	(ii) 6 h at 1.08–9.0	
Evidence of tegumental surface membrane changes *in vitro*[22]		
(i) adult		(i) 6 h at 50.0
(ii) 3-week-old juvenile		(ii) 3 h at 20.0
Evidence of internal tegumental changes *in vitro*[23]		
(i) adult		(i) 6 h at 15.0–50.0
(ii) 3-week-old juvenile		(ii) 3 h at 20.0
Disruption of secretory activity in tegumental cells *in vitro*[23]		
(i) adult		(i) 6 h at 15.0–50.0
(ii) juvenile		(ii) 3 h at 20.0
Disruption of spermatogenesis *in vitro*[24]		6 h at 15.0; 3 h at 50.0

Table 7.5 (*continued*).

Parameter tested	Triclabendazole	Triclabendazole sulphoxide	Albendazole	Albendazole sulphoxide	Mebendazole
Disruption of secretory activity in vitelline cells *in vitro*[25]		6 h at 50.0			
Disruption of vitellogenesis *in vitro*[25]		6 h at 50.0			
Inhibition of protein synthesis by adult flukes *in vitro*[26]		6 h at 15.0–50.0			
Inhibition of embryonation of fluke eggs[27]			LD_{50} 0.004		LD_{50} 0.005
(i) degenerative changes in reproductive organs *in vivo*[28] (ii) decreased hatchability of eggs[28]			(i) In ovary, testis and vitellaria after 39–40 days (dose: 10 mg kg^{-1}) (ii) After 1–7 days (dose: 10 mg kg^{-1})		

References: 1. R.J. Richards, Cambridge, 1983 personal communication; 2. Hennessy *et al.* (1987); 3. Marriner and Bogan (1980); 4. Prichard *et al.* (1985); 5. Karlaganis *et al.* (1979); 6. Van den Bossche *et al.* (1982); 7. Behm *et al.* (1983); 8. Boray *et al.* (1983); 9. Smeal and Hall (1983); 10. Turner *et al.* (1984); 11. Bennett and Köhler (1987); 12. Coles (1986); 13. Fairweather *et al.* (1984); 14. McCracken and Stillwell (1991); 15. Carr *et al.* (1993); 16. Cornish and Bryant (1976); 17. Rahman *et al.* (1977); 18. Fetterer and Rew (1984); 19. Bryant *et al.* (1976); 20. Köhler *et al.* (1978); 21. Fetterer (1986); 22. Stitt and Fairweather (1993a); 23. Stitt and Fairweather (1994); 24. Stitt and Fairweather (1992); 25. Stitt and Fairweather (1996); 26. Stitt *et al.* (1995); 27. Coles and Briscoe (1978); 28. Lang *et al.* (1980).

(Cornish and Bryant, 1976) (see Table 7.3). ATP levels show a reduction of 33–40% after 36 h, but total nucleotide levels do not fall until after 48 h *in vitro* (Bryant *et al.*, 1976; Cornish and Bryant, 1976). The studies were carried out at a concentration of 400 µg ml^{-1}, which is approximately 4000 times the maximum blood level *in vivo* (see Table 7.5). *In vivo*, changes in intermediary metabolites and end products do not occur until after 18 h; ATP levels do not fall until after 30 h, total nucleotide levels showing a decrease of 13% at this time (Rahman *et al.*, 1977). So, these changes are long term in nature and may follow detachment and removal of the flukes *in vivo* (Chevis, 1980; Rahman *et al.*, 1977).

There are interesting differences between *in vitro* and *in vivo* studies: for example, glycogen depletion occurs *in vivo* but not *in vitro* and lactate production decreases *in vitro* but increases *in vivo* (see Table 7.5). For glycogen, the difference has been attributed to a protected environment *in vitro* as against a more stressful situation *in vivo* which requires the mobilization of glycogen reserves; this point has already been made with regard to the action of rafoxanide. There is little evidence for disruption of energy metabolism in *F. hepatica* by albendazole, although it has been suggested that it, along with other 'classical' benzimidazoles, is capable of uncoupling oxidative processes in rat liver mitochondria (McCracken and Stillwell, 1991).

The impact of TCBZ on energy-producing pathways in the fluke has yet to be resolved. On the one hand, a stimulation of acetate and propionate production has been observed, the increase surprisingly coinciding with a decrease in motility (Bennett and Köhler, 1987). Prolonged incubations and higher concentrations were required before any major drop in ATP levels takes place, even when the fluke is immobile (Bennett and Köhler, 1987). On the other hand, experiments with rat liver mitochondria have shown that not only is triclabendazole capable of uncoupling oxidative phosphorylation in the mitochondria, but so too (and to a greater extent) are its sulphoxide and sulphone metabolites (Carr *et al.*, 1993).

It is generally accepted that benzimidazole anthelmintics bind to, and cause depolymerization of, cytoplasmic microtubules, thus disrupting microtubule-based processes in helminths (Lacey, 1988; Lubega and Prichard, 1990, 1991). Colchicine-binding data to support such a role for TCBZ are contradictory, Fetterer (1986) reporting a lack of inhibition of [^{3}H]colchicine binding to tubulin from homogenized flukes, while Bennett and Köhler (1987) demonstrated an inhibition of [^{3}H]colchicine binding to purified fluke tubulin. The discrepancy between the two studies has been attributed by Bennett and Köhler (1987) to non-specific binding of TCBZ to fluke protein in the study of Fetterer (1986), thus reducing drug availability to microtubular protein. However, a variety of other, more typical benzimidazoles – including mebendazole and albendazole (together with its sulphoxide and sulphone metabolites) – were shown to inhibit colchicine binding in the *in vitro* study by Fetterer (1986). Having said that, the picture for albendazole is confused, in that no inhibition of colchicine binding was evident in flukes recovered from sheep 12 h and 24 h after treatment with albendazole (Fetterer, 1986; see also Table 7.5). Morphological data in support of a microtubule-targeted

action for mebendazole and albendazole in *F. hepatica* itself are lacking, but mebendazole treatment has been shown to disrupt microtubule-dependent movements of secretory vesicles in the intestine of nematodes (e.g. Borgers *et al*., 1975a) and the tegument of cestodes (e.g. Borgers *et al*., 1975b). Microtubules are involved in many processes within cells and their disruption could have wide-ranging effects on parasites, thus accounting for some of the long-term metabolic changes observed.

In contrast to mebendazole and albendazole, morphological data regarding the action of triclabendazole on *F. hepatica* are available. The ultrastructural changes induced by TCBZ-SX *in vitro* are compatible with a microtubule-based action. Thus, there is a block in the transport of secretory bodies from the tegumental cell bodies to the apical surface of the tegument. The block occurs at their site of formation by the Golgi complex in the cell body, in their movement through the cytoplasmic connections to the syncytium, and in their movement from the base to the apex of the syncytium (Stitt and Fairweather, 1994). The disruption of secretory activity occurs fairly rapidly (3–6 h) in both adult and juvenile flukes (Table 7.5). The presence of a microtubule system in the tegument of *F. hepatica* has been demonstrated by Stitt *et al*. (1992). Since maintenance of the integrity of the tegumental surface membrane is dependent on the continual turnover of secretory bodies, it is reasonable to assume that the disruption of secretory activity evident in the tegument leads to the progressively severe surface damage visible externally, culminating in the total loss of the tegument (by 24 h in the adult) (Stitt and Fairweather, 1993a). Inhibition of proteolytic enzyme secretion in *F. hepatica* by TCBZ has also been attributed to disruption of microtubule-based secretory processes (Bennett and Köhler, 1987).

TCBZ-SX has been shown to inhibit the mitotic division of spermatogenic cells, in particular the early spermatogonial stages (Stitt and Fairweather, 1992). Fragmentation and autophagy of the central cytophore region of the rosette stages takes place, leading to disruption of the spermatocyte and spermatid stages. Spermiogenesis is completely inhibited and few spermatozoa are formed (Stitt and Fairweather, 1992). Again, the changes occur quite quickly (3–6 h *in vitro*: see Table 7.5). Division of the stem vitelline cells is also inhibited by TCBZ-SX, preventing them from undergoing their normal developmental sequence, which involves the production of eggshell material and the laying down of glycogen reserves for the developing embryo. Inhibition is fairly rapid: 6 h *in vitro* (Table 7.5) (Stitt and Fairweather, 1996).

The changes observed in the tegument, testis and vitelline cells of *F. hepatica* following TCBZ-SX treatment are what might be expected following microtubule inhibition. Moreover, they bear close similarity to those induced by microtubule inhibitors, especially tubulozole-C (Stitt and Fairweather, 1992, 1993b). The liver fluke is less susceptible to colchicine, relatively high concentrations (1×10^{-3} M) being required to bring about any morphological changes (Stitt and Fairweather, 1993b). The differential sensitivity to the two microtubule inhibitors may have an important bearing on the mode of action of TCBZ in relation to other benzimidazoles. Thus, the latter are

known to act by binding to the colchicine-binding site on the tubulin molecule (Lacey, 1988). Colchicine binds to tubulin heterodimers and inhibits their polymerization, thereby progressively depolymerizing microtubules (Mareel and De Mets, 1984). In contrast, tubulozole-C inhibits tubulin polymerization by inducing the formation of aggregates of the tubulin monomers (Mareel and De Mets, 1984; De Brabander *et al.*, 1986). So it is possible that TCBZ-SX binds to a separate (non-colchicine) binding site on the tubulin molecule of the fluke, a site that it shares with tubulozole-C. The colchicine-binding site may be less sensitive to disruption, explaining why more typical benzimidazoles are poorly active against *F. hepatica*, while TCBZ lacks activity against helminth parasites other than *F. hepatica*. It may be of significance that the TCBZ-SX molecule is U-shaped rather than the L-shape of other benzimidazoles (Lipkowitz and McCracken, 1991). Only *F. hepatica* among helminth parasites may possess a binding site on the tubulin molecule that can accommodate such a shape of drug. So, the combined data argues for disruption by TCBZ-SX of microtubule-based processes in the fluke, a conclusion that disagrees with the views of Coles (1986), Fetterer (1986) and Guralp and Tinar (1984).

The ultrastructural changes induced by TCBZ-SX have indicated an additional possible mode of action, namely, inhibition of protein synthesis. In the tegumental cells, for example, there is a decline in the number of secretory bodies, a reduction in the amount of GER and a gradual disappearance of the Golgi complex in the cytoplasm and a condensation of chromatin and disappearance of the nucleolus in the nucleus (Stitt and Fairweather, 1994). Similar nuclear changes were evident in the vitelline cells, which also showed a reduction in shell protein production, as evidenced by a decrease in the number of shell protein globules produced, together with a swelling of the GER cisternae and a decrease in their ribosomal covering (Stitt and Fairweather, 1996). In a separate study, it has been shown that TCBZ-SX causes a marked inhibition of the incorporation of [^{14}C]leucine into *F. hepatica* proteins over a 6 h period, thus confirming the conclusion from the morphological studies (Stitt *et al.*, 1995). Moreover, the same study showed that TCBZ-SX is a more potent inhibitor of protein synthesis than tubulozole, suggesting that it inhibits protein synthesis in a manner that is not based on microtubule inhibition (Stitt *et al.*, 1995). Which aspect of protein synthesis is inhibited by TCBZ-SX is not known, although it has been suggested that the action is directed against RNA synthesis (Stitt *et al.*, 1995).

In conclusion, many of the available data support the notion that TCBZ acts against microtubule-based processes in the liver fluke, albeit in a different way from other, more typical, benzimidazole anthelmintics. A second action is directed against protein synthesis. The two actions may be separate, though interrelated because disruption of the microtubule-dependent organization of organelles such as the GER and Golgi complex that are involved in synthetic mechanisms is likely to affect protein synthesis. Effects on energy metabolism are likely to be secondary as they are very long term in nature, occurring long after effects on the cytoskeleton and protein synthesis become apparent and at a time when morphological ‘damage’ is very severe. A direct action on the

neuromuscular system is also unlikely as the suppression of motility is very gradual.

The severe disruption of spermatogenesis and vitellogenesis by TCBZ-SX has already been described. Albendazole also causes marked damage to the reproductive system of *F. hepatica*. Thus, flukes recovered from cattle 5–6 weeks after treatment showed gross degenerative changes to the testes, ovary and vitelline cells (as well as to the gut) and reduced numbers of eggs in the uterus were observed. In addition the eggs were abnormal and exhibited reduced hatchability for 3 weeks post-treatment (Lang *et al.*, 1980). It remains to be resolved whether these changes represent a direct action of albendazole or whether they are simply a manifestation of the axiom proposed by Dawes (1968) and discussed previously in relation to bithionol. In a separate study, it has been demonstrated that both albendazole and mebendazole inhibit the embryonation of fluke eggs (Coles and Briscoe, 1978).

Group 4: sulphonamides

Clorsulon is the only fasciolicide believed to act against glycolysis, which is the main energy-producing pathway in the fluke (Coles, 1975; Barrett, 1976, 1981; Van Vugt, 1979/80). It has been shown to cause inhibition of the glycolytic enzymes 3-phosphoglycerate kinase and phosphoglyceromutase (Schulman and Valentino, 1980). However, the inhibition of the former was 40% at a concentration of 399.53 µg ml^{-1}, 47.7 times that of the maximum blood level attained *in vivo* (8.37 µg ml^{-1} in the rat following a dose of 12.5 mg kg^{-1}; Schulman *et al.*, 1979). Inhibition of phosphoglyceromutase was 43% at a concentration of 197.86 µg ml^{-1}, 23.6 times that of the maximum blood level. Consequently, the disruption of glycolysis *in vivo* may not be very great. Inhibition of glycolysis is potentially very serious for the fluke because it would block the early steps in the respiratory pathway and the downstream reactions would become inoperable; energy production would become severely, if not completely, impeded. In support of this idea, flukes treated for 1 h in clorsulon at a concentration of 500 µg ml^{-1} show a 60% decrease in glucose utilization, a 54% and 85% inhibition of the formation of the metabolic end products acetate and propionate, respectively, and a 67% reduction in ATP levels (Schulman and Valentino, 1980).

Clorsulon causes a gradual suppression of motility, leading to paralysis of the fluke in a flaccid condition (Fairweather *et al.*, 1984). This might be expected to occur with the depletion of energy reserves following glycolysis inhibition; established inhibitors of glycolysis such as sodium fluoride and sodium iodoacetate produce a similar suppression of motility (Holmes and Fairweather, 1985). The motility data agree with pharmacokinetic studies on clorsulon, in which a cessation of feeding was seen to coincide with, or just precede, the onset of elimination of the drug from the fluke, a process which begins 8–12 h following drug administration (Schulman *et al.*, 1979). It is possible that the cessation of feeding coincides with the sudden drop in activity that occurs after 10–12 h *in vitro* (Fairweather *et al.*, 1984).

Morphological studies have shown that changes in the gastrodermis induced by clorsulon occurred more rapidly than those in the tegument and were of greater severity, in both *in vivo* (following an oral dose of 12.5 mg kg^{-1}) and *in vitro* (10 µg ml^{-1}) studies. This may be a reflection of the means by which the drug enters the fluke: clorsulon binds to red blood cell carbonic anhydrase and enters the fluke with ingested erythrocytes (Schulman *et al*., 1979). Surface damage to the fluke, as visualized by SEM, was confined primarily to the oral cone region and was more severe following *in vivo* treatment. By 48 h *in vivo*, the tegument was completely sloughed off the anterior region of the fluke. After 24 h, the tegumental cells appeared to be synthetically inactive and this may have caused the tegumental loss. Also, the gastrodermal cells showed signs of necrosis within 24 h (Fairweather and McDowell, 1995). Within the gut, the apical region of the gastrodermal cells showed signs of vacuolation and disruption after 12 h treatment *in vitro*; by 24 h the cells were showing signs of breakdown (Fairweather and McDowell, 1995). Some of the morphological changes observed may account for the postulated biochemical effects of clorsulon, which were only apparent at extremely high concentrations. For example, once paralysed the fluke ceases to feed and enters a state of starvation. The severe disruption of the gut and, to a lesser extent, that of the tegument then exacerbates the damaging situation for the fluke. Thus, for clorsulon, with its unusual mechanism of uptake, it may well be a case of disruption from the inside out rather than from the outside in, as occurs with other compounds.

Group 5: phenoxyalkanes

Diamphenethide is the only member of this group. It is a unique fasciolicide in that it is more active against juvenile than adult flukes, even against flukes as young as 1 day old (Annen *et al*., 1973; Rowlands, 1973). As such, it offers the possibility of preventing much of the liver damage caused by the migrating juvenile flukes. Diamphenethide itself is rapidly metabolized by deacetylation in the liver of the host to an amine compound (Harfenist, 1973). This deacetylated (amine) metabolite of diamphenethide (DAMD) is responsible for the flukicidal activity of diamphenethide, its locally high concentration in the liver explaining why diamphenethide is so effective against juvenile flukes. Despite its significance, diamphenethide has remained something of an enigma, because little is known about its precise mode of action. The results of *in vitro* studies involving diamphenethide and DAMD are summarized in Table 7.6. Perhaps more is known of what it does *not* do than what it does.

A number of possible actions have been eliminated. Thus, there is general agreement that it does *not* disrupt energy metabolism in the fluke, in contrast to the postulated action of many commonly used fasciolicides, as discussed previously (Campbell and Montague, 1981; Edwards *et al*., 1981a). On the basis of elevated internal Na^+ levels in the fluke, it has been suggested that DAMD acts as an inhibitor of Na^+/K^+-ATPase activity or as a sodium ionophore (Rew *et al*., 1983). There is no pharmacological evidence to support the former

Table 7.6. A comparison of studies on the mode of action of diamphenethide against the liver fluke, *Fasciola hepatica* (concentrations expressed as μg ml^{-1}) (studies carried out *in vitro* unless stated otherwise).

Parameter tested	Diamphenethide	Deacetylated (amine) metabolite of diamphenethide	Reference
Maximum blood level *in vivo* in sheep	30 Dose: 400 mg kg^{-1}	12 Dose: 100 mg kg^{-1}	R.C. Parker, Berkhamsted, 1981 (personal communication)
Greater activity against juvenile than adult *F. hepatica* in sheep	Dose: 100 mg kg^{-1}	—	Armour and Corba (1972); Kingsbury and Rowlands (1972); Annen *et al.* (1973); Kendall and Parfitt (1973); Rew *et al.* (1978)
Activity against flukes as young as one-day-old in sheep	Dose: 100 mg kg^{-1}	—	Annen *et al.* (1973); Rowlands (1973)
Inhibition of malate dehydrogenase activity in adult *F. hepatica*	None at 372.4	—	Lwin and Probert (1975)
Minimum concentration for maximum uncoupling of *Hymenolepis diminuta* mitochondria	No uncoupling at 74.48	-	Yorke and Turton (1974)
Minimum concentration for uncoupling of rat liver mitochondria	372.4–∞	288.4–∞	Campbell and Montague (1981)
Reduction of ATP levels in adult *F. hepatica*	—	47% at 28.84 after 24 h	Rew and Fetterer (1984)
Changes in levels of ATP, respiratory intermediates and end products in adult *F. hepatica*	—	Only increased malate at 2.0 (after 3 h)	Edwards *et al.* (1981a)
Changes in respiratory end products in adult *F. hepatica*	—	Increase in acetate, propionate and lactate between 6 and 24 h at 28.84	Rew and Fetterer (1984)
Inhibition of glucose transport in juvenile (3–5-weeks-old) and adult *F. hepatica*	—	39% in juvenile at 28.84 (after 2 h). None in adult	Rew *et al.* (1983)

Inhibition of glucose transport in adult *F. hepatica*	—	30% at 10 (after 2 h)	Edwards *et al*. (1981a)
Minimum concentration for paralysis of adult *F. hepatica*	No paralysis within 12 h at 100	10 (flaccid paralysis within 1.5 h)	Fairweather *et al.* (1984)
Change in motility of 4-week-old juvenile and adult *F. hepatica*	No change in either adult or juvenile over 2 h period (concentration not given)	Spastic paralysis within 2 h at 28.84	Rew *et al*. (1983)
Inhibition of cholinesterase activity in adult *F. hepatica*	20.8% at 372.4	—	Durrani (1980)
Protection of *F. hepatica* by dopamine against flukicidal action	—	5.0	Edwards *et al*. (1981b)
Depolarization of tegumental membrane potential of 4-week-old juvenile *F. hepatica*	—	Within 0.5 h at 28.84	Rew *et al*. (1983)
Change in ion content of adult *F. hepatica*	—	15% increase in Na^+ concentration at 28.84 (after 3 h)	Rew *et al*. (1983)
Change in ion levels in adult *F. hepatica*	— —	Only initial and short-lived drop in Na^+ over a 6 h period at 10	Caseby *et al.* (1991)
Change in wet weight of adult *F. hepatica*	—	12% increase at 28.84 (after 3 h)	Rew *et al*. (1983)
Evidence of tegumental surface changes (leading to sloughing of tegument)	—	Adult: 3 h (24 h) 5-week-old: 3 h (6 h) 3-week-old: 1.5 h (9 h) Freshly-excysted metacercaria (day 0): 1 h (all at 10)	Anderson and Fairweather (1988); Fairweather *et al.* (1987)

Table 7.6 (*continued*).

Parameter tested	Diamphenethide	Deacetylated (amine) metabolite of diamphenethide	Reference
Inhibition of tegumental Na^+/K^+-ATPase activity	—	No inhibition after 18 h at 10	Skuce *et al.* (1987)
Evidence of internal tegumental changes (and onset of flooding)	—	Adult: 6 h (9 h) 5-week-old: 3 h (3 h) 3-week-old: 1.5 h (3 h) 0-day: 0.5 h (1 h) (all at 10)	Anderson and Fairweather (1995); Fairweather *et al.* (1986)
Disruption of secretory activity in tegumental cells	—	Adult: 9 h 5-week-old: 3 h 3-week-old: 3 h 0-day: 1 h (all at 10)	Anderson and Fairweather (1995); Fairweather *et al.* (1986)
Disruption of secretory activity in gut cells	—	Adult: 6 h 5-week-old: 6 h 3-week-old: 3 h 0-day: — (all at 10)	Anderson (1989)
Disruption of secretory activity in vitelline cells	—	Stem cell: 6 h It1 cell: 6 h It2 cell: 9 h Mature cell: 9 h (all at 10)	Fairweather *et al.* (1988a)
Inhibition of protein synthesis	—	After 6 h at 10	Anderson *et al.* (1993)
Disruption of spermatogenesis *in vivo*	Within 4 h (dose: 20 mg kg^{-1} in rabbit)	—	Stammers (1975b)

suggestion, because ouabain, a known inhibitor of Na^+/K^+-ATPase activity, induces a spastic rather than flaccid paralysis of the fluke (Fairweather *et al.*, 1988b). A similar neuromuscular response to ouabain has been cited for *S. mansoni* (Fetterer *et al.*, 1980). The increased muscle tension is probably the result of the elevated intracellular sodium concentration following sodium pump inhibition; this acts on the membrane Na^+/Ca^{2+} exchange to increase the intracellular level of calcium (Allen and Navran, 1984; Repke and Schönfeld, 1984; Allen *et al.*, 1985).

There is some evidence to support an ionophore role for DAMD, although the data are not straightforward. Monensin, a sodium ionophore, also induces a flaccid paralysis of the fluke (Fairweather *et al.*, 1988b). This is the opposite of what might be expected: the influx of sodium ions should lead to a depolarization of the membrane potential, triggering muscle contraction and leading to a spastic paralysis. However, the paradox of membrane hyperpolarization by monensin has been observed in a variety of cell types, including muscle cells, and has been attributed to the stimulation of sodium pump activity by raised intracellular Na^+ levels, leading to their reduction (Brock and Smith, 1982). Prior inhibition of pump activity by ouabain allows subsequent monensin treatment to generate the anticipated depolarization (Fahim *et al.*, 1983). The same is true for *F. hepatica*, both monensin – and, more significantly, DAMD – elicit a rapid spastic paralysis following ouabain pretreatment (Fairweather *et al.*, 1988b).

DAMD induces a swelling of the infoldings of the basal plasma membrane of the tegument of *F. hepatica*; the swelling leads to flooding and eventual sloughing of the tegument (Fairweather *et al.*, 1986; Anderson and Fairweather, 1995). The changes are suggestive of a disruption of the osmoregulatory role of the tegument, with the swelling of the basal infolds being what might be expected of an ionophore action. Thus, the large influx of Na^+ ions might overwhelm the capacity of the Na^+/K^+-ATPase-driven ion pumps located along the apical plasma membrane, the ions would penetrate deeper into the syncytium and be pumped into the basal infolds by the ion pumps situated along their membranes. The lumen of the infolds would become hypertonic with respect to the surrounding cytoplasm and water would be drawn into the infolds from the cytoplasm, making them swell and thus causing the flooding. Unfortunately for this idea, the sodium ionophore monensin does not cause any swelling of the fluke basal infolds, even after prolonged (24 h) incubation *in vitro* (Skuce and Fairweather, 1989). This unexpected result may be due to stimulation of the ion pumps because of increased internal levels of Na^+ following monensin treatment, the activity of the pump thus effectively masking the normal action of monensin. 'Unmasking' of monensin activity by prior inhibition of the pumps with ouabain does cause vacuolation of the tegument of *F. hepatica*, but this has nothing to do with the basal infolds (Skuce and Fairweather, 1989). Ouabain could mimic the effect of monensin by inhibiting the ion pumps, thus allowing Na^+ to enter down its concentration gradient. However, ouabain does not induce any swelling of the basal infolds (Skuce and Fairweather, 1989), although it has been shown to inhibit Na^+/K^+-ATPase activity in the

tegument (Skuce *et al*., 1987). With regard to a potential role as an ATPase inhibitor, it has been shown that, while DAMD does induce swelling of the basal infolds, it does not affect the ATPase activity associated with the tegumental ion pumps (Skuce *et al*., 1987).

Additional analytical studies have demonstrated that DAMD causes little change in internal ion levels in *F. hepatica* over a 6 h period (Caseby *et al*., 1991). The sodium ionophore monensin induces a decrease in K^+ levels to a value below that of Na^+, the reverse of the normal condition. There is a short-lived drop in Na^+ levels early on, but otherwise there is no difference from controls. Ouabain, an inhibitor of Na^+/K^+-ATPase activity, causes a marked reduction in K^+ levels; Na^+ and Ca^{2+} levels also fall. Pretreatment with ouabain followed by monensin does not affect the decline in K^+ levels, but prevents the short-lived Na^+ decline observed with monensin alone (Caseby *et al*., 1991). The results with monensin and ouabain were somewhat unexpected and consistent with the results of the pharmacological and morphological studies discussed previously. Neither pattern matched that produced by DAMD, so the ion analysis data add further weight against a Na^+ ionophore or Na^+/K^+-ATPase inhibitor role for DAMD.

DAMD induces a rapid flaccid paralysis of the fluke, but the basis of this action remains unclear (Fairweather *et al*., 1984). DAMD may have a neuropharmacological action, although a cholinesterase inhibitor role appears unlikely because there is only a 20.8% inhibition at a concentration of 372.4 µg ml^{-1}, although this result was obtained with the parent compound (Durrani, 1980). There is a possibility of disruption of dopaminergic mechanisms because dopamine, an excitatory transmitter in *F. hepatica* (Holmes and Fairweather, 1984), can (at 200 µg ml^{-1}) protect the fluke against the metabolic effects (elevated malate and succinate levels) of DAMD (5.0 µg/ml^{-1}) (Edwards *et al*., 1981b). This possibility needs to be explored further.

DAMD causes extensive damage to the tegumental surface as evident with SEM. Studies involving different developmental stages, from freshly excysted metacercaria to mature adult, have shown that the early stages are more severely and more rapidly affected than the adult (Fairweather *et al*., 1987; Anderson and Fairweather, 1988). There are also interesting regional differences in the pattern of drug-induced surface damage: in the adult, it is the dorsal surface that is more severely affected, whereas it is the ventral surface in the case of the earlier stages. These differences have been linked to developmental changes in tegumental architecture and Na^+/K^+-ATPase-driven ion pumps (Fairweather *et al*., 1986). In the adult fluke, there is an anterior–posterior spread of damage (Fairweather *et al*., 1987; Anderson and Fairweather, 1988).

Internal changes within the tegumental syncytium initially take the form of a 'stress' reaction (an apical concentration and increased exocytosis of secretory bodies, formation of microvilli and blebbing of the surface membrane), leading to the swelling of the basal infolds described previously. It is the latter that is responsible for the sloughing of the tegument. Age-related differences in the time sequence of tegumental changes are apparent and consistent with the SEM observations (Fairweather *et al*., 1986; Anderson and Fairweather, 1995). The trends parallel the results of *in vivo* efficacy

studies (see Table 7.6). The flooding of the tegument spreads internally to involve the tegumental cell bodies. A number of distinct changes in cell structure take place and will be discussed below in relation to the mode of action of DAMD. Here, perhaps, it is relevant to point out that the changes observed are *not* indicative of an ionophore or ATPase inhibitor role. The sodium ionophore monensin causes osmotic dilation of the *cis* Golgi cisternae, whereas the action of DAMD is directed more against the *trans* cisternae. Unmasking of monensin activity by ouabain pretreatment does lead to a typical swelling of the Golgi cisternae, although ouabain alone only causes the complex to become diffuse, even after 18 h incubation (Fairweather *et al.*, 1986; Skuce and Fairweather, 1989).

DAMD induces marked disruption of the gut of *F. hepatica*: changes include reduced numbers of secretory bodies, increased autophagy, vesiculation of the GER, swelling of the mitochondria, accumulation of lipid, and cellular necrosis in extreme cases. There are clear age-related trends in the onset and extent of the changes, with juvenile flukes being more susceptible than adult flukes (Anderson, 1989). The trends mirror those observed in the tegument. Again, the changes observed are not compatible with either an ionophore or ATPase inhibitor action (Skuce, 1987).

Diamphenethide treatment leads to disruption of the reproductive system of *F. hepatica*. The parent compound, diamphenethide itself, affects spermatogenesis *in vivo* fairly rapidly (within 4 h) (Stammers, 1975b). Treatment with the active metabolite, DAMD, *in vitro* causes changes to the vitelline cells, particularly the undifferentiated stem cells and the intermediate cells in the early stages of protein synthesis; the changes occur fairly quickly (within 6 h) and will be discussed in more detail later (see Fairweather *et al.*, 1988a). The vitelline cells appear to become inactive, being prevented from proceeding with their normal developmental sequence: as time progresses, there is a change in the cell population within the follicle, with relatively more stem, early It1 and mature cells and a decline in numbers of characteristic It1 and It2 cells (Fairweather *et al.*, 1988a). With regard to a possible ionophore or ATPase inhibitor action for DAMD, the profile of monensin- and ouabain-treated cells is very different from that for DAMD, adding further morphological evidence against such roles (for details, see Skuce and Fairweather, 1988a).

The morphological data from studies on the vitelline, tegumental and gut cells of *F. hepatica* indicate an alternative action for DAMD, namely, inhibition of protein synthesis in the fluke. In the vitelline cells, for example, there is condensation of chromatin within the nucleus and disappearance of the nucleolus, a progressive loss of ribosomes from the GER cisternae and reduction in the number of eggshell protein globules produced (Fairweather *et al.*, 1988). The action of DAMD is directed against the stem and early It1 cells, preventing them from proceeding with their normal developmental sequence. Similar, though less dramatic, changes are evident in the tegumental and gut cells (Fairweather *et al.*, 1986; Anderson, 1989).

In order to test this idea further, the effect of DAMD on the uptake and incorporation by adult *F. hepatica* of radioactively labelled precursors of DNA, RNA and protein synthesis has been determined by a liquid scintillation

counting technique. DAMD caused a significant decrease in the overall uptake and incorporation of [^{3}H]uridine after 6 h incubation *in vitro*, decreased the incorporation of [^{3}H]leucine from 6 h onwards and also caused a significant decrease in the overall protein content of the fluke, although it had no significant effect on the uptake or incorporation of [^{3}H]thymidine (Anderson *et al*., 1993). The results indicate that DAMD inhibits protein synthesis in *F. hepatica* by inhibition of RNA synthesis. Inhibition of protein synthesis would have serious consequences for the fluke. This is not only with regard to processes such as egg production and the maintenance of tegumental integrity (which is essential for nutrient uptake, immunoprotection and osmoregulation). It would also affect the synthesis of enzymes involved in metabolic pathways within the fluke. This could account for the metabolic changes which are known to occur following treatment with DAMD, but are not considered to be the primary effect of the drug (Edwards *et al*., 1981a; Rew *et al*., 1983; Rew and Fetterer, 1984; see also Table 7.4).

An inhibition of protein synthesis might explain the high activity of DAMD against juvenile flukes. Juveniles are in a very active phase of growth and differentiation and require higher rates of production of tegumental secretory bodies and glycocalyx turnover than the adult flukes to protect them from the immune response in the host liver (Hanna, 1980). Therefore, the juvenile flukes might be expected to be more susceptible to a drug which inhibits protein synthesis.

Furthermore, inhibition of protein synthesis would be a novel mode of action for a fasciolicide, although emetine dihydrochloride, an inhibitor of protein synthesis, has been used to treat liver fluke infections in rodents, sheep and man (Duriez *et al*., 1964; Grant and Jaggers, 1969). It is only effective against intrahepatic juvenile flukes, not against adult flukes in the bile duct, so it shows some similarity with DAMD. Emetine also shows greatest activity against juvenile flukes in *in vitro* screens (Ibarra and Jenkins, 1984). However, the antischistosomal drug hycanthone is known to act against protein synthesis in *S. mansoni*. Hycanthone treatment brings about a change in the population of cells within the vitelline follicle as a result of inhibition of division of the stem cells. The number of stem cells declines and mature cells accumulate, while intermediate stages disappear. This was interpreted as being due to inhibition of nucleic acid synthesis (Erasmus and Popiel, 1980). Initial studies indicated that hycanthone inhibited RNA synthesis *in vitro* (Pica-Mattoccia *et al*., 1981). *In vivo*, however, hycanthone action is directed predominantly towards DNA synthesis (Pica-Mattoccia and Cioli, 1983). More recent work has shown that hycanthone-sensitive schistosomes are able to convert the drug to a reactive ester, which spontaneously dissociates to an electrophilic species which is capable of alkylating macromolecules, preferentially DNA (Cioli *et al*., 1985; Pica-Mattoccia *et al*., 1988). Deoxyguanosine is the site of covalent binding and alkylation in the DNA molecule (Archer *et al*., 1990). Oxamniquine is believed to act in the same way against schistosomes (Pica-Mattoccia and Cioli, 1985; Pica-Mattoccia *et al*., 1989; Archer *et al*., 1990). Similar studies involving labelled DAMD may represent a possible future step in the elucidation of the mode of action of diamphenethide.

Finally, the morphological data from studies on the tegument and gut of *F. hepatica* have highlighted interesting age-related trends in the onset and severity of changes induced by DAMD (Fairweather *et al.*, 1986, 1987; Anderson and Fairweather, 1988, 1995; Anderson, 1989). Thus, the fluke becomes less susceptible to DAMD action with increasing age. The trend parallels the situation *in vivo*, efficacy studies showing that diamphenethide is more active against immature pre-bile duct stages than against adult flukes (for references, see Table 7.6).

From the data available, it is possible to establish a time course of events during DAMD treatment *in vitro*. Taking the adult fluke as an illustration, the fluke is paralysed within 1.5–2.0 h, surface alterations are evident from 3 h onwards and internal tegumental changes after 6 h. Flooding of the tegument begins after 9 h and leads to the tegumental sloughing observed after 24 h. Inhibition of secretory activity in the vitelline and gut cells is evident after 6 h and that in the tegument after 8 h. Inhibition of protein synthesis has been recorded from 6 h onwards. These changes occurred at a concentration of 10 µg ml^{-1}, which corresponds very closely to the maximum blood level *in vivo*. Of the metabolic changes observed, malate levels were elevated after 3 h (at 2.0 µg ml^{-1}), levels of the end products acetate, propionate and lactate were increased between 6 and 24 h (at 28.84 µg ml^{-1}) and ATP levels had dropped by 47% after 24 h (at 28.84 µg ml^{-1}).

The time sequence indicates that the fluke enters a state of starvation fairly quickly, as a result of the induced paralysis. This condition is compounded by the tegumental damage and gut changes observed and may account for some of the longer-term metabolic changes. Inhibition of protein synthesis will certainly exacerbate the situation even further and can be considered as a major target for the drug. However, it is probably not possible to conclude that this is the primary mode of action of DAMD. The neuromuscular effect, being the most immediate, may be of paramount importance, and this action remains to be resolved. Consequently, DAMD remains something of an enigma in terms of its mechanism of action.

Drug Resistance in *Fasciola hepatica*

Resistance to anthelmintics among helminth parasites is a global problem of increasing concern. It is particularly prevalent in nematode parasites (see reviews by Jackson, 1993; Shoop, 1993; Prichard, 1994), but is also evident in other trematodes such as schistosomes (Cioli *et al.*, 1993; Brindley, 1994). Drug resistance in *F. hepatica* is not yet a major problem, but resistance to a number of fasciolicides has been identified in the field and in the laboratory (Boray and De Bono, 1989; Boray, 1990, 1997). Data pertaining to individual compounds are discussed below.

Salicylanilides

It has been shown that long and regular use of salicylanilide compounds, particularly rafoxanide and closantel for the treatment of fasciolosis in sheep,

has selected resistant strains of *Fasciola hepatica* in endemic areas of New South Wales. These two compounds are also used for the control of the nematode *Haemonchus contortus* and there is evidence for resistance in the worm to them (Van Wyk and Malan, 1988; Rolfe *et al*., 1990). The fluke strains retained their resistant status in cattle and through several passages in sheep. In about 60% of the properties, and from different geographical regions, surveyed in New South Wales, flukes showed resistance to rafoxanide at recommended dose rates in *F. hepatica* and side resistance to closantel was evident (Fig. 7.2). There was also cross-resistance to nitroxynil, a halogenated phenol (Boray, 1997). Resistance manifested against immature, but rarely against adult, fluke. A degree of salicylanilide resistance has also been reported in western England and Wales, respectively (Boray, J.C. and W.M. Allen, unpublished data). Fluke strains resistant to rafoxanide and closantel show no side resistance to another salicylanilide, oxyclozanide. This may be due to differences in the age-related susceptibility of the fluke to the drugs (Coles, 1975) or to the pharmacokinetic characteristics of the latter. Oxyclozanide quickly reaches its peak concentration in the blood after treatment and its excretion is equally rapid. Rafoxanide and particularly closantel are strongly bound to plasma protein and persist in the blood at subtherapeutic concentrations for up to 90 days and are more likely to select for resistance.

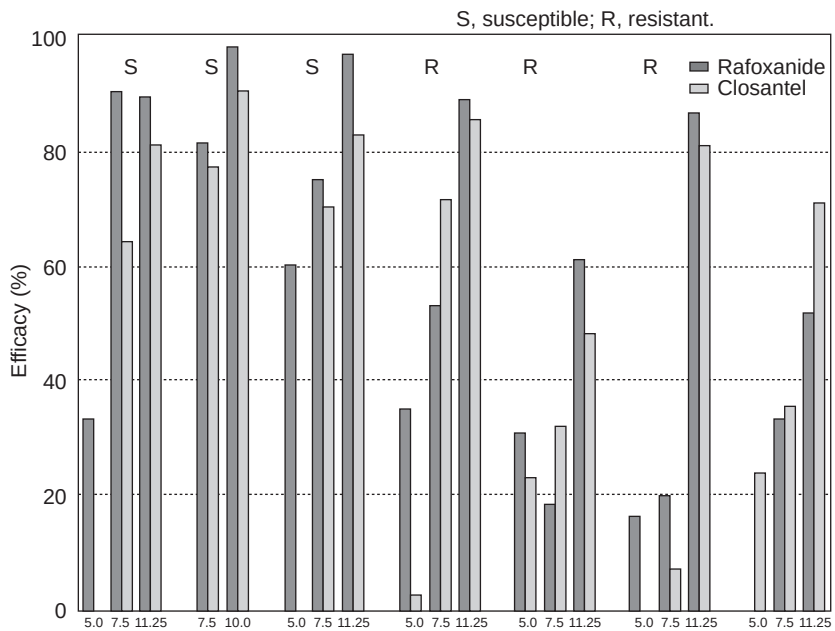

Fig. 7.2. Efficacy of rafoxanide and closantel against fluke aged 6 weeks.

Triclabendazole

An *F. hepatica* isolate was selected by drenching every 8 weeks with triclabendazole in the field for a period of 3 years and further selected by discriminative doses in the laboratory. Efficacy of the drug at comparable dose rates against the strain 4 weeks after infection was appreciably lower than against strains never exposed to triclabendazole (Fig. 7.3). Further selections at the recommended dose rate of 10 mg kg^{-1} significantly reduced the efficacy of the drug against flukes aged 2 weeks from 98% to 60% (Fig. 7.4). Recent studies confirmed the occurrence of serious triclabendazole resistance of an undefined intensity in the field in Australia (Overend and Bowen, 1995). In one sheep farm in the same area, triclabendazole at the recommended dose rate of 10 mg kg^{-1} was ineffective against the fluke isolate aged 2, 4, 6 and 12 weeks. Increased dose rates of 20, 45 and 67 mg kg^{-1} were ineffective against flukes aged 4 to 6 weeks and treatment at the dose rate of 100 mg kg^{-1} resulted in less than 80% efficacy against flukes aged 6 weeks (Boray *et al.*, 1997: Fig. 7.5). Anecdotal evidence suggests the occurrence of resistance to the drug in Counties Sligo and Cavan in Ireland (Anon., 1995).

Luxabendazole

Two isolates were selected in the laboratory by luxabendazole, a new broad-spectrum benzimidazole carbamate. Using discriminative dose rates, the

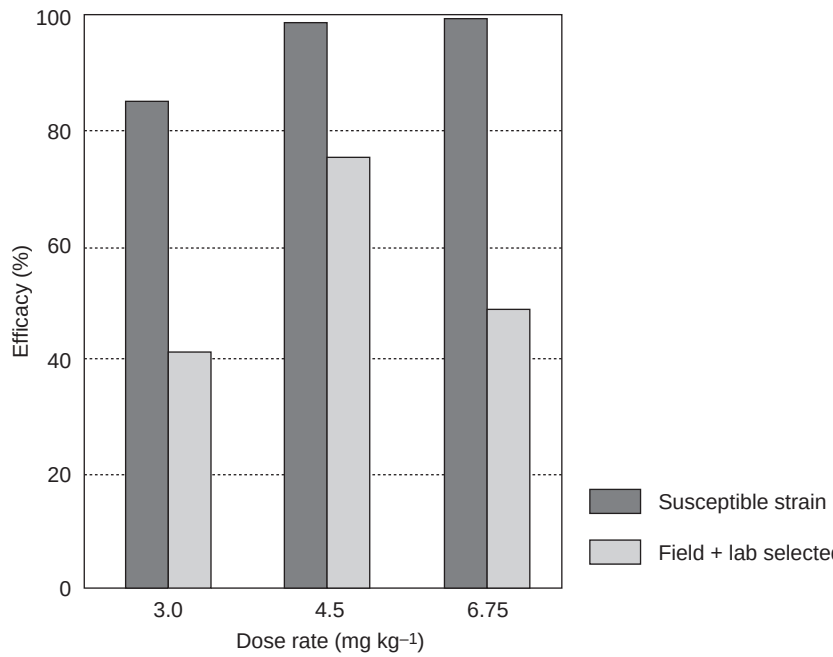

Fig. 7.3. Efficacy of triclabendazole against fluke aged 4 weeks.

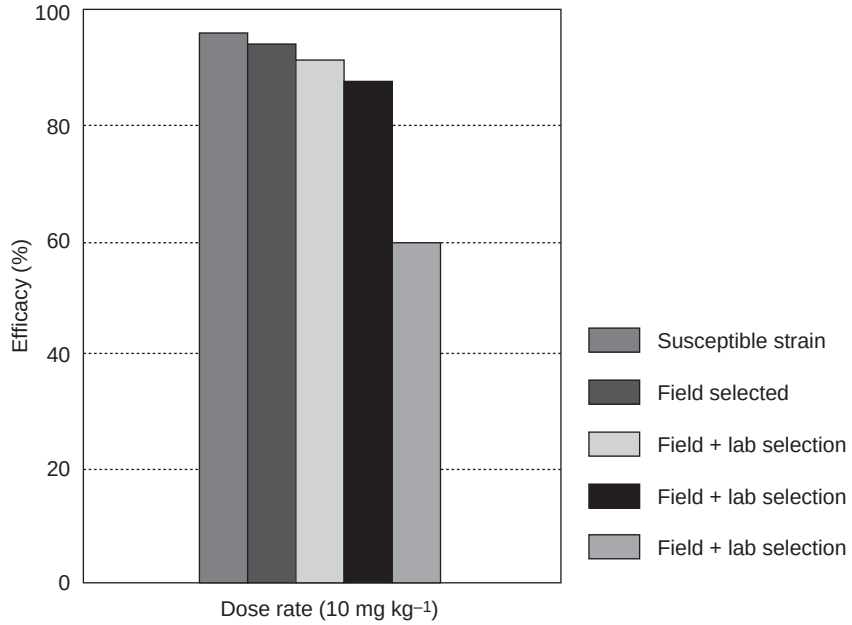

Fig. 7.4. Efficacy of triclabendazole against fluke aged 2 weeks.

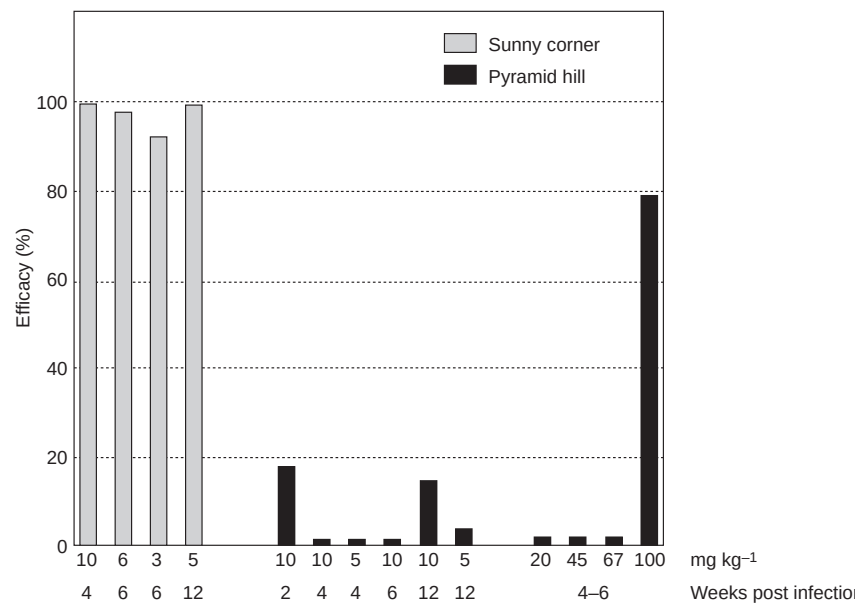

Fig. 7.5. Efficacy of triclabendazole drench against *Fasciola hepatica* in sheep.

efficacy of the drug against the selected strains in sheep 6 weeks after infection was appreciably reduced compared with the usually high efficacy achieved at similar dose rates against susceptible isolates (Boray, 1997).

Clorsulon

Attempts to induce resistance to clorsulon in the laboratory have failed: thus three laboratory selections with clorsulon did not result in reduced efficacy against the strain.

Management of Drug-resistant *Fasciola hepatica*

The resistance of *F. hepatica* to anthelmintics has been shown to be genetically controlled but the selection mechanisms involved are unknown. Through preferential selection for resistance in the immature stage of *F. hepatica*, drugs effective against early immature fluke could lose their advantage in chemoprophylaxis of fasciolosis. Demonstration of an unexpected 'total' resistance to triclabendazole against immature and adult flukes in the field suggests that more serious problems in controlling fasciolosis may occur in the future. Wide usage of a single highly effective anthelmintic is undesirable since resistance may develop against several chemically unrelated drugs.

A strategic programme with alternating drug use and specific farm management with minimum use of drugs should be implemented for the prevention of resistance, when resistance has been established or is suspected. However, this method may prove to be too complicated or not attainable. An alternative strategy is to use a combination of drugs.

Synergistic drug combinations

True synergism occurs when a combined efficacy of two or more chemotherapeutic agents is greater than the sum of their individual activities. The combinations are particularly useful when the efficacy of individual drugs has been reduced by the appearance of resistance, but they are still highly effective in synergistic combinations. In recent studies in Australia the successful use of synergistic combinations of drugs of different chemical groups has been reported for the prevention of resistance and for the treatment of fasciolosis due to resistant strains of *F. hepatica* (Boray, 1993, 1997).

The results of experimental studies have shown that combinations of drugs from different chemical groups, such as triclabendazole and clorsulon or triclabendazole and luxabendazole, achieve high efficacy against susceptible strains of *F. hepatica* aged 6 weeks at a fraction of their respective recommended dose rates by a synergistic effect. The combination of clorsulon and luxabendazole showed strong synergistic action. These or similar combinations would be useful for the prevention of resistance (Fig. 7.6). The combination of triclabendazole and clorsulon at one-fifth of their recommended dose rates or triclabendazole and luxabendazole at one-fifth and one-third of their respective

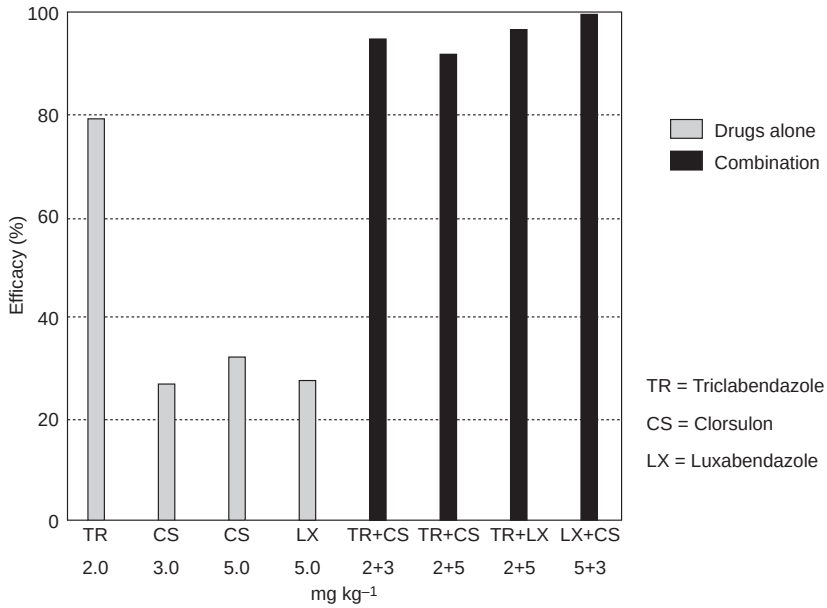

Fig. 7.6. Efficacy of drug combinations against susceptible *F. hepatica* aged 6 weeks.

recommended dose rates were highly effective against triclabendazole-resistant *F. hepatica* aged 6 weeks by a true synergistic action (Fig. 7.7). A strong synergistic effect was demonstrated when closantel at the dose rate of 7.5 mg kg^{-1}, which is lower than the effective dose rate against flukes aged 6 weeks, was combined with either triclabendazole, clorsulon or the benzimidazole carbamate, luxabendazole at reduced dose rates (Fig. 7.8). Nitroxynil at lower than its recommended dose rate has been used successfully against strains of 6-week-old *F. hepatica* resistant to both closantel and luxabendazole when the drug was combined with low dose rates of closantel or clorsulon in injectable formulations (Fig. 7.9).

When closantel was combined with other benzimidazole carbamates, such as albendazole and fenbendazole, no synergism was observed. Oxfendazole showed a moderate synergistic action but luxabendazole achieved a strong synergistic effect against a salicylanilide-resistant strain aged 6 weeks (Fig. 7.10). Against another salicylanilide-resistant strain, the efficacy of closantel was enhanced by combining the drug with either clorsulon or luxabendazole when the drugs were used at a fraction of their respective recommended dose rates (Fig. 7.11). The combination of a slightly increased dose rate of closantel with a low dose rate of clorsulon showed strong synergistic effect and achieved high efficacy against a salicylanilide-resistant strain of *F. hepatica* aged 4 weeks (Fig. 7.12). With some adjustments of dose rates, combination products can be developed which are highly effective against *Fasciola* spp. aged 2 weeks and older.

In certain combinations, one or both of the active components have additional effect against parasitic infections other than fasciolosis. Some

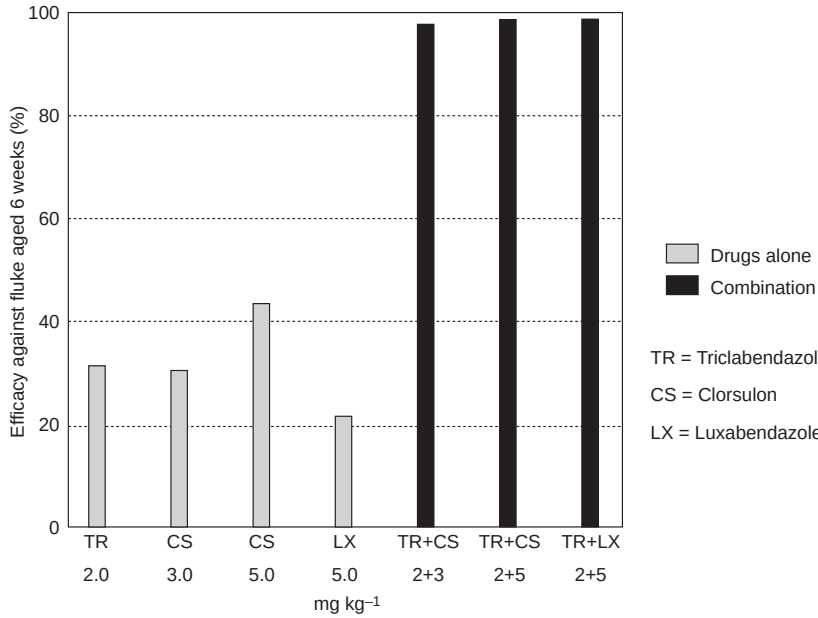

Fig. 7.7. Anthelmintic efficacy against triclabendazole-resistant *F. hepatica*.

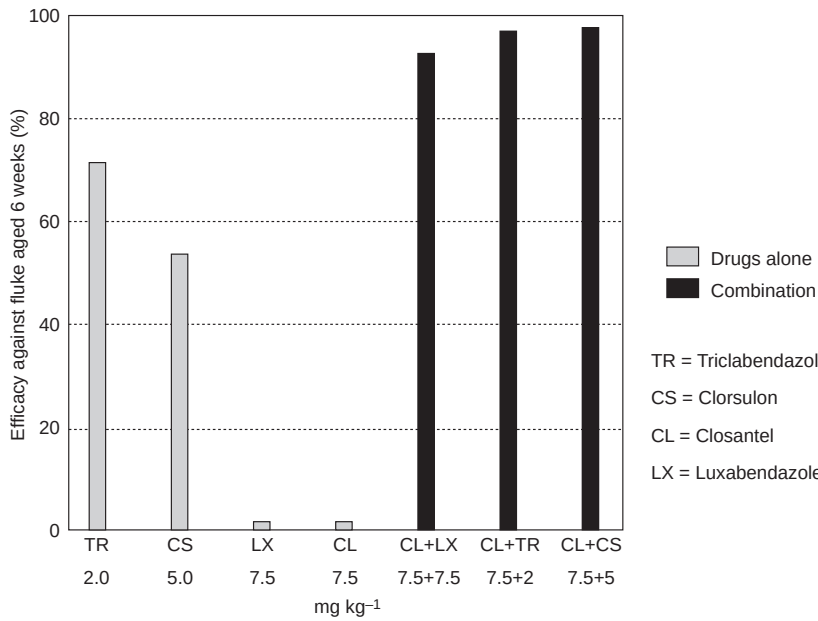

Fig. 7.8. Anthelmintic efficacy against closantel + luxabendazole-resistant *F. hepatica*.

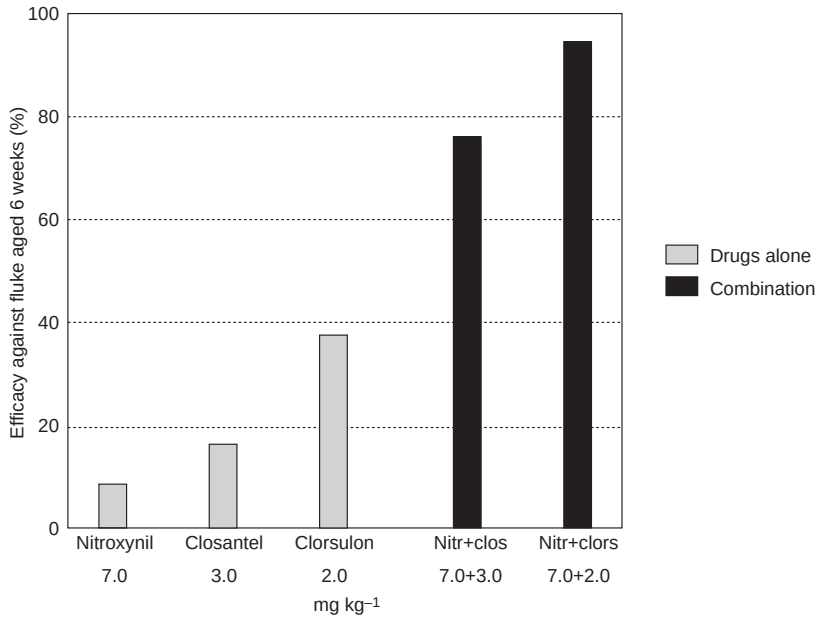

Fig. 7.9. Anthelmintic efficacy against salicylanilide-resistant *F. hepatica*; applied as a subcutaneous injection.

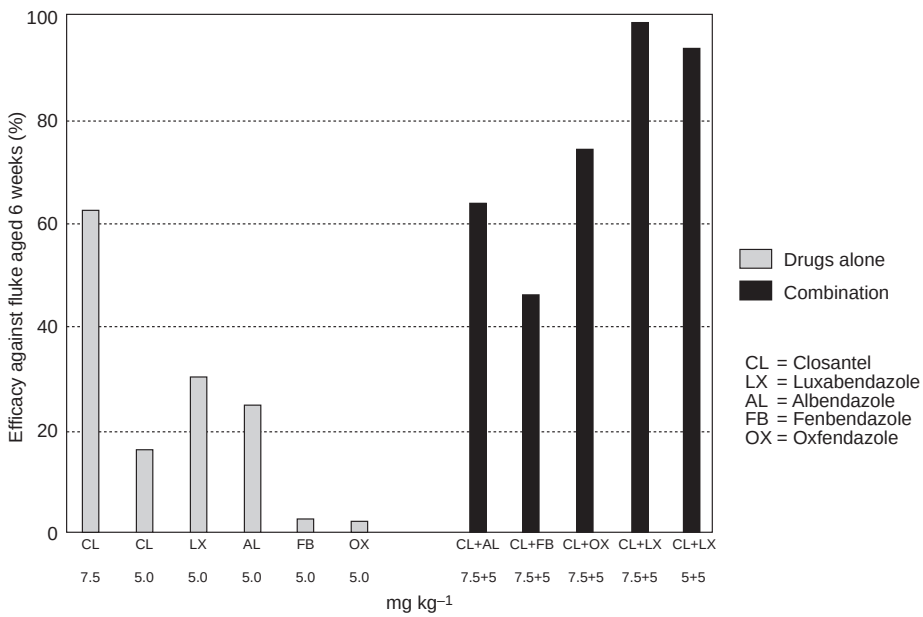

Fig. 7.10. Efficacy of closantel and benzimidazoles against rafoxanide-resistant *F. hepatica*.

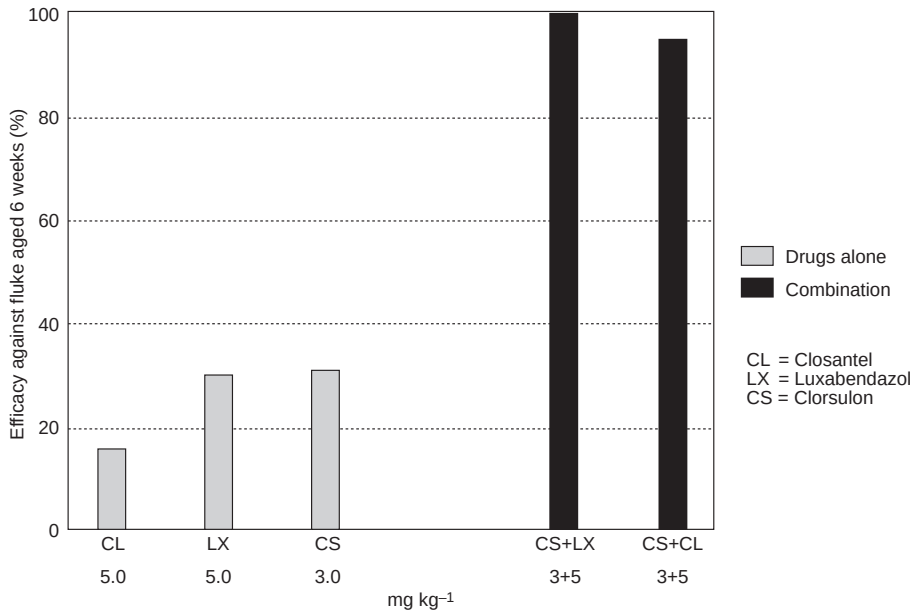

Fig. 7.11. Efficacy of clorsulon + anthelmintics against rafoxanide-resistant *F. hepatica*.

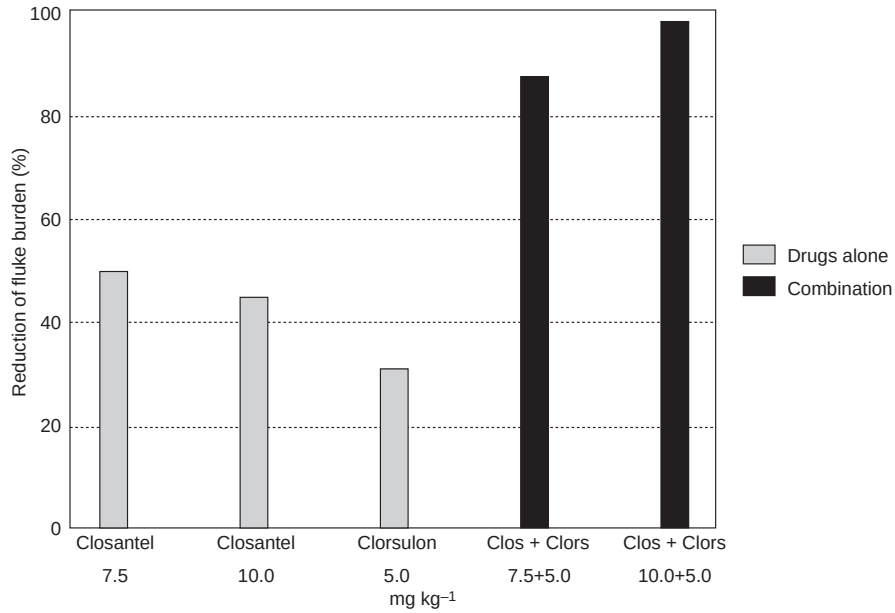

Fig. 7.12. Efficacy of clorsulon and closantel against *F. hepatica* aged 4 weeks; resistant to salicylanilides.

combinations would be suitable for the treatment of resistant and susceptible strains of trematodes (*Fasciola* spp., *Dicrocoelium* spp. and *Eurytrema pancreaticum*) as well as gastrointestinal nematodes, lungworms, tapeworms and *Oestrus ovis* in sheep (see references in Boray, 1990).

Salicylanilides act on both *F. hepatica* and *H. contortus* by uncoupling oxidative phosphorylation and related reactions of the mitochondrial membranes involved in electron transport. In the development of resistance a permeability barrier may operate (for references see Boray, 1990). The regular use of these drugs may play an important role in the development of salicylanilide resistance for both parasites. A level of resistance of *H. contortus* to rafoxanide and closantel has been reported with references to previous investigations by Rolfe *et al.* (1990). Since the mode of action of salicylanilides is similar in either *Fasciola* spp. or *Haemonchus contortus*, the closantel–luxabendazole combination would be effective against the salicylanilide-resistant strains of the two parasites, with an additional broad-spectrum activity against gastrointestinal nematodes. All combinations with closantel would give persistent efficacy against susceptible *H. contortus*.

By using synergistic combinations, three major aims may be achieved:

1. A combination product that is highly effective against both immature and adult *F. hepatica* at reduced dose rates, and which results in reduced tissue residues of the synergistic components and possibly reduced cost of production.
2. A product that would be effective against a variety of fluke strains resistant to one or both components, and which may prevent the development of resistance in susceptible fluke populations.
3. A product which would be suitable for the treatment of resistant and susceptible strains of trematodes (*F. hepatica*, *F. gigantica*, *Fascioloides magna*, *Fasciolopsis buski*, *Dicrocoelium* spp., *Eurytrema pancreaticum*) and some intestinal nematodes, lungworms and tapeworms in sheep and bovines and *Oestrus ovis* in sheep.

Conclusions

When all the disparate data from studies on fasciolicides are collected together, it is evident that they represent a fair body of work. More has been done than might have been imagined. Individual researchers have adopted a variety of approaches and examined different targets and processes within the fluke, namely, energy metabolism, neuromuscular activity, the tegument and tegument-based secretory processes, the osmoregulatory system and ionic regulation, reproduction and egg production and the cytoskeleton. On the basis of the evidence available is it possible to state unequivocally what is the precise action of any particular fasciolicide? The disappointing answer is no. We can say that closantel and possibly other related salicylanilides interfere with energy metabolism, that triclabendazole interacts with the microtubular cytoskeleton and that diamphenethide disrupts protein synthesis. However, they may not be the definitive actions of these drugs and other possibilities exist.

Why is this so? There are a number of reasons. Any fasciolicide will interact with a number of different systems within the fluke and exert a variety of effects. It is unrealistic and probably foolish to focus on any single target to the exclusion of others. An effect on one biochemical system is likely to have a knock-on effect on other systems and so it is very difficult to determine whether fasciolicidal action is due to a single effect or to a combination of effects. For example, a major disturbance of the neuromuscular system may not only cause detachment of the fluke, but also lead to the cessation of feeding. The fluke will enter a state of starvation and this will impose a severe metabolic stress upon it, draining its energy reserves. The paralysis induced by a number of fasciolicides is rapid at physiological concentrations and so may be more important than any long-term metabolic changes. In a similar way, disruption of gut cells and their production of digestive enzymes will impair the uptake of nutrients and trigger a state of starvation, with the consequences just described. A number of fasciolicides will enter the fluke in the ingested blood and thus have a direct impact on the gastrodermal cells. The tegument of *Fasciola* has a number of important roles, including nutrient uptake (sugars and amino acids), immunoprotection and osmoregulation. It represents the first line of defence against drug attack (excretion of the fasciolicide in the bile bringing it into contact with the tegument) and its integrity is essential for the continued viability of the fluke. Once this defence has been breached, the drug may be capable of penetrating to many of the innermost tissues of the fluke and, in the *in vivo* situation, external factors such as bile and an immune response become more significant in inducing severe damage. The surface damage caused by fasciolicides can be extreme and this will have serious consequences for the fluke. Moreover, maintenance of the integrity of the surface membrane is dependent on the turnover of secretory bodies produced by the underlying tegumental cell bodies, so any disruption of secretory processes in the cells would only exacerbate the situation. Finally, the microtubular component of the cytoskeleton is important for many synthetic and secretory processes, as well as for cell division; its disruption would have wide-ranging effects on the parasite.

So, an 'integrated systems' approach rather than an 'isolationist' view is required when trying to understand fasciolicidal action. Having said that, it is true to say that so far no such 'broad-spectrum' study for any single fasciolicide has been carried out under uniform conditions at a single concentration to obtain a complete profile of the morphological, biochemical and physiological effects of the drug both *in vivo* and *in vitro*. Such a study would enable the time course of drug-induced changes to be built up and so help to establish the mechanism of action of the fasciolicide concerned. The nearest attempt along these lines has been achieved for diamphenethide, as discussed above.

Another problem in elucidating fasciolicide actions concerns the difficulty of comparing different studies – not just comparisons between *in vivo* and *in vitro* studies, where the more protected environment *in vitro* may underplay the effect of the drug on the parasite. Studies *in vivo* may involve different hosts, different dosing regimes and different time courses, for example. *In*

vitro, drug concentrations, culture media, solvents and timings are some of the factors that have to be taken into account. Even the test system that is used is important because it may involve non-fluke material. Too many *in vitro* studies have been carried out at concentrations far in excess of maximum blood levels *in vivo* and so the results they generate may have little bearing on the actual mode of action of the fasciolicide concerned.

Having presented a slightly jaundiced view of studies aimed at unravelling the mechanisms of action of different fasciolicides, the reader may be excused for wondering what purpose they have served and whether the data they have generated have any value. After all, the basic motivation for such studies is that a better understanding of drug actions could be used for the development of more effective and perhaps safer drugs in the future. For *F. hepatica* there is a continuing need to develop compounds against the immature, intrahepatic stage that causes the pathology associated with fasciolosis during its migration through the liver parenchyma to the bile duct. Any search for new compounds also has to take into account that *F. hepatica*, *F. gigantica* and *Fascioloides magna* are unusual parasites, seemingly requiring drugs that are specific for themselves – diamphenethide, clorsulon and triclabendazole being cases in point. It is to be hoped that 'mode of action'-type studies will help to identify potential 'targets' within the fluke – perhaps an enzyme or receptor that can be linked to a process – and that such information could be used to generate lead compounds for further evaluation and screening. From an academic point of view, drug studies have the added bonus of stimulating fundamental research into particular aspects of parasite biology, improving our overall knowledge of liver fluke physiology, which in turn may highlight further possibilities for therapeutic exploitation.

In conclusion, the future for fasciolicides as the main form of fluke control is assured, at least in the short term. Even when reliable and effective vaccines come on stream, which is unlikely to materialize in the near future (see Chapter 15 of this volume), there is likely to be a complementary role for drugs. Drug resistance is not a major problem at present, but that is not a reason for complacency; constant vigilance and monitoring are needed to avoid the problems that bedevil the control of nematode parasites. The authors hope that studies on drug mechanisms will continue, generating information for the more rational development of fasciolicides in the future.

Acknowledgement

A particular debt of thanks is due to Mrs Karen Moore who has managed to transform my disorganized manuscript into the final polished article – even to the point of correcting my mistakes!

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8 Metabolism

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Introduction

Parasitic helminths, including *Fasciola hepatica*, have many interesting properties in their biochemistry which have stimulated research not only to enlarge our fundamental biochemical knowledge in general, but also for the development of new chemotherapeutics. In contrast to infections with bacteria, viruses and protozoa, a continued helminth infection in the host is not dependent on replication of the parasite; the parasite itself must be removed from the body of the host. Potential targets for anthelmintic drugs are therefore the metabolic pathways of the parasite and the attention has to be especially focused on differences in metabolism between the host and the parasite. Another aspect, which has stimulated research on parasite biochemistry, is the adaptation of the metabolism of parasitic helminths to the changing environment during their life cycle.

There are several, so far unresolved difficulties in the study of the biochemistry of *F. hepatica*. The parasitic stages have to be studied isolated from their host; therefore, the interaction between host and parasite is lost and, consequently, essential requirements for proper physiological functioning might be lacking. Most studies have been performed on adult liver flukes obtained from the bile ducts of infected final hosts, as the parasitic stages inside the intermediate host are too difficult to obtain pure, and in quantities large enough to allow biochemical experiments. Another limitation to biochemical investigations is the fact that even the size of adult liver flukes is so small that intact or homogenized parasites are used instead of isolated organs or tissues. The results obtained will, therefore, represent the overall activity of the whole animal. This will mask typical, so far unknown metabolic processes of specific organs or tissues of the parasite. It is most likely that, as in higher animals, distinct differences in metabolism exist between the cells of different tissues and that products of one cell type can be metabolized by other cells. These questions can only be answered when culture methods for certain cell types of *F. hepatica* are established.

Fasciolosis (ed. J.P. Dalton)

In this chapter an overview is given of some selected aspects of the biochemistry of *Fasciola*, with emphasis on its energy metabolism and related subjects. For other aspects and further details, the reader is referred to recent reviews on the biochemistry of proteins (North and Lockwood, 1995), purines and pyrimidines (Berens *et al.*, 1995), polyamines (Bacchi and Yarlett, 1995), *trans*-splicing (Davis, 1995) and neuropeptides (Halton *et al.*, 1990; Fairweather and Halton, 1991; see also Chapter 9 of this volume) in parasitic helminths.

Nutrients and Digestion

Uptake of substrates

F. hepatica has, like all other parasitic helminths, various developmental stages in its life cycle and the availability of substrates varies widely during this life cycle. When they reside inside their definitive or intermediate host, *Fasciola* can and will obtain substrates from the host, as the term 'parasite' itself signifies. The free-living stages, (meta)cercariae, eggs and miracidia, on the other hand, live on their endogenous reserves, which they stored in their previous parasitic stage. As these reserves are limited, the free-living stages are either short living and in frantic search for a new host (miracidia), or they are a dormant stage, waiting to be reactivated by external triggers (metacercariae and eggs).

The parasitic stages inside their respective hosts need substrates for the biosynthesis of structural elements as well as for the generation of energy. *F. hepatica*, like all other parasitic helminths, has a very limited anabolic capacity (see 'Synthetic capacities', below), they also need more complex building blocks for their biosynthetic machinery for maintenance and repair as well as for reproduction.

Adult flukes inside their final host possess two surfaces that can potentially absorb nutrients: the external surface, called tegument, and the intestinal epithelium of the gut. The relative contributions of both surfaces are still unclear, but both intestine and tegument are considered to be important in the uptake of substrates by *F. hepatica* (Arme, 1988; Pappas, 1988; Thompson and Geary, 1995).

The digestive tract of *F. hepatica* is blind-ended, with a single opening that functions both as entrance and as exit. With its oral sucker the liver fluke can browse the bile duct epithelium and the underlying tissues. The incomplete gut is first filled with nutrients and after digestion the undigested remains are regurgitated through the same oral opening. The intestine of the fluke is covered with a cellular single layer of epithelial cells through which the nutrients are absorbed after degradation by digestive enzymes. Proteins of the host, including haemoglobin, are degraded by proteolytic enzymes, whereafter the resulting amino acids can be taken up by the parasite. It has been suggested that glutathione *S*-transferase might play a role in the absorptive function of the gut of adult *F. hepatica* (Creaney *et al.*, 1995). Earlier, glutathione *S*-transferases had been identified as novel vaccine candidates that protect sheep against *F. hepatica* infections (Sexton *et al.*, 1990, 1994).

The tegument of *F. hepatica* plays an important role in the uptake of nutrients and consists of a standard lipid bilayer from which a thin glycoprotein coat, the glycocalyx, extends. Simple substrates like glucose, amino acids and lipids are mainly absorbed via the tegument.

Substrates of energy metabolism

In contrast to the host, in *F. hepatica* a clear distinction exists between substrates for energy generation and substrates for biosynthetic purposes. The mammalian host can adapt its metabolism to the availability of substrates. Depending on the supply, carbohydrates, lipids or proteins can be the main source of energy. *F. hepatica*, on the other hand, is completely dependent on carbohydrates for its energy metabolism. Because of the very limited aerobic capacity of adult flukes, their metabolism has to be mainly fermentative. Carbohydrates are suitable substrates for fermentation, as both oxidation and reduction of this substrate can occur. Lipids are too reduced for this purpose and can therefore not be used for the generation of energy by parasitic helminths in general. The free-living stages of *F. hepatica* probably have a classical aerobic metabolism and could, in principle, be able to utilize lipids for the generation of energy. Very little research has been done on the lipid metabolism of these stages of parasitic helminths, but up to now no evidence was found for the degradation of lipids for energy generation by *F. hepatica*. Generally, free-living stages of trematodes are supposed to depend on their endogenous glycogen reserves.

Amino acids are necessary as precursors of protein synthesis for normal growth and reproduction in *F. hepatica*, but oxidation of amino acids is considered to contribute little to the overall energy metabolism (Coles *et al*., 1980; Barrett, 1991). A possible role in energy metabolism could be an anaplerotic one, as in schistosomes, where degradation of amino acids, and especially glutamine, provides Krebs cycle intermediates (Foster *et al.*, 1989).

Synthetic Capacities

In accordance with their opportunistic way of living as a parasite, *F. hepatica* has limited biosynthetic capacities. As described above, the liver fluke obtains many simple substrates from the host. More complex molecules that the parasite cannot obtain directly from the host are synthesized from these simpler building blocks. Obviously, the parasite has to synthesize complex structures like proteins and DNA by itself, for a start because the amino acid and nucleotide sequences of these molecules are different from those in the host. In general, the biosynthetic pathways of parasitic helminths have a close resemblance to those of their mammalian hosts. The enzymes of these pathways, however, often possess different properties, and in some cases where parasites produce unique end products, certain distinct final parts of a pathway – and thus the enzymes involved – are completely absent in the host.

Carbohydrates such as glucose are not synthesized *de novo* by *F. hepatica*, as gluconeogenesis has never been demonstrated in parasitic helminths except

from intermediates at the level of triose phosphates (Bryant and Behm, 1989). Simple carbohydrates are obtained from the host and are then used by the parasite to synthesize the complex ones, for instance the glycoproteins of the surface glycocalyx or glycogen, the storage carbohydrate used by *F. hepatica* (Tielens *et al*., 1990).

Lipids, like phospholipids, triacylglycerols and cholesterol, are not synthesized *de novo* by *F. hepatica*, but are obtained directly from the host (cholesterol and fatty acids) or are synthesized from building blocks obtained from the host (certain fatty acids and in the case of phospholipids, the fatty acids and also the head groups).

Purine and pyrimidine nucleotides are essential components of many biochemical molecules, from DNA and RNA to ATP and NAD^+. In recent years the pyrimidine and especially the purine metabolism of parasitic helminths have been extensively investigated, because of the differences with comparable pathways in the mammalian host, which make them potential targets for chemotherapeutic attack. Parasitic helminths do not synthesize purines *de novo* but obtain them from the host. They possess, however, elaborate purine salvage pathways for a more economical management of this resource. Pyrimidines, on the other hand, are synthesized *de novo* by all parasitic helminths studied so far and, as in mammalian cells, their ability to salvage pyrimidines is limited. For an extensive review of purine and pyrimidine pathways in parasitic helminths and protozoa see Berens *et al.* (1995).

Polyamines such as spermidine and spermine, which bind tightly to nucleic acids and are abundant in rapidly proliferating cells, are present in parasitic helminths in amounts comparable to those in vertebrate cells. It is assumed, however, that these compounds are obtained from the host as the enzymes necessary for their synthesis are lacking in adult parasitic helminths (Bacchi and Yarlett, 1995). In *F. hepatica* a polyamine *N*-acetyltransferase has been characterized, and is suggested to play a major role in the polyamine metabolism of this parasite by inactivating excess amines (Aisien and Walter, 1993).

Lipid Metabolism

Lipids such as phospholipids, triacylglycerols and cholesterol play various important roles in living organisms. Phospholipids and cholesterol are the main components of biological membranes, which not only form the boundaries of the various compartments inside cells and organisms, but also act as the interface between organism and environment, and in the case of *F. hepatica*, the outer membrane is therefore the site of interaction between parasite and host.

Very little of the lipid metabolism of *F. hepatica* has been studied, although it is in several aspects distinctly different from that of the mammalian host. A schematic and partly hypothetical overview of the main pathways of the lipid metabolism in *F. hepatica* is shown in Fig. 8.1. Fatty acids are not degraded by *F. hepatica* and are thus not used for energy

generation. *De novo* fatty acid synthesis also does not occur in *F. hepatica* and, therefore, fatty acids have to be obtained from the host. *F. hepatica* contains a protein with homology to mammalian fatty-acid-binding proteins, which is supposed to play a role in the uptake and transport of fatty acids from the host (Rodriguez-Perez *et al.*, 1992). A similar fatty-acid-binding protein is present in *S. mansoni* and *S. japonicum* (Moser *et al.*, 1991; Becker *et al.*, 1994), and could form the basis of the protective immune cross-reactivity between liver flukes and schistosomes, as a recombinant fusion protein stimulates a protective response against both *F. hepatica* and *S. mansoni* infection (Tendler *et al.*, 1996).

Although *F. hepatica* obtains fatty acids from its host, comparison of the fatty acids present in the host and parasite revealed the presence of some fatty acids in adult flukes which are virtually absent in the host (Oldenborg *et al.*, 1975, 1976). These parasite-specific fatty acids are not synthesized *de novo*, but are produced by modification of fatty acids obtained from the host. *F. hepatica* is unable to desaturate fatty acids, but can use acetate for the elongation of the host-derived fatty acids (Oldenborg *et al.*, 1976). This chain elongation has a specificity for certain fatty acids, of which the elongation of oleic acid (18:10) to eicosenoic acid (20:1) is quantitatively the most important one. In general, two different pathways exist for the elongation of fatty acids: one is in principle a reversal of β-oxidation and in the other acetyl-CoA is carboxylated to malonyl-CoA and subsequently added to pre-existing fatty acids. It has not yet been fully established which pathway occurs in *F. hepatica*, but acetyl-CoA carboxylase is present in this parasite whereas there is no indication for the occurrence of a reversal of β-oxidation (Brouwers, Schmitz and Tielens, unpublished results). The reason for the persistence during evolution of chain elongation of large amounts of fatty acids in parasitic helminths, while all other synthesizing and catabolic pathways of fatty acids are lost, is unclear.

Fatty acids, with or without prior chain elongation, are incorporated into phospholipids and triacylglycerols (Fig. 8.1). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major phospholipid classes present in *F. hepatica*, and both the head groups and the fatty acids are obtained from the host and used as precursors. Pathways of the other phospholipids are not yet thoroughly investigated in *F. hepatica*, but are most likely comparable to those in other parasitic helminths, of which *S. mansoni* is the most extensively studied to date. Generally, the phospholipid classes present in the host are also found in parasitic helminths, although sometimes in different ratios. Next to phospholipids, *F. hepatica* contains a large amount of glycerophosphocholine, a degradation product of phosphatidylcholine (Mansour *et al.*, 1982; Tielens *et al.*, 1982; Matthews *et al.*, 1985; Rohrer *et al.*, 1986). The presence of this degradation product indicates a high turnover of phospholipids, but the role of this putative rapid turnover in the lipid metabolism of *F. hepatica* has not yet been further investigated.

Sterols such as cholesterol are not synthesized *de novo* by parasitic trematodes, but the so-called mevalonate pathway is active in parasitic helminths (Fig. 8.1) (Coppens and Courtoy, 1996). This pathway was mainly

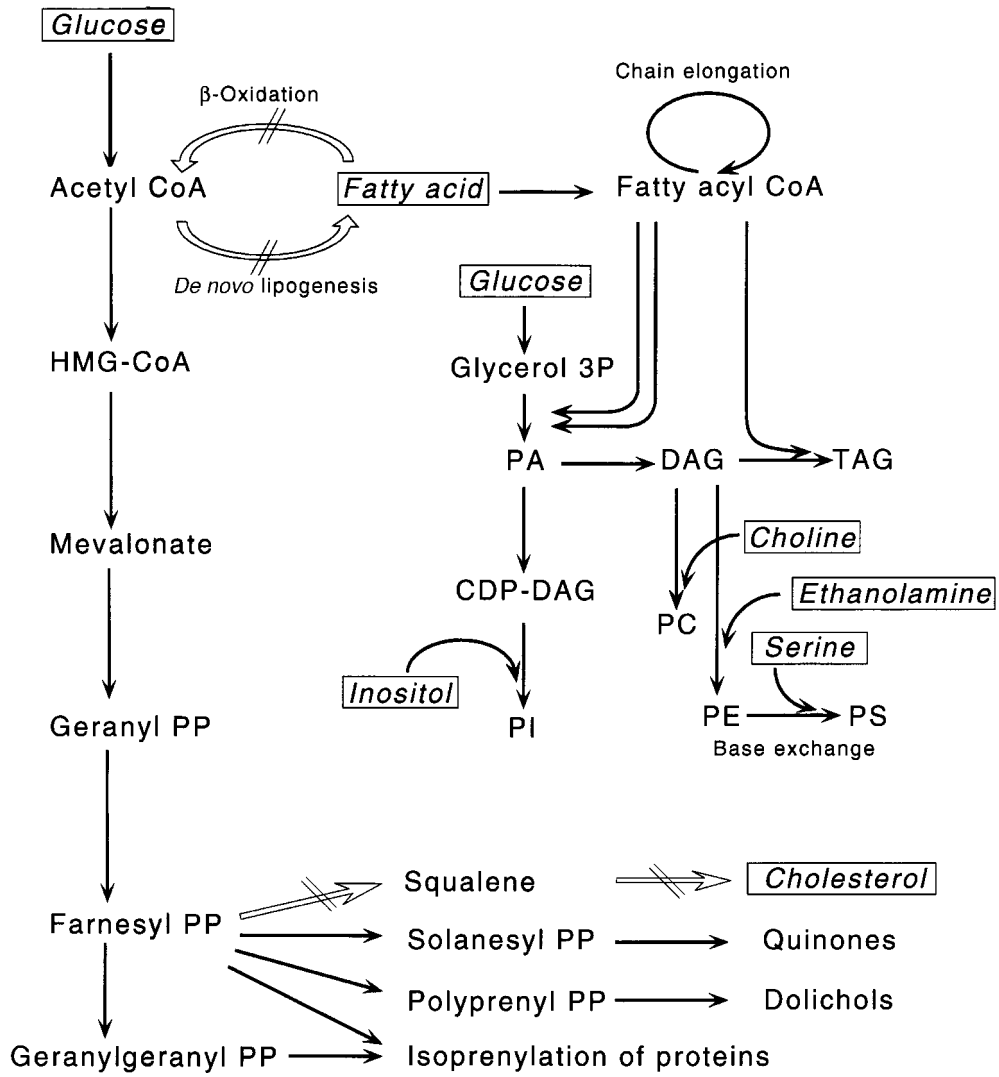

Fig. 8.1. Schematic representation of the main pathways in the lipid metabolism of adults of parasitic helminths. Boxed substrates are supplied by the host. Pathways present in mammalian systems but absent in *Fasciola hepatica* are shown with open arrows. Abbreviations: DAG, diacylglycerol; CDP–DAG, cytidine diphosphodiacylglycerol; Farnesyl PP, farnesyl pyrophosphate; Geranyl PP, geranyl pyrophosphate; Geranylgeranyl PP, geranylgeranyl pyrophosphate; Glycerol 3P, glycerol 3-phosphate; HMG-CoA, hydroxymethylglutaryl-coenzyme A; TAG, triacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

studied in *S. mansoni*, but up to now all available evidence indicates that the lipid metabolism of *F. hepatica* is very comparable in this respect. The mevalonate pathway was shown to be used by *S. mansoni* for the synthesis

of dolichols as cofactors in protein glycosylation, of quinones as electron transporters in the respiratory chain, and of farnesyl and geranylgeranyl pyrophosphates as substrates for the isoprenylation of proteins (Chen and Bennett, 1993; Foster *et al.*, 1993). A key enzyme in the mevalonate pathway is 3-hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase) and it was shown that the schistosomal enzyme differs from the mammalian type, both structurally and in its regulatory properties (Rajkovic *et al.*, 1989; Chen *et al.*, 1991). Farnesyl pyrophosphate plays a key role in the mevalonate pathway as it is the last common substrate for the synthesis of all end products (Fig. 8.1). As mentioned already, the branch leading from farnesyl pyrophosphate via squalene to cholesterol is not operative in parasitic helminths, whereas the other branches are active, at least in *S. mansoni* and probably also in *F. hepatica*.

One of the branches from farnesyl pyrophosphate leads to the formation of quinones, essential lipids in electron transfer chains where they carry electrons from one protein complex to the next. In an aerobic energy metabolism when the quinone has to transfer the electrons to complex III of this chain, the required quinone is a ubiquinone. Adult *F. hepatica*, however, has an anaerobic energy metabolism and this requires a quinone with a lower standard redox potential (see below). It has been known for a long time that many parasitic helminths indeed contain a quinone with such a lower standard redox potential, rhodoquinone, which was suggested to play a role in their anaerobic energy metabolism (Sato and Ozawa, 1969; Allen, 1973). Recently it was shown that rhodoquinone is an essential component in eukaryotes that have a metabolism comparable to that of parasitic helminths (Van Hellemond *et al.*, 1995). Rhodoquinone was shown to play an essential role in the anaerobic energy metabolism during the entire life cycle of *F. hepatica* (Van Hellemond *et al.*, 1996). This study also demonstrated that in *F. hepatica* adults, rhodoquinone was not produced by modification of ubiquinone obtained from the host but was synthesized *de novo* by the parasite. Most likely, ubiquinone and rhodoquinone are derived from the same precursor and only the last step in rhodoquinone synthesis differs from that in ubiquinone synthesis. As rhodoquinone is essential for the anaerobic energy metabolism of parasitic helminths and absent in their hosts, rhodoquinone synthesis (and especially the enzyme catalysing the last step) is a key target for anthelmintic drug design.

As mentioned above fatty acids obtained from the host are to some extent modified. In addition, cholesterol obtained from the host is not only directly used as a component of the membranes of *F. hepatica*, but is also used as a substrate for the synthesis of other compounds, like ecdysteroids (Foster *et al.*, 1992). Sphingomyelin synthesis in *F. hepatica* occurs probably by a pathway similar to that found in mammals (Bankov and Barrett, 1990).

In summary, *F. hepatica* has discarded some main pathways of synthesizing lipids *de novo*, but has selectively retained several biosynthetic pathways, mainly to modify lipids obtained from the host. Lipids such as fatty acids and cholesterol, which are abundant in the environment of *F. hepatica* when they reside in their hosts, are not synthesized *de novo* but are obtained from the

host. On the other hand, lipids such as specific unsaturated fatty acids, eicosanoids, ecdysteroids and quinones, which are difficult to obtain because the concentration in the host is very low, are synthesized by the parasite, often by modification of substrates that are easier to obtain from the host. In this way the lipid metabolism of *F. hepatica* is adapted to an opportunistic way of living, just like its energy metabolism (Brouwers *et al.*, 1996).

Protein Metabolism

Amino acids are not an important energy substrate for *F. hepatica*, but it should be realized that they are of course essential for normal development, just like they are for the host. Amino acids in parasitic helminths are mainly used for biosynthetic purposes. The basic features of the protein and amino acid metabolism in parasitic helminths, including *F. hepatica*, resemble those of their mammalian hosts (for reviews see: Barrett, 1991; North and Lockwood, 1995). *F. hepatica* obviously needs to synthesize many different proteins, not only to function as enzymes in the various metabolic pathways but, for instance, also for structural elements like tubulin and actin, parts of the cytoskeleton that have recently been partially characterized for *F. hepatica* (Stitt *et al.*, 1992a, b).

Protein synthesis plays also an important role in the process of egg laying of *F. hepatica*. Large amounts of proteins have to be synthesized by adult flukes not only for the proteins inside their offspring, the prospective miracidia, but also for the formation of the eggshells. As mature liver flukes produce thousands of eggs per day, a large proportion of their total biosynthetic capacity (and energy budget) is devoted to egg production. Each egg is enclosed in a tough and chemically resistant shell made of tanned proteins. Eggshell precursor proteins are synthesized and stored within the extensive vitellaria of the adult fluke. Some of these proteins are now characterized and were shown to be unique in containing rather high levels of the amino acid 3,4-dihydroxyphenylalanine (DOPA) (Waite and Rice-Ficht, 1987, 1992; Rice-Ficht *et al.*, 1992; Rzepecki, 1993; Waite, 1995).

Fasciola spp. obtain amino acids by hydrolysis of proteins from the host, utilizing proteases excreted by the parasite. Several types of proteases have been described in *F. hepatica*: they are used not only in protein degradation to provide substrates for biosynthetic purposes, but are also involved in host–parasite interactions, the invasion of host tissues and the evasion of immune attack mechanisms (Carmona *et al.*, 1993). Most likely, cysteine proteases have an essential role in the degradation of connective tissue by liver flukes, like they have in *Haemonchus contortus* (Rhoads and Fetterer, 1997). Cysteine proteases (cathepsin L and cathepsin B) and aspartyl proteases also play a role in digestion of haemoglobin and other proteins of the host (Yamasaki *et al.*, 1992; Carmona *et al.*, 1994; Heussler and Dobbelaere, 1994). Several of these proteases (earlier called 'haemoglobinases') are now characterized, also for *S. mansoni* (Smith *et al.*, 1993; Dalton *et al.*, 1995).

Energy Metabolism

Free-living versus parasitic stages

The free-living stages of *F. hepatica* (eggs, miracidia, cercariae and metacercariae) are self-supporting: they do not obtain food or substrates other than oxygen from the environment and are completely dependent on their endogenous energy stores acquired in the previous host. Glycogen is present as storage carbohydrate in these stages of the life cycle of *F. hepatica* and it is used to span the gap in food supply until the next host is reached. Miracidia will die if the glycogen reserves are exhausted before the next host, a snail, is entered. Metacercariae can be considered – biochemically speaking – to be a dormant stage, and therefore their substrate reserves are not as rapidly exhausted as those of miracidia.

It is generally accepted that free-living stages of parasitic helminths have an aerobic energy metabolism, although this has only been studied in very few cases. For *S. mansoni* it has been shown that cercariae as well as miracidia degrade their stored glycogen reserves via the classical glycolytic pathway to pyruvate. Inside mitochondria this pyruvate is then broken down to carbon dioxide via the Krebs cycle (Tielens *et al.*, 1991, 1992). The main part of the energy is generated via the mitochondrial respiratory chain and oxidative phosphorylation. The energy metabolism in these free-living stages is not different from the standard aerobic metabolism in mammals. Recently, metabolic experiments showed that *F. hepatica* miracidia are metabolically very comparable to those of *S. mansoni* and are also mainly dependent on the aerobic degradation of glycogen (Boyunaga, Van Hellemond and Tielens, unpublished results). The consumption of oxygen by these miracidia as well as their motility was strongly reduced in the presence of cyanide, which indicates that energy generation via electron transfer to oxygen plays an essential role in their energy metabolism. Histological studies have indicated that Krebs cycle activity plays a less pronounced role in the generation of energy in *F. hepatica* sporocysts compared to miracidia (Humiczewska, 1975).

Adult parasitic helminths have an energy metabolism strikingly different from that in the free-living stages. Although the pattern of their end products varies greatly between different species of adult trematodes, none of them degrades carbohydrates completely to carbon dioxide, as the free-living stages do. In general, parasitic helminths do not use oxygen as final electron acceptor, but have a fermentative metabolism instead. When oxygen cannot function as terminal electron acceptor, the degradation of substrates will have to be in redox balance: the number of NADH-producing reactions will have to equal the number of NADH-consuming ones (without the use of oxygen).

Malate dismutation

The classical adaptation to metabolism without oxygen, fermentation of carbohydrates to lactate or ethanol, is not the main pathway used in adult *F. hepatica*. Instead, adult liver flukes use a different pathway for the fermentation

of carbohydrates: malate dismutation. In this pathway carbohydrates are degraded to phosphoenolpyruvate (PEP) via the normal Emden–Meyerhof pathway. PEP is then carboxylated by phosphoenolpyruvate carboxykinase (PEPCK) to form oxaloacetate, which is subsequently reduced to malate (Fig. 8.2). This part of the pathway occurs in the cytosol and is comparable to the formation of lactate or ethanol: it is in redox balance and yields 2 mol of ATP per mole of glucose degraded. This end product of the cytosol is not excreted like lactate, but is transported into the mitochondria for further degradation. In a split pathway a portion of the substrate (malate) is oxidized to acetate, and another portion of it is reduced to succinate (Fig. 8.2). The oxidation occurs first to pyruvate by malic enzyme and then to acetate by pyruvate dehydrogenase and a succinate/succinyl-CoA cycle. In the other part of the pathway, reduction of malate occurs in two reactions which reverse part of the Krebs cycle. Many parasites, including *F. hepatica*, metabolize succinate further to propionate which is then excreted. This so-called malate dismutation is in redox balance when twice as much propionate as acetate is produced. Apart from the electron-transport-associated ATP formation in the reduction of fumarate (see below), malate dismutation is also accompanied by substrate-level phosphorylations (Fig. 8.2). In total the anaerobic production of propionate and acetate yields approximately five moles of ATP per mole of glucose degraded.

Selected aspects of glycolysis

In the search for possible targets for chemotherapeutics, several glycolytic enzymes of *F. hepatica* have been studied in more detail. In these studies most emphasis, of course, has been put on the main regulatory enzymes, and some interesting differences with the corresponding ones of the final host have been found.

Phosphofructokinase (PFK) is the main regulatory site of glycolysis, and PFK of *F. hepatica* has been extensively studied. In general, the activity of PFK is regulated by several effectors. The most potent activators of mammalian as well as *Fasciola*'s PFK are fructose 2,6-bisphosphate and AMP, whereas ATP is inhibitory (Van Schaftingen *et al.*, 1981; Kamemoto *et al.*, 1987). In contrast to mammalian PFKs, the enzyme of *Fasciola* is relatively insensitive to inhibition by citrate (Kamemoto *et al.*, 1987). The most remarkable difference from mammalian PFKs is that the kinetic properties of PFK of *Fasciola* are strongly influenced by phosphorylation (Kamemoto and Mansour, 1986). Phosphorylation by a cAMP-dependent protein kinase results in activation of PFK and it was proposed that this phosphorylation plays, at least in part, a functional role in the regulation of PFK of the liver fluke under *in vivo* conditions (Kamemoto *et al.*, 1989). *Fasciola* PFK is phosphorylated with cAMP-dependent protein kinase to 0.2 mol P per mole subunit and this results in a threefold increase in maximal enzyme activity compared to the unphosphorylated enzyme (Mahrenholz *et al.*, 1991). This phosphorylation occurs at a threonine residue in a phosphorylation site which shows no homology to the phosphorylation site of PFK of *Ascaris*, an enzyme also

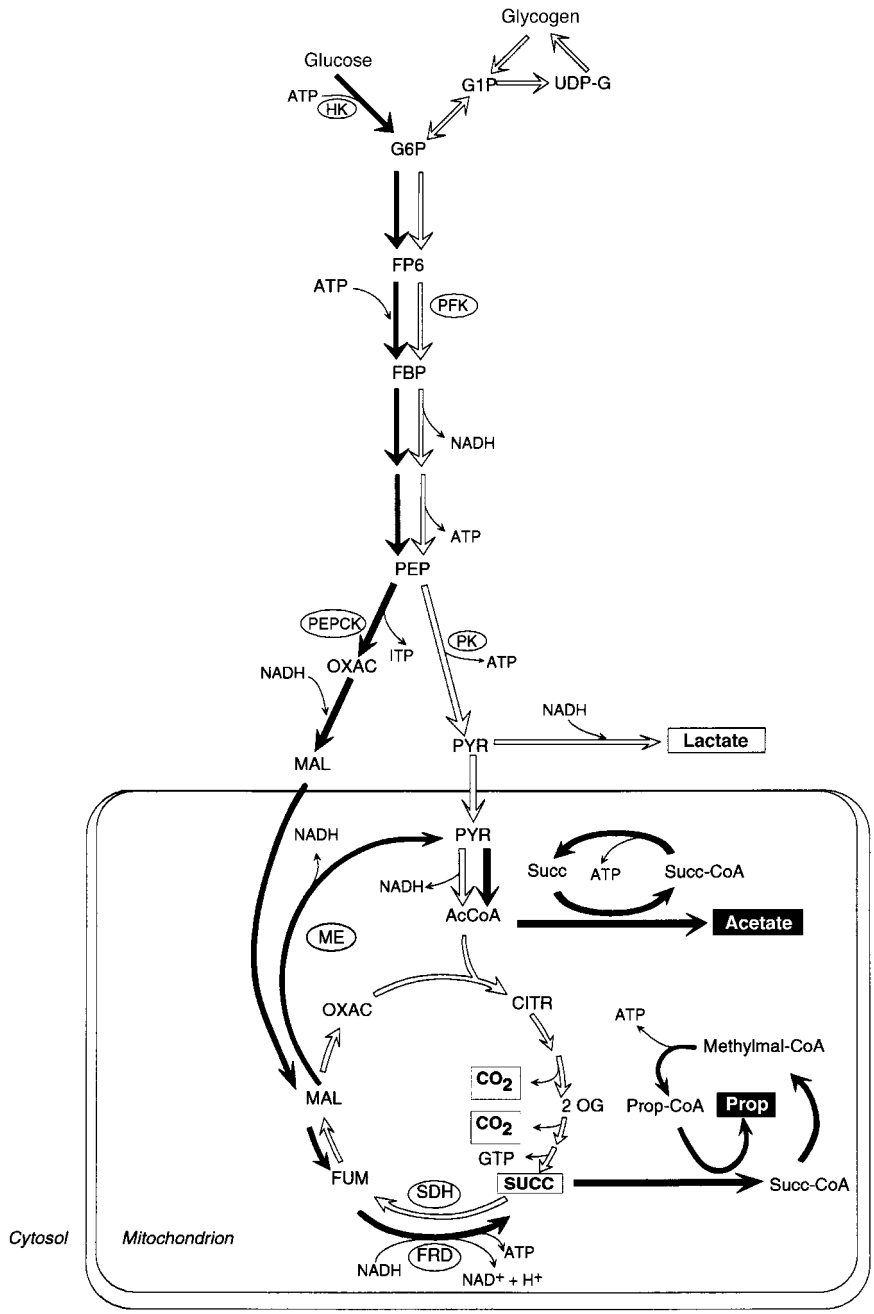

Fig. 8.2. Main pathways of the aerobic and anaerobic carbohydrate degradation in *F. hepatica*. The aerobic degradation by newly excysted juveniles and free-living stages is indicated by open arrows, whereas the malate dismutation occurring in adult flukes is indicated by filled arrows. Abbreviations: AcCoA, acetyl-CoA; CITR, citrate; FRD, fumarate reductase; FUM, fumarate; MAL, malate; Methylmal-CoA, methylmalonyl-CoA; ME, malic enzyme; 2 OG, 2-oxoglutarate; OXAC, oxaloacetate; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; PROP, propionate; Prop-CoA, propionyl-CoA; PYR, pyruvate; SDH, succinate dehydrogenase; SUCC, succinate; Succ-CoA, succinyl-CoA.

known to be stimulated by phosphorylation (Kulkarni *et al.*, 1987; Mahrenholz *et al.*, 1991). Further structural data to determine the differences between mammalian PFKs and those of parasitic helminths are not yet available.

Fructose 1,6-bisphosphatase (FBPase) is also active in adult *F. hepatica* and together with the activity of PFK this can result in extensive substrate cycling. ^{13}C-NMR experiments showed that substrate cycling in the glycolytic pathway of adult flukes occurs to a large extent *in vitro* (Matthews *et al.*, 1986). This cycling consumes ATP, but it is generally assumed that the occurrence of futile cycling enables the cell to regulate its net glycolytic flux more efficiently.

Pyruvate kinase (PK) from *F. hepatica* closely resembles L-type PK from mammalian liver (Prichard, 1976; Behm and Bryant, 1980). It shows cooperative kinetics with phosphoenolpyruvate, but in the presence of fructose 1,6-bisphosphate (FBP) this changes to Michaelis–Menten kinetics. The enzyme is inhibited by ATP and this inhibition can be relieved by FBP. Regulation of PK from *Fasciola* by more recently discovered effectors of mammalian-type PKs, such as glucose 1,6-bisphosphate and phosphorylation/dephosphorylation, has not yet been reported.

Phosphoenolpyruvate carboxykinase (PEPCK) functions in *F. hepatica*, as in other parasitic helminths, as a CO_2-fixing enzyme. This is remarkably different from the decarboxylating anabolic role of PEPCK in mammalian gluconeogenesis. Parasitic helminths are not unique, however, in this respect, because PEPCK functions as CO_2-fixing enzyme in the glycolytic degradation of glucose in many other invertebrates, such as lower marine organisms. PEPCK activity appears to be controlled primarily by the concentration of enzyme, substrates and products, in contrast to PK activity, which is under tight allosteric control (Behm and Bryant, 1982).

PEPCK is a key enzyme in the cytosolic production of malate, an important process in the anaerobic energy metabolism of *F. hepatica* adults, whereas PK is a key enzyme in the glycolytic pathway to pyruvate, which is operative in the aerobically functioning juvenile liver fluke (Fig. 8.3). Therefore, during the change from an aerobic to an anaerobic energy metabolism that occurs during the development of *F. hepatica* in the final host, a change in the relative importance of PK and PEPCK may be expected. Such a change was indeed observed, as the development of the fluke in the host liver was accompanied by an almost complete disappearance of PK activity (Tielens *et al.*, 1987). One should bear in mind, however, that the fate of PEP at the PK/PEPCK branchpoint cannot simply be estimated by measuring the ratio of PK and PEPCK activities *in vitro*, as the use of PEP in each reaction is determined by K_m values and substrate concentrations, by the extent of phosphorylation of PK, by the presence of (in)activators, and by the rate of the following reactions.

Regulation of the flux through glycolysis occurs probably mainly in the hexokinase and phosphofructokinase reactions, but in *F. hepatica* additional regulatory processes are operative. In *F. hepatica* the product of the PFK reaction, fructose 1,6-bisphosphate (FBP), activates PK as well as lactate

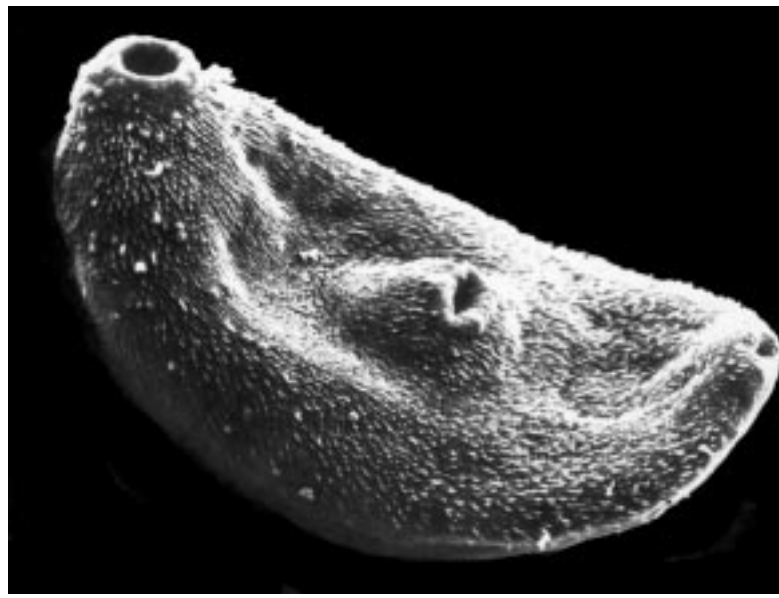

Fig. 8.3. Scanning electron micrograph of a newly excysted juvenile liver fluke, *F. hepatica*. A method developed for large-scale *in vitro* excystment of metacercariae and subsequent isolation of juvenile liver flukes was used for the preparation of this specimen (Tielens *et al*., 1981b). (Photograph © A.G.M. Tielens & C.J.A.H.V. van Vorstenbosch.)

dehydrogenase (LDH), thereby providing feed-forward control (Behm and Bryant, 1982; Lloyd, 1986). However, the metabolic relevance of this regulation by FBP is uncertain, because in *F. hepatica* the flux through this pathway is very low as lactate is only a minor end product.

The effects of serotonin (5-hydroxytryptamine, 5-HT) on the rate of glycolysis have been studied extensively in the past, in *F. hepatica* as well as in other parasitic helminths (Mansour, 1984; Mettrick, 1989; Smart, 1989). Serotonin increases the motility, glycogen degradation and glycolysis in intact liver flukes. *F. hepatica* has serotonin receptors that function through a transmembrane signalling system requiring GTP which activates adenylate cyclase (Mansour and Mansour, 1986). The formed cAMP activates a protein kinase that is probably involved in activation of glycogen phosphorylase and phosphofructokinase thus stimulating glycogen breakdown and glycolysis (Kamemoto *et al.*, 1989). The role of serotonin in the regulation of the carbohydrate metabolism of *F. hepatica* is still unresolved. The presence of serotonin in the tissues of *F. hepatica* was demonstrated, however, and the amine is concentrated in the head region of this organism where many of the serotonin-containing neurons appear to be motor neurons that are responsible for the innervation of the musculature of the digestive tract and body wall (Fairweather *et al.*, 1987; Sukhdeo and Sukhdeo, 1988).

Selected aspects of mitochondrial processes

Krebs cycle activity is used by the juvenile liver fluke after excystment from the metacercarial cyst, by the miracidia and, most likely, also by cercariae for the final part of carbohydrate catabolism. For most of the Krebs cycle enzymes of parasitic helminths, including *F. hepatica*, no differences in properties from the classical Krebs cycle enzymes are known. Recently, however, it was described that the α-ketoglutarate dehydrogenase complex (KGDC) from *F. hepatica* exhibits remarkable kinetic properties (Diaz and Komuniecki, 1996). In contrast to the mammalian type of enzyme the complex from *F. hepatica* is calcium insensitive. In addition, the enzyme is insensitive to regulation by adenine nucleotides, which has been reported for other calcium-insensitive KGDCs. The effect of calcium on the pyruvate dehydrogenase phosphatase of *F. hepatica* is not yet known, but in *Ascaris suum* this enzyme is also insensitive to calcium (Song and Komuniecki, 1994). The exact role of KGDC and pyruvate dehydrogenase in the regulation of metabolism in *F. hepatica* awaits further research, but possibly calcium ions do not serve as a regulatory signal in the mitochondrial metabolism of this parasite.

In *F. hepatica* the substrate for the Krebs cycle, acetyl-CoA, is mainly the product of the oxidation of pyruvate resulting from cytosolic carbohydrate breakdown, and not from the breakdown of amino acids or fatty acids.

In the anaerobically functioning mitochondria of adult liver flukes, malate dismutation is the main final pathway of carbohydrate degradation and not the Krebs cycle (Fig. 8.2). Malate, the major end product of the cytosolic carbohydrate degradation, is transported into the mitochondria for further degradation. Partly, it is oxidized to pyruvate via malic enzyme, which is NADP-linked in *F. hepatica* (Prichard, 1978; Tielens *et al.*, 1987). The resulting pyruvate is oxidized and decarboxylated to acetyl-CoA by the pyruvate dehydrogenase complex. This enzyme has not yet been characterized in *F. hepatica*, but the complex from *A. suum* was shown to be specially adapted to anaerobic functioning (Song and Komuniecki, 1994). Its activity is controlled by phosphorylation and dephosphorylation of the α-subunit of pyruvate dehydrogenase, effected by intrinsic kinase and phosphatase activities. The high $NADH/NAD^+$ ratio characteristic for these anaerobically functioning mitochondria does not result in activation of the kinase, which would result in inactivation of the pyruvate dehydrogenase complex (Thissen and Komuniecki, 1988).

Further degradation of acetyl-CoA to acetate occurs via a succinate/succinyl-CoA cycle with the concomitant production of ATP (Van Vugt *et al.*, 1979; Saz *et al.*, 1996). Recently, the presence of the first enzyme of this cycle, the acetate:succinate CoA transferase which catalyses the transfer of CoA from acetyl-CoA to succinate, was unequivocally demonstrated in *F. hepatica* as well as in Trypanosomatidae (Van Hellemond *et al.*, 1998).

The above-mentioned oxidation of part of the incoming malate, which results in formation of NADH, is balanced by the reduction of another part of malate to succinate. This reduction of malate to succinate occurs via fumarate

by two reactions which reverse part of the Krebs cycle (Fig. 8.2). The reduction of fumarate by NADH is coupled to an electron-transport-linked phosphorylation of ADP at site I of the respiratory chain (see below).

In *F. hepatica*, succinate is not a main end product as it is further metabolized to propionate. This occurs by a reversal of the reactions involved in the formation of succinate from propionate in mammalian tissues. The decarboxylation of succinate to propionate in *F. hepatica* is coupled to substrate-level phosphorylation (Köhler *et al.*, 1978; Pietrzak and Saz, 1981).

Electron transport chain

In the juvenile liver fluke (Fig. 8.3) and in the miracidia, a respiratory chain up to cytochrome oxidase is active and all evidence obtained so far indicates that this electron transport chain is not different from the classical one present in mammalian mitochondria (Fig. 8.4). In the aerobically functioning stages, electrons are transferred from NADH and succinate to ubiquinone via complexes I and II of the respiratory chain, respectively. Subsequently, these electrons are transferred from the formed ubiquinol to oxygen via complexes III and IV of the respiratory chain.

In the anaerobically functioning mitochondria of the adult liver fluke, however, this electron transport chain is altered as oxygen is not used as terminal electron acceptor. Endogenously produced fumarate functions as terminal electron acceptor during malate dismutation. In this case, electrons

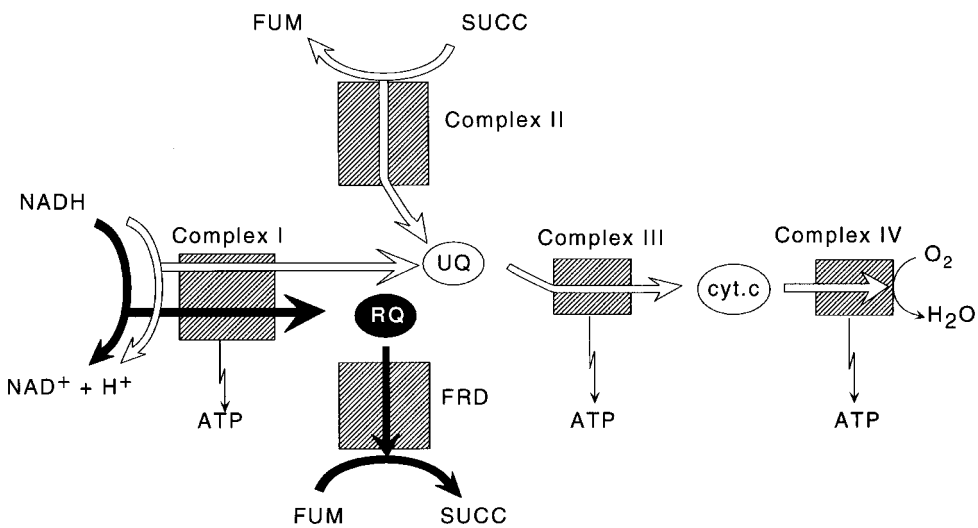

Fig. 8.4. Schematic representation of the electron transport chain in *F. hepatica*. Electron flow to oxygen during aerobic respiration is indicated by open arrows, whereas the electron flow during malate dismutation is indicated by filled arrows. Abbreviations: cyt. c, cytochrome c; FUM, fumarate; SUCC, succinate; RQ, rhodoquinone; UQ, ubiquinone.

are transferred from NADH to fumarate via complex I and fumarate reductase (Fig. 8.4). This implies that during the development of *F. hepatica* in its final host, a transition occurs from succinate oxidation via succinate dehydrogenase in the Krebs cycle of the juvenile liver fluke to the reverse reaction: reduction of fumarate to succinate in the adult. Bacteria contain two homologous but distinct enzyme complexes: one to oxidize succinate (succinate dehydrogenase) and one to reduce fumarate (fumarate reductase), although each enzyme will catalyse both reactions *in vitro*. *In vivo*, distinct enzyme complexes are needed for these opposite reactions as the electron flow through the complex is reversed, which implies differences in the affinity for electrons (standard electron potential) of the electron-binding domains of these enzyme complexes (Ackrell *et al.*, 1992; Van Hellemond and Tielens, 1994). Recently, distinct enzyme complexes have been described in the parasitic nematodes *Haemonchus contortus* (Roos and Tielens, 1994) and *A. suum* (Saruta *et al.*, 1995). These complexes were shown to be differentially expressed during the life cycle of the parasites and are suggested to function either as a succinate dehydrogenase or as a fumarate reductase. Evidence for differential expression of two different enzyme complexes in *F. hepatica* is still lacking, but it has been shown that the kinetic properties of the complex from adult liver flukes resemble those of other organisms known to reduce fumarate (Van Hellemond *et al.*, 1995). Mitochondria of adult *F. hepatica* had a low SDH/FRD activity ratio, which corresponds to their main *in vivo* function. On the other hand, significant differences in activity ratios were not detected between different stages of *F. hepatica* (adult and metacercariae), although *in vivo*, the free-living stage oxidizes succinate, whereas the adult reduces fumarate. Apparently, the observed change in the activity ratio that was observed between free-living and parasitic stages of *A. suum* does not occur in *F. hepatica* (Saruta *et al.*, 1995; Van Hellemond *et al.*, 1995). The low SDH/FRD activity ratio, which was observed in *F. hepatica* and in all other fumarate-reducing eukaryotes (parasitic helminths as well as lower marine organisms), is caused by a markedly increased fumarate reduction activity compared with mammalian type mitochondria, which correlates with the significant capacity for fumarate reduction of the mitochondria from fumarate-reducing eukaryotes.

In addition to distinct enzyme complexes for the succinate oxidation and fumarate reduction, distinct quinones are also involved in these processes in parasitic helminths. The electron transport chains of many bacteria employ menaquinone when fumarate is the final electron acceptor (Collins and Jones, 1981). In parasitic helminths, however, the presence of rhodoquinone was demonstrated and because rhodoquinone is present mainly in anaerobic, fumarate-reducing stages, it was suggested that rhodoquinol functions as electron donor in fumarate reduction, similar to menaquinol in fumarate reduction of bacteria (Allen, 1973). It was shown that rhodoquinone is an indispensable component for efficient electron transport in the anaerobic electron transport chain of all eukaryotic organisms investigated so far and which reduce fumarate during anoxia (Van Hellemond *et al.*, 1995). Rhodoquinone with its relatively low redox potential ($E'_0 = -63$ mV) is comparable to menaquinone in this respect and transfers electrons via

fumarate reductase to fumarate (E'_0 = 30 mV), whereas ubiquinone (E'_0 = 110 mV) preferentially donates electrons to complex III of the electron transport chain.

The essential function of rhodoquinone in the anaerobic energy metabolism during the entire life cycle of *F. hepatica* was demonstrated as the amount of rhodoquinone present reflected the importance of fumarate reduction in various stages (Van Hellemond *et al.*, 1995, 1996). Throughout the life cycle of *F. hepatica* a strong correlation is found between the quinone composition and the type of metabolism: the amount of ubiquinone is correlated with the use of the aerobic respiratory chain, and the amount of rhodoquinone with the use of fumarate reduction. The amount of both quinones is thus adapted to the energy metabolism during the life cycle, and as both quinones are synthesized *de novo* by *F. hepatica*, the rate of synthesis of both quinones is apparently adapted to their use. Furthermore, it was demonstrated that in *F. hepatica* adults, the rhodoquinone contains mainly ten isoprenoid units and is not produced by modification of ubiquinone obtained from the host, but that rhodoquinone and ubiquinone are synthesized *de novo* via the mevalonate pathway (Fig. 8.1). Further analyses of the rhodoquinone synthesis prompted the suggestion that rhodoquinone is synthesized by *F. hepatica* via a pathway nearly identical to that of ubiquinone biosynthesis: possibly only the final reaction differs (Van Hellemond *et al.*, 1996).

Transitions in Energy Metabolism

In the different stages of its life cycle, *F. hepatica* has to adapt to different environments. Among other chemical and physical parameters, the availability of oxygen and substrates varies widely during the life cycle. Therefore, transitions have to occur, for instance, from the fully aerobic functioning of the juvenile liver fluke to the almost completely anaerobic functioning of the adult parasite in the bile duct. Most likely a comparable switch from an aerobic to an anaerobic energy metabolism will also occur during the development of miracidia into sporocysts and rediae, but this switch has not yet been studied at the metabolic level. It should be realized that these transitions in *F. hepatica* are definitive, as the same organism will not encounter its previous environment again. The change to a fermentative metabolism is permanent and is not forced by a temporarily hypoxic environment or burst-type of exercise, and therefore, these metabolic switches are not comparable to the transient switches that occur in, for instance, skeletal muscle and lower marine organisms like *Mytilus edulis*.

After emergence from the metacercarial cyst, the juvenile liver fluke (Fig. 8.3), which is almost exclusively dependent on Krebs cycle activity, develops gradually into a fermenting adult that lives in the bile ducts of the definitive host (Tielens, 1994). During this development three different pathways of glucose breakdown successively provide the major part of ATP production (Fig. 8.5). Krebs cycle activity, which is by far the main source of energy of the juvenile fluke, gradually decreases during the development of *F. hepatica*

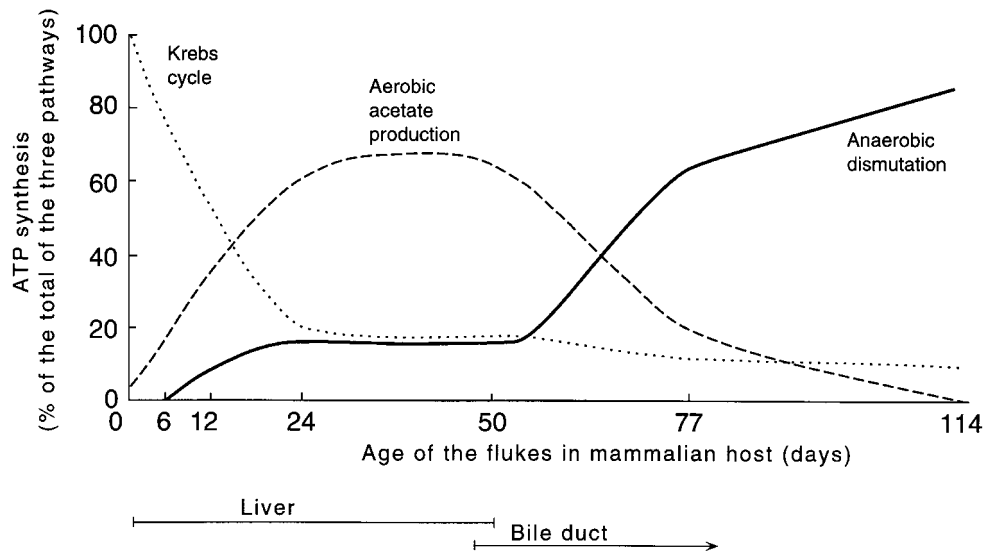

Fig. 8.5. Changes in energy metabolism during the development of *F. hepatica* in the final host. Contribution of the three pathways of glucose breakdown to ATP synthesis is shown (Krebs cycle, aerobic acetate production and anaerobic dismutation). (After Tielens *et al.*, 1984.)

in the liver parenchyma. Concomitantly, acetate becomes the major end product of the late parenchymal and early bile stages of the worm (Tielens *et al.*, 1982). This formation of acetate requires oxygen for the re-oxidation of the formed NADH, and is the most important source of energy for the developing fluke in the liver parenchyma. Finally, in the bile ducts, the adult type of energy metabolism develops when the anaerobic production of propionate and acetate provides essentially all the ATP required (Tielens *et al.*, 1984). Apparently, the aerobic capacity of *F. hepatica* decreases during its development in the liver of the host. The observed decrease in Krebs cycle activity per milligram of total protein cannot be explained by a slow decrease in the number of remnant mitochondria from an earlier, more aerobic, stage. It was shown that, when calculated per fluke, the Krebs cycle activity actually increased immensely during development and that this increase was directly proportional to the surface area of the fluke (Tielens *et al.*, 1984). This correlation holds for the entire development from the newly excysted juvenile to the mature fluke from the bile ducts (Plate 1). These observations support the view that Krebs cycle activity is limited by the diffusion of oxygen and occurs only in the outer layer of the parasite. Parasitic helminths possess neither respiratory organs nor a circulatory system. Therefore, the availability of oxygen inside the tissues is dependent on its slow diffusion, and growth of the parasite limits its aerobic capacity. In the adult stage the aerobically functioning outer layer is far less than 1% of the total volume, making the metabolism of *Fasciola* appear to be completely anaerobic.

Oxidative phosphorylation is, however, probably essential for the functioning of adult flukes as they are sensitive to uncouplers like the anthelmintic closantel (Skuce and Fairweather, 1990; see Fairweather, Chapter 7 of this volume).

The metabolism of the developing liver fluke is thus altered by a lack of oxygen. This happens in two steps. First, as outlined above, in the aerobic liver parenchyma of the host the limited diffusion of oxygen in the growing fluke will result in a lack of oxygen in the innermost tissues of the parasite. Second, after arrival in the bile ducts, the entire fluke will have to function anaerobically because of the very low oxygen content of the bile. The prolonged stay in this anaerobic environment will ultimately result in metabolic adaptations both in the cytosol and in the mitochondria (Lloyd, 1986; Tielens *et al.*, 1987). A striking example of such an adaptation is the change in quinone composition that occurs during the development of *F. hepatica* (Van Hellemond *et al.*, 1995, 1996). Mitochondria of adult *Fasciola gigantica* showed weak or no activity for cytochrome *c* oxidase activity, indicating that this parasite is also adapted to the anaerobic environment in the bile duct (Fujino *et al.*, 1995). The nematode *A. suum* is also known to undergo a metabolic transition from an aerobic to an anaerobic energy metabolism, accompanied by adaptations in enzyme levels (Vanover-Detling and Komuniecki, 1989; Duran *et al.*, 1993; Saruta *et al.*, 1995; Klingbeil *et al.*, 1996).

Cytochemical staining of cytochrome oxidase in tissues of parasitic nematodes indicated that in these parasites a metabolic gradient exists, as in *F. hepatica*: an aerobic energy metabolism near the outside and a more anaerobic one inside (Fry and Beesley, 1985). Furthermore, it is known that nematode species with a large diameter have a more anaerobic metabolism than the thinner ones (Atkinson, 1980). Apparently, the diffusion of oxygen is a limiting factor for an aerobic energy metabolism in many parasitic helminths.

During the development of *Fasciola* changes also occur in the cytosolic metabolism. In the adult the cytosolic degradation of glucose via PEPCK is the most important route, whereas in the juvenile a large part is degraded via PK. However, the adult probably does not exclusively use the PEPCK pathway, nor does the juvenile exclusively use the PK pathway (Tielens *et al.*, 1987). In both stages a mixture of malate and pyruvate is the substrate for the mitochondria (Tielens *et al.*, 1981a, 1987; Kane and Bryant, 1984). The presence of malic enzyme, both in the cytosol and the mitochondria, enables the use of this mixture in any ratio as substrate for the mitochondrial production of acetate and propionate.

The pH profiles of PK and PEPCK of *Fasciola* show that metabolism at the PK/PEPCK branch point could be regulated by the pH (Tielens *et al.*, 1987). Such a pH effect possibly complements regulation by (de)phosphorylation of PK during the aerobic/anaerobic transitions of, for instance, bivalve molluscs (Zwaan and Dandoo, 1984). When, during the development of *Fasciola*, the inner layers of the parasite will gradually be forced to anaerobic functioning, the acid end products will lower the cytosolic pH. After arrival in the bile ducts, the excretion of these end products together with the restricted flow of

bile and the limited buffer capacity will acidify the environment of the fluke and, as ^{31}P-NMR studies have shown, this will contribute to the decrease in intracellular pH (Tielens *et al.*, 1982). This lower internal pH will favour a partitioning of the flux towards malate formation at the PK/PEPCK branch point. Eventually, a lasting predominance of the PEPCK pathway occurs as PK activity almost completely disappears (Tielens *et al.*, 1987).

Although the juvenile liver fluke degrades glucose to carbon dioxide in the presence of oxygen, it is already fully equipped for anoxic functioning: in the absence of oxygen the newly excysted juvenile produces – just like the adult – propionate and acetate, in a molar ratio of 2:1 (Tielens *et al.*, 1981a). This readiness for anoxic functioning, and hence fumarate reduction, is also revealed by the observed presence of rhodoquinone in the metacercariae.

Transitions in energy metabolism between other stages in the life cycle of *F. hepatica* have not yet been studied metabolically, but it is known that in *S. mansoni* a transition in energy metabolism occurs during the transformation of miracidia into sporocysts, which is comparable to the transition occurring during the transformation from cercaria to schistosomulum (Tielens *et al.*, 1992).

Excretory–Secretory Products

Apart from for instance eggs and the end products of the degradation of substrates, *F. hepatica* adults are known to excrete or secrete other substances, of which at least some are used in the interaction with the host. These substances are often referred to as excretory–secretory products (or ESP). Removal of waste products, like the end products of metabolism, is considered to be excretion, whereas for instance the discharge of substances that play a role in the interaction with host or food is called secretion.

Many of the excretory–secretory products are immunogenic and can, therefore, be used for the diagnosis of fasciolosis in humans and animals. Immunological assays are powerful diagnostic tools for the detection of fasciolosis, because the reactions are very specific and sensitive, which enables the rapid differentiation between various parasites or *Fasciola* species, often at a very early stage of the infection (Osman *et al.*, 1995; Qureshi *et al.*, 1995; Rodriguez-Perez and Hillyer, 1995; Martinez *et al.*, 1996; Sampaio-Silva *et al.*, 1996).

Fasciola hepatica is known to synthesize haemoglobin, which is a predominant molecule in extracts of adult liver flukes (McGonigle and Dalton, 1995). Antibodies to this haemoglobin can be detected in sera of infected cattle as early as one week after infection. Inside the parasite this haemoglobin might play a role in oxygen transport and might function as a reservoir of oxygen necessary for tanning of the eggshells. A possible function of this haemoglobin as an excretory product is unknown, and it is pointed out that it is uncertain whether the haemoglobin is actively excreted or is liberated from dying parasites (McGonigle and Dalton, 1995).

As discussed earlier, *F. hepatica* produces proteases which are excreted to catalyse the degradation of proteins from the host (Yamasaki *et al.*, 1992;

Smith *et al.*, 1993; Carmona *et al.*, 1994). Many of these proteases are now being characterized and are also tried as vaccines (Wijffels *et al.*, 1994; Dalton *et al.*, 1996).

The amino acid proline is also an important excretory–secretory product of liver flukes. This proline is thought to induce physiological reactions in the host; it induces the hyperplasia of the bile duct epithelium that accompanies fasciolosis (Isseroff *et al.*, 1977; Sawma *et al.*, 1978). Already during the period when the young flukes are travelling through the liver parenchyma of the infected host, the bile duct epithelium shows evidence of hyperplasia (Dawes, 1963). Eventually the duct endothelium becomes extensively folded and the duct lumina enlarge to enable entry and establishment of the large adult flukes. Starting about 25 days after infection the concentration of proline in bile of infected animals increases steadily and it was shown that the flukes are the most likely source of this excessive proline (Isseroff, 1980). Other putative roles of the observed high proline levels inside adult liver flukes are still unknown.

The tegument of *F. hepatica* is also supposed to play an important role in another aspect of excretion: the osmoregulation of the fluke and the dynamic response to changes in osmolality of the environment. Ion pumps in the tegumental membrane are believed to regulate ion levels inside the fluke. However, recently it was observed that *F. hepatica* obtained from bovine, ovine or rat liver differed: they had different ion compositions (Caseby *et al.*, 1995). Furthermore, in contrast to earlier studies this study also showed that the levels of potassium, magnesium and calcium cations are actually greater in the liver fluke than in bile. These findings might indicate that in *F. hepatica* ion levels are dependent on the environment and are not as strictly regulated as supposed earlier in the tegumental osmoregulatory model (Threadgold and Brennan, 1978).

Concluding Remarks

Fasciola hepatica has many interesting properties in its biochemical processes and especially in its energy metabolism. In the different stages of its life cycle, *F. hepatica* has to adapt its metabolism to the different environments it encounters. Free-living stages do not gather food and are therefore completely dependent on the endogenous stores they acquired in the previous host. Oxygen, on the other hand, is present in the environments of the free-living stages (cercariae and miracidia) and the newly excysted juvenile liver fluke. This enables these stages to live very economically: they use Krebs cycle activity and oxidative phosphorylation to obtain as much energy as possible from the breakdown of carbohydrates. In the free-living stages, this thrifty metabolism serves their only mission very well: becoming a parasitic stage in the next host.

The adult parasitic stage in the final host (and most likely also that in the intermediate host) uses mainly a fermentative process for the generation of energy. For these stages an uneconomical energy metabolism is not detrimental as the host provides the food. Adult liver flukes produce mainly

propionate and acetate as end products of the fermentative degradation of carbohydrates. These end products are formed via malate dismutation, a fermentative pathway that is common in parasitic helminths, but occurs also in lower animals like freshwater snails, mussels, oysters and other lower marine organisms.

It should be realized that all parasitic helminths have an (at least partly) anaerobic, and hence wasteful, energy metabolism when parasitic, but a thrifty (aerobic) one when they become free living and external substrates are no longer freely available. It is not yet known whether the specialized anaerobic pathways of parasites have evolved as a reaction to the hypoxic conditions that occur in many of their habitats, or whether the anaerobic energy metabolism itself has certain unidentified advantages. The excretion of anaerobic end products could be important for the parasite either in its continuous struggle with the defence mechanisms of the host or by the effect of these products on the habitat of the parasite. Otherwise, when glucose is abundant and free, it could be advantageous for an organism to use high-power, low-efficiency pathways. Thus reasoning, anaerobic functioning is not a defect – as it is often regarded – but a positive property, made possible by the literal meaning of parasitism: eating the food of others.

Fasciola hepatica has also in other biochemical pathways interesting features that reflect their parasitic way of living. They have lost several main anabolic pathways, for instance the ability to synthesize lipids and purines *de novo*. Therefore, many substances have to be obtained from the host, but *F. hepatica* is then able to modify them to suit its own needs. *F. hepatica* is apparently adapted in many ways to the opportunistic way of living of a parasite.

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9 Neurobiology

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Introduction

The neurobiology of trematodes has, until quite recently, received very little attention as a topic for research. One obvious reason for this is rooted in the impracticalities of analysing the physiology of the nervous systems of relatively small, acoelomate animals. Another reason is simply the lack of interest, perpetuated by the long-held view that the parasitic way of life engenders simplification, and that the neurosensory abilities and neuromuscular control systems of flatworm parasites are largely reduced or degenerate. Today, as a result of a multidisciplinary approach to the subject, it is evident that trematodes such as *Fasciola*, far from being simple, exhibit often complex behaviour patterns during which they recognize and respond to a plethora of environmental and host-derived cues, implying the presence of well-developed sensory modalities. Thus, electron microscopy has exposed an unsuspected diversity of putative sense organs in the worms; cytochemical and biochemical studies have demonstrated an unprecedented complexity in the chemical composition of their nervous systems; and physiological data on receptors and ion channels are beginning to emerge from the application of voltage-clamp technology and the use of isolated muscle fibres. As a result of this upsurge of exploration, it is becoming increasingly apparent that while the habit of parasitic flatworms may in some respects seem regressive, compared to the more overt activities of their free-living counterparts, many aspects of trematode neurobiology appear to be just as well developed, or even more so. Research developments on the neuromusculature of trematodes, and in particular those of economic importance like *Fasciola*, offer the prospect of identifying pharmacologically important receptors and ion channels, and of determining which are potential drug targets for therapeutic exploitation.

Fasciolosis (ed. J.P. Dalton)

Neuroanatomy of Adult and Larval Stages

Historically, the nervous system of *Fasciola* has been the subject of a number of classical light microscope observations (Lang, 1880; Sommer, 1880; Bettendorf, 1897; Havet, 1900). The advent of transmission and scanning electron microscopy in the 1960s and 1970s, respectively, saw the emergence of numerous ultrastructural studies of the innervation and putative sense organs of both the adult and larval stages. Staining procedures have also advanced the understanding of the trematode nervous system, such as those involving methylene blue and Gomori stains to highlight nerve cells, and silver and gold impregnations for resolving the distribution patterns of putative sensory endings. More recently, effective use has been made of enzyme cytochemistry to localize cholinesterase (ChE) activity in these worms and thereby identify acetylcholine (ACh)-related components. Furthermore, immunocytochemistry, in which labelled antibodies are employed as highly specific cytological probes to localize neuroactive substances, has been used to identify biogenic amines and neuropeptides at both light and electron microscopic levels. Immunocytochemical techniques in particular have proved to be highly effective in localizing putative transmitters and modulators and imaging their neuronal pathways in worms, especially when used in conjunction with confocal scanning laser microscopy (see Halton *et al*., 1994, 1997; Halton and Gustafsson, 1996). Collectively, these approaches have enabled researchers to better define the neuroanatomical components, and thus distinguish the cholinergic, aminergic and peptidergic elements and their relative distribution patterns in a wide range of trematode species, including *Fasciola hepatica*.

Gross structure – adult

In common with other parasitic flatworms, the basic organization of the nervous system of *Fasciola* is bilateral and differentiated into a central nervous system (CNS), comprising a brain, longitudinal nerve cords and transverse connectives in an orthogonal arrangement, and a peripheral nervous system (PNS) that essentially provides motor and sensory innervation to the body wall, adhesive organs, pharynx, and reproductive tract (Fig. 9.1A, B). In these respects, it resembles the turbellarian nervous system. The adult brain consists of a pair of cerebral ganglia, situated either side of the pharynx, which are connected by a broad and largely fibrous dorsal commissure. From each ganglion, three nerve tracts proceed anteriorly to innervate the oral sucker, pharynx and mouth region, and three well-differentiated nerve cords proceed posteriorly. The disposition of the posterior cords are dorsal, lateral and ventral, of which the ventral cords are the best developed and are fused posteriorly. Laterally, all three nerve cords give rise to numerous nerve fibres, many of which cross-link with their opposite numbers at irregular intervals to form transverse connectives. Others extend to the ventral sucker and body musculature where they divide and anastomose, giving rise to acetabular and subsurface nerve plexuses, respectively. There is a similar arrangement of

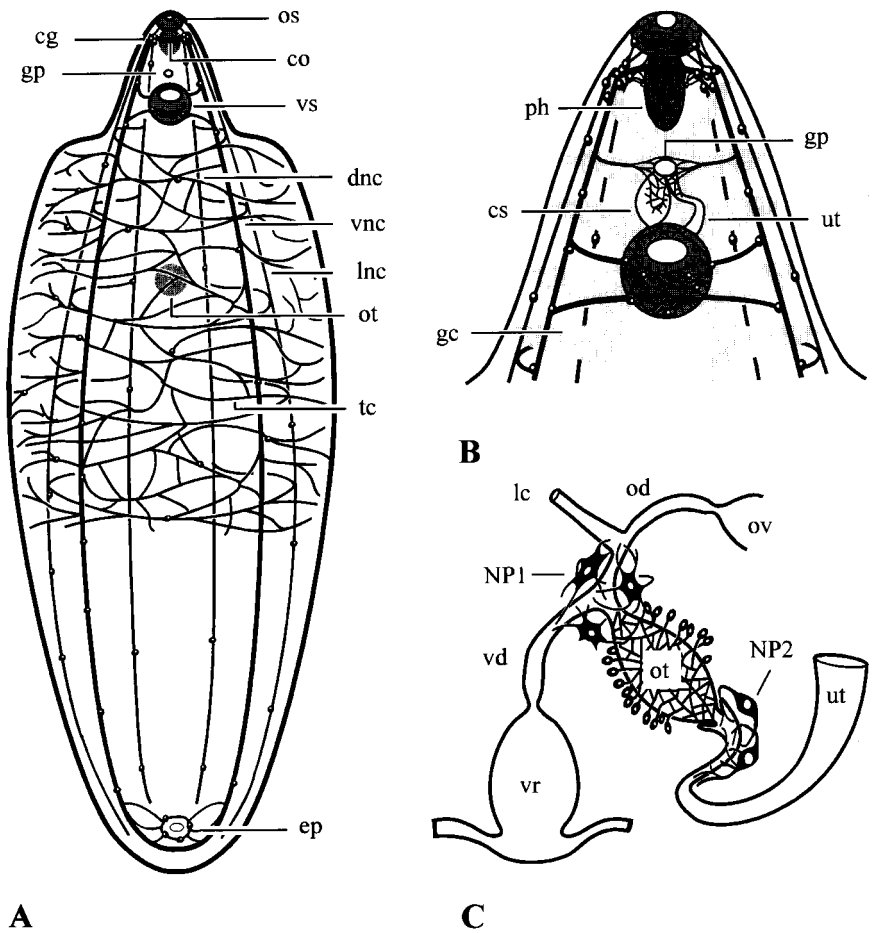


Fig. 9.1. (A) A generalized schematic of the nervous system of *Fasciola hepatica*. (B) Detail of the innervation of the forebody. (C) Peptidergic innervation of the egg chamber (ootype). Note plexuses of peptidergic cells (NP1, NP2) at the entrance and exit of ootype. *Key*: cg, cerebral ganglion; co, commissure; cs, cirrus sac; dnc, dorsal nerve cord; ep, excretory pore; gc, gut caecum; gp, gonopore; lc, Laurer's canal; lnc, lateral nerve cord; od, oviduct; os, oral sucker; ot, ootype; ov, ovary; ph, pharynx; ut, uterus; vd, vitelline duct; vnc, ventral nerve cord; vr, vitelline reservoir; vs, ventral sucker; tc, transverse connective.

plexuses in the oral sucker and pharynx, derived from the anterior nerves, and in the copulatory apparatus and ducting of the reproductive system.

Cholinergic system

Extensive eserine-sensitive ChE activity has been demonstrated cytochemically throughout nerves in both the CNS and PNS of adult *Fasciola* (Halton, 1967;

Krvavica *et al.*, 1967; Ramisz and Szankowska, 1970; Magee, 1990). The strongest staining was evident in the cerebral ganglia and ventral nerve cords, along which bipolar nerve cells were located at intervals. In contrast, very few cholinergic nerve cells were found in the cerebral ganglia, the latter appearing to be composed largely of masses of fibres converging from the adjacent nerve cords and commissure. ChE activity has been demonstrated ultrastructurally in the cerebral ganglia of *Fasciola*, both within the ER–Golgi apparatus in neuronal cell bodies and, extracellularly in the neuropile, at sites associated with synaptic endings containing small, clear vesicles (S.C. Sukhdeo *et al.*, 1988b). The distribution of ChE is consistent with it functioning as a degrading enzyme in cholinergic transmission. Branches from the main nerve cords anastomose throughout the body of the fluke and form a network of fibres, connected by densely staining bipolar nerve cells, and extending from the level of the pharynx to just anterior to the excretory pore. The dendritic nature of the nervous system, as seen by ChE-staining, is particularly evident in the posterior half of the worm. At the level of the ventral sucker, two well-developed nerve tracts leave each of the ventral nerve cords and extend centrally towards the acetabulum where they divide and encircle the sucker as a marginal ring complex. There is comparable staining for ChE in the oral sucker and pharynx. Cholinergic fibres also innervate the dorsoventral body muscles, and the muscle fibres surrounding the uterus and gastrodermis. In the excretory system there is a similar arrangement of fine ChE-reactive fibres innervating the muscles of the excretory bladder; several unipolar neurons occur in this region, with their axons extending into the bladder wall. In the body wall region, there is an extensive subsurface plexus of fibres associated with the circular and longitudinal muscle layers and, in places, fine cholinergic fibres have been observed in the tegument itself, particularly in the anteriolateral margins of the worm, where they terminate in the form of bulbs. Strong reactivity for ChE has been reported as present in the muscles themselves, particularly those of the pharynx, suckers and the muscular sheath of the cirrus sac (Halton, 1967).

Aminergic system

Nerve fibres containing catecholamines (CAs) and serotonin (=5-hydroxytryptamine, 5-HT) are widespread in both the CNS and PNS of *Fasciola*, as are those that are immunoreactive with antibodies to the amino acids, glutamate, and, to a lesser extent, γ-aminobutyric acid (GABA). Using a modified Falck and Owman (1965) fluorescence histochemical method, Bennett and Gianutsos (1977) demonstrated reactivity for dopamine in neuronal cells and fibres of the nervous system of immature specimens of the worm, and showed that its distribution was similar to that described by Shishov *et al.* (1974) for CAs in the adult fluke. Moreover, the pattern of reactivity correlated to some extent with that for ChE activity, as described above, i.e. occurring throughout much of the CNS and PNS.

Reports by Fairweather *et al.* (1987), S.C. Sukhdeo and M.V.K. Sukhdeo (1988) and Magee (1990), each using immunocytochemical methodologies, recorded similar distribution patterns for 5-HT, but differences in the numbers of 5-HT-immunoreactive cell bodies in the CNS were observed. Thus,

Fairweather *et al.* (1987), using conventional fluorescence microscopy, described numerous bipolar nerve cell bodies in the cerebral ganglia and fewer immunoreactive cells along the nerve cords, whereas with the improved resolution of confocal microscopy Magee (1990) observed a single multipolar neuron per ganglion and many immunoreactive cells occurring in groups along the nerve cords. The study by S.C. Sukhdeo and M.V.K. Sukhdeo (1988) revealed five immunoreactive cell bodies in each cerebral ganglion, the majority being spindle-shaped (25–30 µm in length) and comprising a mixture of uni-, bi- and multipolar types with processes extending from the ganglia to innervate the muscle fibres of the oral sucker and pharynx (Fig. 9.2). In addition, numerous small, bilaterally arranged bipolar neurons (10–15 µm in size) were observed in the vicinity of the cerebral ganglia, with many of them innervating the pharynx and others innervating the body wall muscles.

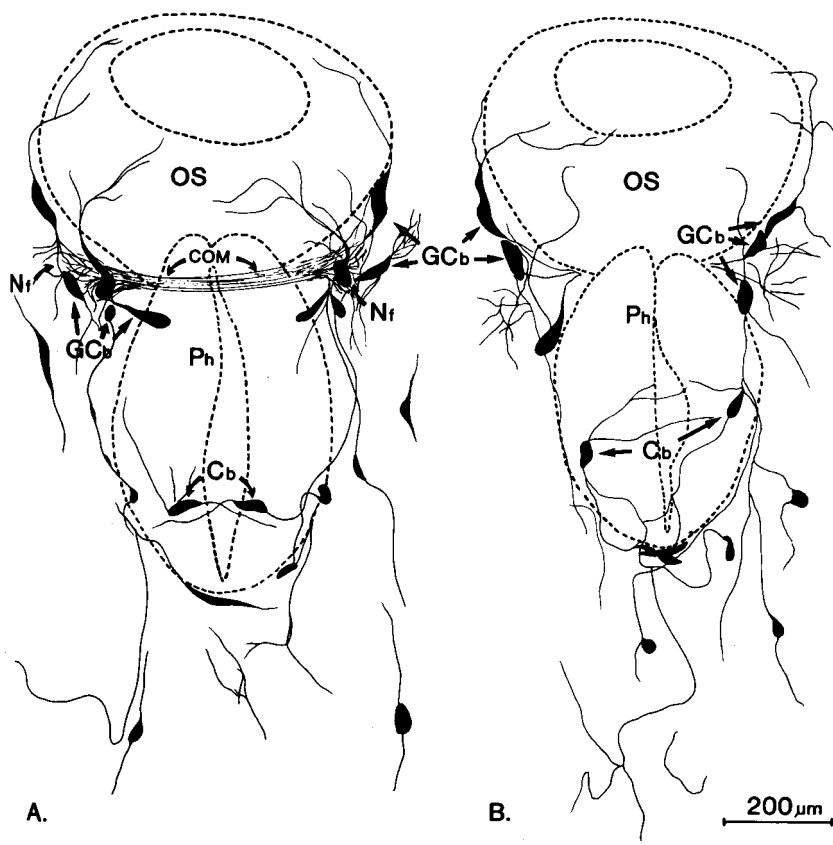


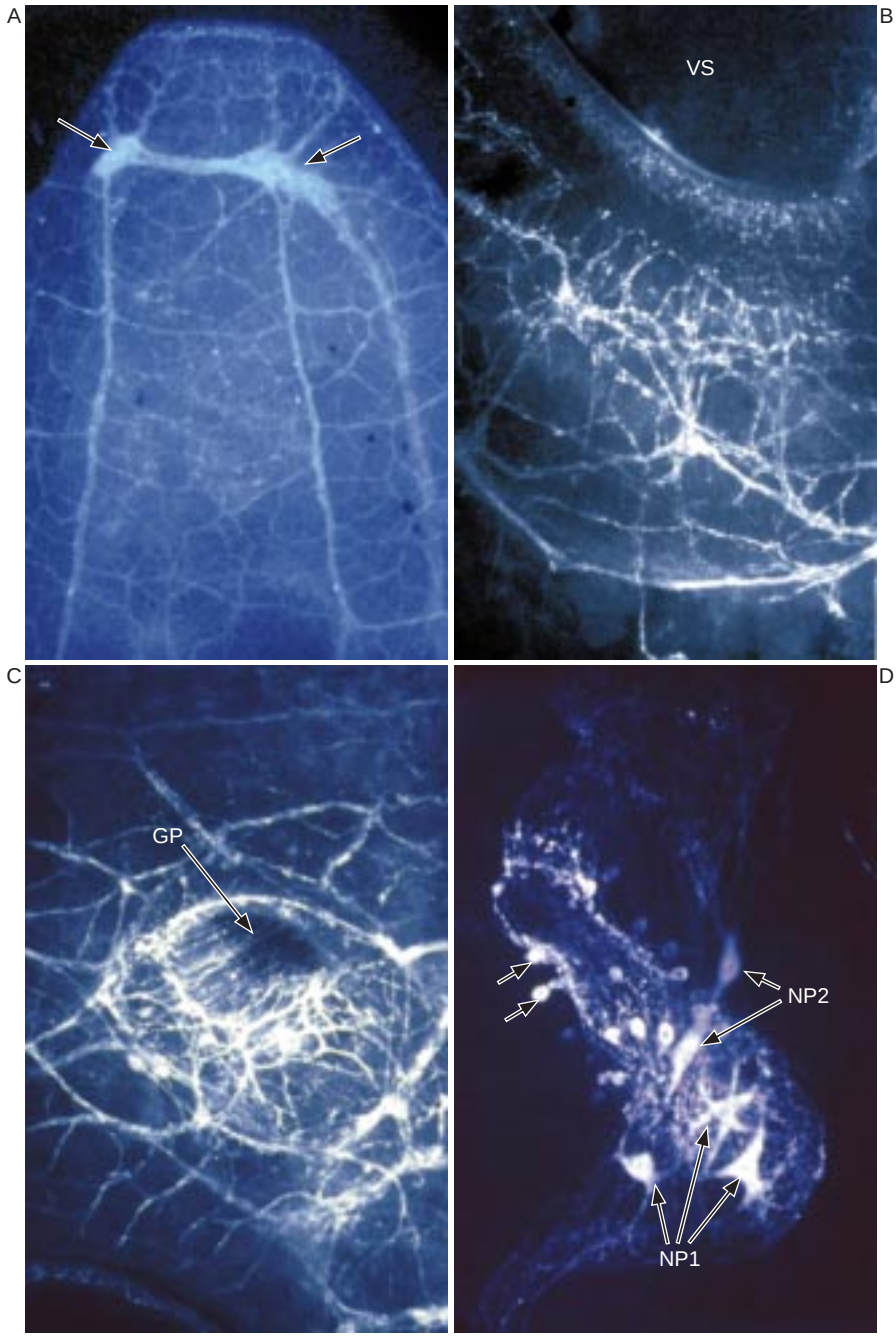
Fig. 9.2. Camera lucida drawings of 5-HT-immunoreactive cells in the forebody of *Fasciola hepatica*. (A) Dorsal view, showing five cell bodies in each ganglion (GCb). (B) Ventral view, from which can be seen three cell bodies per ganglion. *Key*: Cb, peripheral cell bodies; COM, commissure; Nf, neuropile area; OS, oral sucker; Ph, pharynx. (After S.C. Sukhdeo and M.V.K. Sukhdeo, 1988.)

Reactivity with anti-glutamate antibodies has been demonstrated in nerve cells and fibres in the cerebral ganglia and commissure and in all three longitudinal nerve cords; staining was also evident in the innervation of the suckers and ootype/Mehlis' gland complex (Brownlee and Fairweather, 1996). In contrast, immunostaining with anti-GABA antibodies has been found only in the longitudinal cords and lateral nerves in the posterior half of the worm, and in thin-fibre bundles under the tegument (Eriksson *et al*., 1995).

Peptidergic system

The existence of a peptidergic component in the nervous system of *Fasciola* was first recognized through the application of neurosecretory staining techniques, and from histophysiological evidence of secretory activity in nerve cells (Grasso,1967a,b; Grasso and Quaglia, 1972, 1974; Radlowski, 1975). The first immunocytochemical studies on neuropeptides in *Fasciola* described strong and extensive labelling in the CNS and PNS, using antisera to conserved C-terminal motifs of vertebrate pancreatic polypeptide (PP) and peptide tyrosine YY (PYY) (i.e. members of the PP-fold family of peptides) and to the molluscan peptide, FMRFamide (Magee *et al*., 1989). Following the isolation of neuropeptide F (NPF) and of the FMRFamide-related peptide (FaRP), GNFFRFamide from the tapeworm, *Moniezia expansa* by Maule *et al*. (1991, 1993), it was suggested that this immunostaining was due to cross-reactivity with *native* parasitic flatworm neuropeptides, largely as a result of shared homology in the C-terminus of the peptides involved. Accordingly, using antisera to NPF and to GNFFRFamide, Marks *et al*. (1995) showed that there was widespread neuropeptide immunostaining at all of the sites described by Magee *et al*. (1989) for PP-fold peptides and FMRFamide. Moreover, pre-adsorption (antigen blocking) studies and radioimmunoassay confirmed the presence of a specific NPF-related peptide and FaRP in the worm (for further details, see section on peptidergic components). The study by Marks *et al*. (1995) further revealed that there were no discernible differences in the staining patterns for the two peptides. Staining in the CNS was most apparent in the cerebral ganglia and commissure and in the longitudinal nerve cords, particularly the ventral

Fig. 9.3 (*opposite*). Confocal scanning laser microscope (CSLM) fluorescence images of FaRP-immunoreactivity (IR) in whole-mount preparations of *Fasciola hepatica*. (A) Oral cone region of worm, showing strong peptide-IR in the cerebral ganglia (arrows), commissure and associated anterior and posterior nerve cords. (B) A plexus of fine fibres provides the innervation of the ventral sucker (VS), and is derived from larger fibres that are in continuity with cross-connectives of the ventral nerve cords. (C) Innervation of the musculature surrounding the genital atrium and gonopore (GP). (D) Innervation of the egg-forming apparatus, showing the three immunoreactive, stellate-shaped cells (=NP1 cells) situated at the proximal end of the ootype, and two large, immunoreactive cells (=NP2 cells) at the junction of ootype and uterus. Note the numerous, immunoreactive cell bodies of neurons innervating the ootype wall (arrows). (After Marks *et al*., 1995.)


A
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cords, and their connectives (Fig. 9.3A). The immunostained nerve cell bodies in the cerebral ganglia were a mixture of uni-, bi- and multipolar types, averaging 10 × 6 μm in diameter, with individual cell bodies, largely bipolar in form (*c.* 10 × 14 μm in size), occurring at intervals along the longitudinal cords and peripheral to the central core of fibres. These were most evident in the ventral cords of the forebody region of the worm. A ring-like arrangement of large multipolar neurons (*c.* 30 × 20 μm in diameter) surrounds the excretory bladder. PNS staining for neuropeptides was strongest in the extensive nerve plexuses of the body-wall muscle layers and the two suckers (Fig. 9.3B), and in the innervation to the muscles that invest the ducting of the female reproductive system (Fig. 9.3C,D). Intense immunostaining was found associated with the egg chamber (see section on neurobiology of reproduction below).

Positive immunoreactivity for vertebrate substance P (SP) has also been demonstrated in the cerebral ganglia and commissure, and in the main cords and their connectives (Magee *et al.*, 1989). In the PNS, immunostaining was also observed in a plexus of fibres associated with the circular and longitudinal muscle fibres of the body wall, and in the muscular wall of the cirrus pouch.

Gross structure – miracidium

The nervous system of the miracidium consists of an anterior neural mass or ganglion from which are derived six nerve trunks that extend to the body wall. ChE activity has been detected in the region of the eyespots and ganglion, where it was found concentrated in the rind of cell bodies rather than in the central neuropile (Krvavica *et al.*, 1967; Panitz and Knapp, 1967; Magee, 1990). The ganglionic mass has also been found to be immunoreactive for 5-HT, as have the longitudinal nerve tracts and the associated transverse connectives, with many of the fibres displaying varicosities of varying size (Magee, 1990). Immunoreactivities for PP-fold peptides (PP, PYY) and FMRFamide showed a similar distribution pattern; cell bodies immunoreactive for PYY and FMRFamide were found in or close by the ganglion (Magee, 1990).

Gross structure – redia

Staining for ChE revealed that the nervous system of the redia consists essentially of a pair of cerebral ganglia which are situated beneath the pharynx, and an extensive peripheral network of nerve fibres; the fibres are best developed in the anterior of the larva, gradually thinning out to a diffuse network posteriorly (Magee, 1990). The pharynx and gut caecum also stain for ChE. There is a rich innervation of 5-HT-immunoreactive fibres and cell bodies in the redia, most of which occur in two groups, each of two to three of uni- or bipolar type, forming a pair of ganglia beneath the pharynx. The redial body supports a well-differentiated network of varicose fibres and an occasional cell body (*c.* 5.8 × 4.2 μm in diameter); a multipolar cell body and

associated fibres appear to innervate the musculature of the posterior-lobe lappets. Extensive neuropeptide immunoreactivity has been demonstrated in the redial nervous system, using antisera to PP-fold peptides and FMRFamide. In this respect, immunostaining revealed four to eight cells (each *c.* 5.7 × 3.4 µm in diameter) in the cerebral ganglion, a subsurface varicose nerve net and two multipolar cell bodies (*c.* 7.2 × 6 µm and 9.7 × 7.2 µm in diameter, respectively) located at sites approximately one-third and two-thirds distance from the anterior of the body.

Gross structure – cercaria

The cercarial nervous system has a similar pattern to that described for the adult worm. Typically, it consists of an anterior complex of paired cerebral ganglia and dorsal commissure, in the region of the pharynx, from which three pairs of longitudinal nerve trunks and associated transverse connectives run posteriorly in orthogonal arrangement; secondary branches innervate the oral and ventral suckers, pharynx and tail. The system stains strongly throughout for ChE, including the innervation of the oral and ventral suckers and the tail (Krvavica *et al.*, 1967; Magee, 1990). Similarly, 5-HT immunoreactivity is extensive, with one or two nerve cell bodies (*c.* 4.5 × 3 µm in diameter) in each cerebral ganglion and two or three along each of the ventral nerve cords. A ring of immunostaining marks the junction of the ventral nerve cords and the four varicose nerve fibres that extend the length of the tail; two 5-HT-immunoreactive multipolar cell bodies (*c.* 5.1 × 3.8 µm in diameter) occupy the proximal portion of the tail.

The peptidergic component of the cercarial nervous system is widespread, as evidenced by intense immunoreactivity with antisera to PP-fold peptides and FMRFamide, and occurs throughout much of the innervation of the body and tail (Magee, 1990). Immunostaining also revealed several cell bodies, mostly bipolar in form (*c.* 6.4 × 5.2 µm in diameter), in the anterior ganglia and ventral and dorsal nerve cords; bi- and multipolar cells (*c.* 11.3 × 5 µm in diameter) were observed in the tail (three proximal and two distal). Substance P-immunoreactivity was detected in the CNS and in fibres innervating the pharynx (Magee, 1990).

Fine Structure

Adult

Description of the ultrastructure of the CNS of adult *Fasciola* is based on the studies of S.C. Sukhdeo *et al.* (1988a). The neurons are generally bi- or multipolar in form and are scattered widely along the nerve cords and in the plexuses of the suckers, pharynx and reproductive tract. Those in the cerebral ganglia are located mainly around the periphery of the brain, but their cell bodies are not organized in a clearly defined rind as they are in the brain of the larval stages (see below) or in the migrating juvenile worm (see S.C. Sukhdeo and M.V.K. Sukhdeo, 1990). The cell bodies, or somata, are irregular

in outline and display a large centrally placed nucleus. The surrounding cytoplasm is occupied mainly by inclusions that are typical of those of a neurosecretory cell, namely, mitochondria, ER, Golgi stacks; a variety of both clear and dense vesicles, together with a random array of neurotubules, are also present in the cytoplasm. In places, the cell bodies give rise to one or more cytoplasmic processes (axons, dendrites), and these form the bulk of the nerve fibres in the neuropile, commissure and nerve cords. The fibres are non-myelinated and contain mitochondria, neurotubules (orientated along the long axis) and numerous vesicles. Two distinct sizes of neuronal cell process or fibre have been identified by S.C. Sukhdeo *et al.* (1988a): the smaller have diameters of less than 2 µm, while the larger have diameters greater than 12 µm and display extensive invaginations of the delimiting plasma membrane. The larger fibres ramify into the neuropile and make up much of the commissure; they have been designated 'giant' nerve processes or fibres by these authors. These giant nerve fibres first appear in the CNS of the young, sexually immature worm after it has entered the liver (e.g. 30 days post-infection in rat), and their intrusion into the cerebral ganglion is seen to be largely responsible for the loss of cell-rind integrity and the increase in neuropile volume. By the time the fluke has reached adulthood, giant fibres occupy up to 60% of brain volume and account for the overall increase that has taken place in the size of the brain; there is no evidence of a concomitant increase in the numbers of cell bodies through neurogenesis. In non-myelinated fibres, conduction velocity increases as a function of axon diameter, and fast-conducting giant fibres in higher organisms are generally associated with rapid communication and reflex mechanisms. The significance of giant fibres in adult *Fasciola* is unclear, although M.V.K. Sukhdeo (1992) believes they may provide a network of fast-acting nerves to coordinate the complex alimentary activity associated with feeding in the worm.

At least four morphologically distinct types of neuronal vesicles have been identified in the smaller nerve fibres of *Fasciola*: (i) small clear vesicles (30–46 nm in diameter); (ii) dense-cored vesicles (56–84 nm); (iii) dense vesicles (60–75 nm); and (iv) dense ellipsoidal vesicles (60–75 nm × 130–180 nm). The biochemical nature of the contents of these vesicles is unknown. However, ultrastructural studies using antibodies to vertebrate PP and molluscan FMRFamide (Brownlee *et al.*, 1994), or specific antisera to the flatworm peptides NPF and GNFFRFamide (Marks *et al.*, 1995), have shown that all of the immunogold labelling for the peptides was localized exclusively over dense-cored and ellipsoidal vesicles, suggesting that they contain neuropeptides. Marks *et al.* (1995) further showed by double immunogold-labelling experiments on the peptides that there was an apparent homogeneity of antigenic sites within the vesicles, reflecting cross-reactivity of the FaRP antiserum with NPF. The smaller dense-cored neuronal vesicles are similar in size and appearance to those found in other invertebrate sites that are rich in biogenic amines, and the small, clear vesicles resemble known cholinergic vesicles from mammalian nerves.

Synapses between the smaller fibres are common in the neuropile, commissure and nerve cords and are typically asymmetric and distinguished

by pre and/or postsynaptic thickenings. Aggregations of small, clear vesicles have been seen observed against the presynaptic membrane, but other vesicle types may also be present. The synaptic cleft is approximately 16.5–17.5 nm in width. Many of the synapses are shared in form, with a divergent presynaptic terminal abutting two postsynaptic fibres; others include axo-axonal synapses, especially between adjacent fibres in the nerve cords, and myoneural junctions. No synapses have been found between giant nerve fibres or between giant nerve fibres and small fibres. Typically, the neurons are separated from other cell types by an extracellular matrix of fibrous interstitial material, which not only provides a medium for support but is also thought to serve in the transport and exchange of substances. Cellular coverings of neuronal tissue by non-neuronal cells have been observed in *Fasciola*, in the form of multilayered sheaths of mesenchymal tissue that surround the cerebral ganglia and separate it from adjacent parenchymal cells; similar 'glia-like' structures also intrude into the surface infoldings of the giant nerve fibres, presenting trophospongium-like features, and are thought to be involved in the regulation and/or supply of nutrients to the neurons (S.C. Sukhdeo and M.V.K. Sukhdeo, 1994).

Miracidium

Information on the fine structural composition of the miracidial nervous system derives largely from the detailed observations of Wilson (1970), who used serial sections of the larval central nerve elements prepared for both light and electron microscopy, and silver impregnation techniques for detecting peripheral nerve endings bearing cilia. The large, central nerve ganglion, equivalent to a brain, is situated anteriorly at the level of the second tier of ciliated epithelial cells; estimates indicate that it occupies some 8% of total body volume. The ganglion comprises a central neuropile of fine nerve fibres surrounded by numerous cell bodies of two distinct types. The most common type (3–5 µm in diameter) are scattered over the neuropile surface, while the second type (4.5–6 µm in diameter) consists of a single pair of cell bodies lying beneath the mid-ventral surface of the neuropile. Three pairs of fibre tracts run outward from the ganglion, both anteriorly and posteriorly, to the muscles of the body wall and to presumed sensory endings in the body wall and apical papilla. The cytoplasm of individual fibres is occupied largely by aggregations of vesicles, of which three types are described, all of which correspond to those found in the adult worm. These are: (i) small, clear vesicles (30–45 nm in diameter); (ii) dense-cored vesicles (65–75 nm in diameter); and (iii) large, dense vesicular bodies (up to 165 nm in diameter), with a variety of granular contents, and which are considered to be neurosecretory (peptidergic). Synapses are common, especially in the neuropile, and are characterized by pre- and postsynaptic densities and by aggregations of the small, clear vesicles in the presynaptic side of the cleft. There are specialized neuromuscular junctions, comparable in structure to those described in other flatworms, where nerve cell processes, containing mitochondria, small vesicles (*c.* 25 nm) and dense-cored vesicles (*c.* 55 nm),

abut muscle fibres (Wilson, 1969). The muscle fibres appear to be innervated at several levels in the body, suggesting that neuronal influence on muscle contraction in the miracidium occurs locally.

Cercaria

The nerve tracts in the cercaria consist of bundles of non-myelinated axons containing an assortment of vesicles and granules that can be grouped into four categories, according to their size and appearance (Dixon and Mercer, 1965). Again, these are typically: (i) small, clear vesicles (20–80 nm in diameter), which are the most numerous; (ii) dense-cored vesicles (60–100 nm in diameter); (iii) a rarely occurring dense stellate granule (100–170 nm in diameter); and (iv) large ovoid, dense-cored vesicles (200 nm in diameter). The neuronal cell bodies are concentrated largely at the periphery of the cerebral ganglia and form a well-organized cell rind around a dense central neuropilar meshwork of nerve processes. They contain somewhat featureless nuclei, small mitochondria, Golgi stacks and vesicular inclusions of types i and ii. Also associated with the cerebral ganglia is a presumed neurosecretory (peptidergic?) cell containing secretory vesicles similar to type iv. The synapses observed by Dixon and Mercer (1965) are described as resembling those observed in the mammalian CNS, with dense membranes in close proximity and an asymmetric distribution of the small, clear vesicle type i; examples of what appeared to be *en passant* synapses between adjacent axons have been recognized. Neuromuscular junctions in the cercaria have been difficult to define, although those in the pharynx exhibited closely adherent membranes of neuron and muscle cell and the presence of the small, type-i vesicles in the axon terminal; folding of the subsynaptic membrane, characteristic of vertebrate neuromuscular junctions, was not evident.

Putative sense organs

In common with most other digeneans examined, the motile miracidial and cercarial stages of *Fasciola*, as well as the migrating juvenile form, appear able to perceive and respond to a wide range of environmental stimuli, exhibiting quite complex behavioural patterns. Indeed, scanning and transmission electron microscopy, together with silver staining methods, have revealed the surface and internal architecture of a wide array of peripheral nerve endings in the miracidium, cercaria and juvenile; on the basis of comparative morphology, many of these structures would seem to have a sensory function. Unfortunately, there are no ultrastructural descriptions of adult worm sense organs in the literature.

Miracidium

Excluding the eyespots, there are at least four separate types of specialized nerve endings situated principally around the forebody of the miracidium, with two terminating at the body surface and bearing cilia, and two ending internally (Wilson, 1970). Their descriptions are as follows.

1. *Lateral sheathed ciliated nerve endings.* There are six such structures distributed at regular intervals around the body of the miracidium, each positioned in a notch in the posterior margin of one of the six ciliated epithelial cells in the first tier. The structures comprise a bulbous termination of a nerve fibre, secured to the intercellular ridge by an annulate septate desmosome, and a cilium surrounded by a sheath or collar of non-nervous tissue derived from the ridge; the cilium is short (*c.* 2 μm in length, according to Køie *et al.*, 1976) and does not project beyond the opening of the collar (Fig. 9.4A).

2. *Ciliated pit endings.* These occur only on the apical papilla and are divided into two laterally situated subgroups, each of five or six nerve endings, lying in crescentic arrays around the terminal ducts of the apical gland and the two pairs of accessory gland cells. Each of these presumed sense organs consists of one to eight modified cilia borne within a pit formed by a cup-like extension of the terminal cytoplasm of a single nerve fibre (Fig. 9.4B). The number of cilia per nerve ending relates to its position on the papilla and appears to be fairly constant for each miracidium.

3. *Internal club-shaped endings.* This is a pair of nerve endings situated laterally beneath the body wall and connected to the neuropile of the central ganglion (brain) by a single nerve fibre. The endings themselves are highly convoluted and contain aggregations of vesicles and an array of cilia-like structures that do not project from the cytoplasm (Fig. 9.4C).

4. *Lateral bulbous endings.* These comprise two pairs of bulbous endings which terminate laterally beneath the cytoplasm of the intercellular ridge between the first and second tiers of epithelial cells. There are two nerve endings in each pair, distinctive both in size and contents: the smaller bulb contains large electron-dense vesicles (*c.* 150 nm in diameter); the larger has more numerous small vesicles (*c.* 75 nm in diameter) of less dense content. The nerve fibres extend to the neuropile.

In common with most other trematodes examined, the miracidium of *Fasciola* possesses two dorsally situated eyespots; these occur at a level corresponding to the posterior edge of the first tier of ciliated epithelial cells. Details of the fine structure of these presumed photoreceptors have been described by Kummel (1960) and Isseroff and Cable (1968). Essentially, each eyespot consists of a prominent pigment cell with a pair of rhabdomeres in the pigment cup, with a fifth rhabdomere occupying a posteromedian chamber in the left pigment cell; there is no lens.

The principal biological function of the *Fasciola* miracidium is to locate and enter an appropriate molluscan host, and transform into the sporocyst for the purpose of procreating successive generations of larvae. It is assumed, therefore, that its sense organs and endings include adaptations for photo-, tango-, chemo- and georeception. A number of authors have reported photo-, chemo- and geotactic swimming responses by miracidia, including those of *Fasciola* (see Yasuraoka, 1953; Wilson and Denison, 1970a,b; and review by Smyth and Halton, 1983). Miracidia most probably have a tactile sense, and their ciliated sense endings likely serve in the transduction of environmental

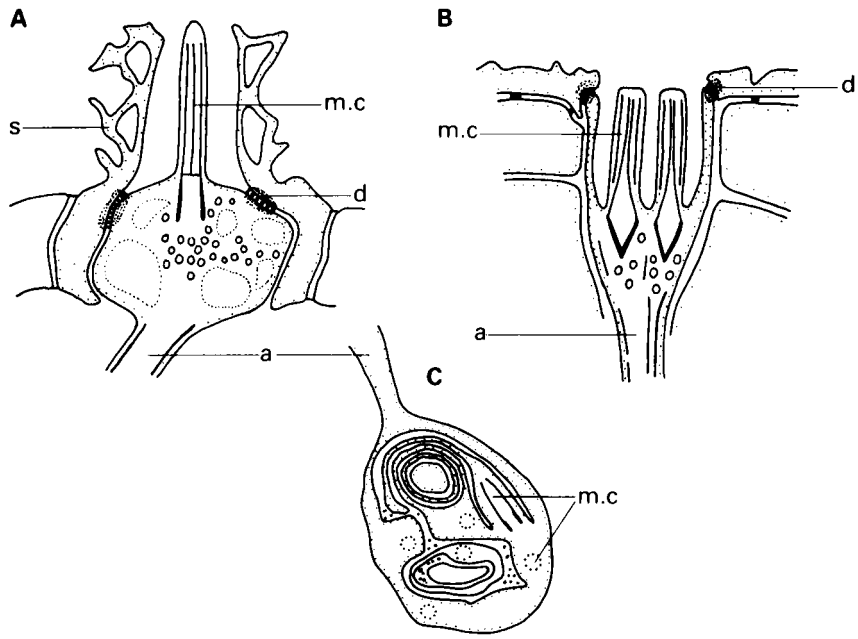

Fig. 9.4. Putative sense organs from the miracidium of *Fasciola hepatica*. (A) Lateral sheathed uniciliated ending. (B) Multiciliated pit ending. (C) Internal club-shaped ending. *Key*: a, axon; d, desmosome; m.c., modified cilium; s, sheath. (After Wilson, 1970.)

stimuli into nerve impulses, although this has yet to be confirmed and, indeed, would be extremely difficult to demonstrate behaviourally. In a scanning electron microscope study of penetrating *Fasciola* miracidia, Køie *et al.* (1976) showed that the sheathed ciliated nerve endings and the lateral papillae are not exposed to the surrounding environment until after the shedding of epithelial cells of the first tier (less than 1 min after attachment to the snail), after which there is extensive contraction and relaxation of the miracidium for some 15 min, prior to the final penetration of the snail and the loss of the remaining tiers of cells. It is believed these observations indicate that the main function of these presumed receptors is to control the penetration process; the structures are lost following the miracidium's entry into the molluscan host and its transformation into the sporocyst.

Cercaria

In the cercarial stage of *Fasciola*, presumed sense organs are spread widely over the body and tail, with a preponderance at the anterior end. However, transmission electron microscopy has thus far revealed only a single type of receptor. This resembles the uniciliated structures in the miracidium and consists of a nerve axon terminating in a bulb of cytoplasm embedded in the tegument and bearing a single cilium that projects freely above the body

surface; the bulb contains numerous small vesicles and is secured to the tegument by a circular desmosome (Dixon and Mercer, 1965). Numerous uniciliate structures, measuring up to about 10 µm in length, have also been observed projecting from the tegumental surface in scanning electron microscope images of the cercaria, the majority of which were found in the anterior region of the larva, both on and around the oral sucker (Køie *et al*., 1977). The longest (*c*. 10 µm in length) of these ciliated structures occur dorsally and on the proximal portion of the cercarial tail; the shortest (*c*. 0.3 µm in length) project from small protuberances on the rim of the oral sucker.

The cercaria is basically a dispersive phase in the life cycle of *Fasciola*, and like the miracidium is well equipped for motility. Its swimming behaviour has been analysed by high speed cinematography and the rate and pattern of the swimming stroke determined (Coil, 1984). However, again, there is no hard experimental evidence that the behavioural events recorded are in fact mediated by any of the sensory structures described. Their microscopic size precludes the application of normal electrophysiological and ablation techniques, leaving any interpretation of their function to be based somewhat tentatively on morphological similarities to receptors of other invertebrates, where the function of such is either known or is suspected.

Juvenile worm

Three types of putative sense organ have been distinguished at the surface of newly excysted juvenile *Fasciola*, using scanning and transmission electron microscopy (Bennett, 1975):

1. A ciliated type, resembling those already described from the miracidium and cercaria, which occurs among the tegumental spines and on the anterior ventrolateral surfaces except for an anterodorsal pair. In section, it consists of a bulb-like structure in continuity with a neuron, and contains a typical ciliary body and rootlet from which arises a short, single cilium (*c*. 0.5 µm in length) that projects above the surface of the tegument.
2. A domed-ended type, which occurs only on the tegument of the suckers and in section resembles the ciliated type in that it has a ciliary basal body and rootlet, but lacks a cilium.
3. A group of three tegumental pits, each shielded by a spiral of tegument, situated on either side of the oral sucker.

The three types of structure described are believed to serve, respectively, as possible tangoreceptors in locomotory movements, in contact communications during attachment, and in chemoreception during migration, but their functions have yet to be proven experimentally (Bennett, 1975).

Studies on habitat selection in juvenile *Fasciola* by S.C. Sukhdeo *et al*. (1987) have revealed that migration from the host gut to the liver appears not to involve orientation responses *per se*, but rather a succession of fixed behaviour patterns, designated as probing, crimping and creeping, and that these behaviours facilitate entry to the abdominal cavity, contact with the inner body wall, and contact with the liver, respectively. An instructive

commentary on the behavioural responses of the migrating juvenile of *Fasciola* is given by M.V.K. Sukhdeo and S.C. Sukhdeo (1994).

Neurochemistry of Larval and Adult Stages

The application of a succession of analytical methodologies, notably radiometric, fluorometric and immunometric, to the study of *Fasciola* neurochemistry has provided data consistent with the results of cytochemical studies for the presence of cholinergic, aminergic and peptidergic substances in the nervous system of the worm. Candidate neurotransmitter or neuromodulatory substances which have been identified include the small molecule transmitters, acetylcholine, 5-hydroxytryptamine (5-HT); the catecholamines, noradrenaline (NAdr) and dopamine (DA); and the amino acids, glutamate and γ-aminobutyric acid (GABA); together with biochemical evidence for the presence of a number of larger putative messenger molecules, the neuropeptides.

Cholinergic and aminergic components

An acetylcholine (ACh)-like substance has been found in especially large amounts in homogenates of *Fasciola*, equivalent to 0.19–1.7 μg ACh g^{-1} wet weight, together with activity for the enzymes that catalyse its synthesis – choline acetyltransferase (ChAT) and degradation – acetylcholinesterase (AChE) (Bacq and Oury, 1937; Chance and Mansour, 1953; Sekardi and Ehrlich, 1962; Mansour, 1964; Frady and Knapp, 1967). Using specific substrates in conjunction with specific inhibitors, Probert and Durrani (1977) characterized and compared cholinesterases in *F. hepatica* with those in *F. gigantica* from four different hosts and found the presence of both AChE (accounting for 74–79% of the ChE activity) and a non-specific (pseudo) cholinesterase (10–41% of activity) in both species.

Quantitative data on four of the five biogenic amines detected in *Fasciola* (5-HT, DA, NAdr, GABA) are summarized in Table 9.1. There are marked regional differences in the amounts of biogenic amine that have been recorded in extracts of the worm. Thus, Gianutsos and Bennett (1977) found the anterior half of *Fasciola* contained approximately three times more dopamine than did the posterior portion, and that the noradrenaline detected in the worm was restricted to the head region; levels of 5-HT in the anterior half were found to be about 20-fold higher than those in the less innervated posterior half (S.C. Sukhdeo and M.V.K. Sukhdeo, 1988). Chromatographic analyses of whole-worm homogenates of adult *Fasciola* have detected GABA in amounts consistent with those evident from immunocytochemical data (Eriksson *et al*., 1995).

Peptidergic components

The discovery showing that the nervous systems of helminths, in common with other metazoan nervous systems, are rich in peptidic components has given new impetus to the study of helminth parasite neurochemistry (Maule

Table 9.1. Amounts of acetylcholine and biogenic amines (total and regional) recorded as present in *Fasciola hepatica*.

	Concentration ($\mu g\ g^{-1}$ wet tissue)	References
Acetylcholine (total worm)	0.19–1.7	Chance and Mansour, 1953
Serotonin (total worm)	0.18–0.31	Mansour and Stone, 1970
Serotonin (anterior end)	0.24–0.31	Shalaeva *et al.*, 1986
Serotonin (anterior end)	0.61 ± 0.07	S.C. Sukhdeo and M.V.K.
Serotonin (posterior end)	0.21 ± 0.01	Sukhdeo, 1988
Dopamine (anterior end)	0.67–2.53	Gianutsos and Bennett, 1977
Dopamine (posterior end)	0.40–0.42	
Dopamine (total worm)	0.33–0.93	Chou *et al.*, 1972
Dopamine (total worm)	0.20–0.80	Terenina, 1991
Noradrenaline (anterior end)	3.46 ± 0.62	Gianutsos and Bennett, 1977
Noradrenaline (posterior end)	0.00	
GABA (anterior end)	1.73 ± 0.51	Eriksson *et al.*, 1995

et al., 1996; Shaw, 1996; Shaw *et al.*, 1996). Evidence to date indicates a degree of chemical complexity hitherto unsuspected in the relatively anatomically-simple nervous systems of flatworms, with multiple peptidergic neurons in all of the major groups examined. Homologues to 26 vertebrate peptides and six invertebrate peptides have been detected immunocytochemically in the nervous systems of some 20 trematode and cestode species (Halton *et al.*, 1994). However, in all but a few cases, the antigens responsible for the immunoreactivity are unknown. At the time of writing, seven native neuropeptides have been isolated and sequenced from flatworms, namely two NPF molecules and four FaRPs (details of their amino-acid sequences are given in Table 9.2).

Table 9.2. Primary structures[a] of platyhelminth neuropeptides.

Neuropeptide F (NPF) peptides	
Fasciola hepatica[1]	[b]PSVQEVEKLLHVLDRNG–KV–AE————————.NH_2
Moniezia expansa[2]	PDKDFIVNPSDLVLDNKAALRDYLRQINEYFAIIGRPRF.NH_2
Artioposthia triangulata[3]	KVVHLRPRSSFSSEDEYQIYLRNVSKYIQLYGRPRF.NH_2
FMRFamide-related peptides (FaRPs)	
Dugesia tigrina[4]	GYIRF.NH_2
Bdelloura candida[5]	GYIRF.NH_2, YIRF.NH_2
Artioposthia triangulata[6]	RYIRF.NH_2
Moniezia expansa[7]	GNFFRF.NH_2

[a]Amino acid sequences shown using single letter notation.
[b]While the number of amino acid residues constituting the *Fasciola* peptide is unknown, the partial structure is aligned so as to present maximum homology with NPF (*M. expansa*).
References: 1. Magee *et al.* (1991b); 2. Maule *et al.* (1991); 3. Curry *et al.* (1992); 4. Johnston *et al.* (1995); 5. Johnston *et al.* (1996); 6. Maule *et al.* (1994); 7. Maule *et al.* (1993).

Several studies have characterized trematode peptide immunoreactivity, using chromatographic and radioimmunometric methodologies (Magee *et al.*, 1989; Skuce *et al.*, 1990; Marks *et al.*, 1995), but the elucidation of the full primary structure of an endogenous trematode peptide has been unsuccessful. The earliest attempt to quantify and characterize a neuropeptide from *Fasciola* was by Magee *et al.* (1991a,b) who showed that the major peptide immunoreactivity in worms from sheep, cattle and rat was due to a PP-fold-like peptide. Starting with an acid–ethanolic extract of some 432 g of *Fasciola* (equivalent to >5000 flukes) from cattle, and using an antiserum directed to the C-terminal hexapeptide amide of bovine PP (i.e -LTRPRYamide), gel-permeation analysis identified a single major immunoreactive molecule of a size comparable to bovine PP (4.2 kDa). Following extensive purification by reverse-phase and ion-exchange HPLC, some 45 ng of pure peptide was recovered for sequence analysis, allowing the first 24 amino acid residues of the N-terminal region of the peptide to be determined. This N-terminal fragment exhibits no apparent homology with PP-fold peptides. Unfortunately, the more conserved and functionally active C-terminal moiety was not sequenced and remains uncharacterized.

The first successful isolation of a PP-immunoreactive peptide in a flatworm was from kilogram quantities of the cestode *Moniezia expansa* by Maule *et al.* (1991), again using antisera to the highly conserved C-terminal hexapeptide amide of PP, and resulted in the discovery of neuropeptide F (NPF). The C-terminal tetrapeptide amide (-RPRFamide) of this 39-residue peptide is identical to that of amphibian and reptilian PP, and all of the physicochemical and molecular genetic evidence points to NPF being the invertebrate equivalent and phylogenetic precursor of vertebrate PP-fold peptides (see Shaw, 1996). Immunocytochemical screening for the presence of NPF in the nervous systems of a range of lower invertebrates, including *Fasciola*, using specific antisera to the conserved C-terminal region, has shown all of the immunostaining for PP-fold peptides to be due entirely to NPF (Maule *et al.*, 1991, 1995; Marks *et al.*, 1995). Confirmation of the presence of an NPF analogue in *Fasciola* has come from radioimmunoassay of acid–ethanol extracts of worms in which 8.3 pmol g^{-1} equivalents of NPF were detected and which, using gel-permeation chromatography, co-eluted with synthetic NPF (*M. expansa*) (Marks *et al.*, 1995). Some 4.7 pmol g^{-1} equivalents of FMRFamide were also found in the extract, with an elution profile comparable to that of flatworm FaRPs.

Neurobiology of Reproduction

In common with most trematodes, *Fasciola* is hermaphroditic and very prolific. The worm is endowed with an elaborate reproductive system, essentially comprising male and female gonads and an intricate series of muscularized ducts and associated glands. The system is capable of producing vast numbers of eggs that serve to compensate for the enormous difficulties faced in achieving successful transmission. Happich and Boray (1969) estimated that a single liver fluke can produce an average of 25,000 eggs per day; that in a light

infection of 1–50 flukes per sheep, the overall egg output is 500,000 per day, rising to some 3.5 million in a heavy infection of >200 flukes. Analysis of the figures for a light infection indicates that each worm is capable of producing on average an egg every 3.45 seconds. How this prodigious rate of egg production is achieved remains a mystery, as do the mechanisms underlying the initiation and control of oviposition in *Fasciola*. Indeed, virtually nothing is known about the reproductive development and physiology in any trematode parasite, although by analogy with other invertebrates it is likely that neurosecretory, i.e. peptidergic processes, are involved in regulating and coordinating major events.

Innervation of reproductive structures

The reproductive system in *Fasciola* is richly innervated with elements of the PNS. Cholinergic, aminergic and peptidergic fibres have been identified in the innervation, with marked regional differences in the sites of staining. Thus, staining for ChE activity is largely associated with the male system, in particular the cirrus sac, seminal vesicle and ejaculatory duct, and in the innervation associated with the musculature around the genital atrium and gonopore; somewhat less reactivity for ChE has been observed in the walls of the vitelline duct and reservoir, uterus, Laurer's canal, oviduct, ootype, and in developing eggs (Halton, 1967; Krvavica *et al.*, 1967; Ramisz and Szankowska, 1970; Probert and Durrani, 1977; Magee, 1990). Any ChE staining of the testes in *Fasciola* has been found to be insensitive to ChE inhibitors (e.g. eserine, DFP (isoflurophate), 62C47) and therefore deemed to be attributable to a non-specific carboxylic esterase (Halton, 1967).

Aminergic elements appear to be concentrated largely in the strongly 5-HT-immunoreactive innervation of the uterus and ootype, and in varicose fibres and nerve plexuses associated with the cirrus sac and genital atrium where bi- and multipolar cell bodies (*c.* 9.7 × 7.2 µm and 14.8 × 9.5 µm in size, respectively) have been identified; an outer and inner nerve ring and connecting fibres surround the gonopore (Fairweather *et al.*, 1987; Magee, 1990). The serotoninergic innervation of the ootype in *Fasciola* is provided by an extensive plexus of varicose fibres derived largely from a closely apposed group of some 40–50 flask-shaped cell bodies (*c.* 11.8 × 5.2 µm in diameter) that closely encircle the ootype wall; in contrast, the innervation of the uterus exhibits fewer fibres and varicosities and the bipolar cell bodies (*c.* 12 × 9.5 µm in diameter) tend to be scattered at intervals along the uterine wall, except near the junction with the ootype where they are more numerous. No immunostaining for 5-HT has been recorded for the uterine valve.

Neuropeptide immunoreactivities, using antisera to NPF and the FaRP, GNFFRFamide, have been demonstrated in the nerve cells and plexuses innervating the muscular walls of the cirrus, cirrus pouch, ootype, uterus and gonopore (Marks *et al.*, 1995), confirming that the immunostaining recorded by Magee *et al.* (1989), using antisera to the vertebrate PP-fold peptides, was likely to be due to non-specific immunoreactivity. A particularly intense

immunostaining for these peptides was found associated with the ootype and localized in three distinct groups of neuronal cells (see below). Substance P has been found in the innervation of the cirrus sac (Magee *et al.*, 1989).

Neuromuscular control of egg production

It has long been established that the mechanism regulating the assembly and production of eggs in trematodes resides in the female genital complex or oogenotop, a collective anatomical term for the oviduct, seminal receptacle, ovovitelline duct, ootype and surrounding Mehlis' gland, and the proximal portion of the uterus (see Gönnert, 1962). The focal point of egg production is the ootype or egg chamber in which each egg is formed as a result of a series of exquisitely controlled reactions, involving the release of a mature oocyte from the ovary, spermatozoa from the seminal receptacle, some 30 vitelline cells from the vitelline reservoir, and secretions of the enigmatic Mehlis' gland. All of the components of the egg are transported to the ootype as a result of a coordinated sequence of peristaltic contractions of the duct muscles and associated sphincters; the circular muscles of the ducting are particularly well developed. It would seem that the innervation of the duct muscles and sphincters likely initiate and control the successive events of egg assembly, and immunocytochemical investigations have shown this innervation to be predominantly peptidergic (Magee *et al.*, 1989; Marks *et al.*, 1995).

In *Fasciola*, there are three stellate-shaped, peptidergic neurons (cell body size *c.* 29.5 × 14.8 μm in size) with fibres innervating the muscle surrounding the entrance to the egg chamber at a point marked by the confluence of the oviduct and vitelline duct, and another group of two, pyriform peptidergic cells (*c.* 32.0 × 21.3 μm) located where the egg chamber leads into the uterus, with fibres terminating in close apposition to the uterine valve (Figs 9.1C and 9.3D). These two groups of cells have been shown to be strongly immunoreactive for PP-fold peptides (Magee *et al.*, 1989) and for both NPF and FaRPs (Marks *et al.*, 1995), and they correspond in location and size to the nerve plexuses, NP1 and NP2, respectively, described by Gönnert (1962). Their strategic position at the entrance and exit of the ootype suggests a functional involvement in regulating the movement of egg material into and out of the egg chamber. The main portion of the ootype in *Fasciola* is richly innervated by a third group of peptidergic cells, again immunoreactive for NPF and FaRPs. These comprise some 40–50 unipolar neurons, whose cell bodies (*c.* 14.6 × 8.3 μm in diameter) are scattered among the S2 cells of the Mehlis' gland, and whose axonal processes extend into the muscle layers of the wall to form an extensive plexus of varicose fibres (Magee *et al.*, 1989; Marks *et al.*, 1995) (Figs 9.1C and 9.3D). No immunostaining for neuropeptides has been recorded in the Mehlis' gland cells themselves.

There is as yet no direct evidence of a neuropeptide involvement in the egg assembly mechanism in trematodes, but some indirect evidence has come from observations on oviposition in a monogenean parasite, *Polystoma nearcticum*, whose reproductive activity is synchronized with the periodic sexual activity of its host, a tree frog. Immunocytochemical studies on worms

recovered before, during and after host spawning have revealed that FaRP expression in the ootype innervation occurs only during host sexual activity (Armstrong *et al.*, 1997). In contrast, immunostaining for 5-HT in the ootype nerves of *P. nearcticum* remained unaltered. The fact that FaRPs have been shown to be strongly myoactive in *Fasciola* (see section on Action of regulatory peptides) points to them having a functional role to play in egg assembly in the worm.

Physiology and Pharmacology of the Neuromuscular System

Neuromuscular function

Fasciola is a relatively large and active worm, and upon removal from the host it invariably displays waves of coordinated muscular activity along the lateral margins of the body. These posterior-directed contractions of somatic muscle enable the worms to move quite quickly over smooth surfaces and, together with the coordinated probing action of the forebody aided by the two suckers, presumably help propel the migrating juveniles along the inside of the body wall to the liver, and thence through hepatic tissue to the bile duct where as adults they become established. Additionally, there is coordination of the action of the muscles of the alimentary and reproductive tracts, including what appears to be quite a sophisticated synchronization of neuromuscular events in both feeding and in egg assembly (see previous section). In contrast to the nematode neuromuscular system, which has been the target of some of the most successful anthelmintics to date, for example avermectin, levamisole, piperazine and pyrantel, only one neuromuscular-directed drug against trematodes has been identified, namely metrifonate (itself ineffective against *Fasciola*) (Geary *et al.*, 1992). Nevertheless, the neuromuscular system in trematodes is clearly essential for attachment, feeding and reproduction, and for this reason it is regarded as an important site for anthelmintic action (Thompson *et al.*, 1996).

Although the flatworm nervous system is anatomically very simple, the mechanisms involved in neuromuscular function are little understood. The main reason for this is that, in contrast to large nematodes such as *Ascaris*, the inaccessibility and small size of flatworm neurons precludes experimentation on isolated nerve–muscle preparations. As a result, most of the research on the neuromuscular activity of trematodes, including *Fasciola*, has involved use of intact worms or of muscle-strip preparations. Many of the methods that have been employed to record motility in *Fasciola* have shown it to consist of alternating periods of intense activity and quiescence, which can last up to 30 h after removal from the host (Fairweather *et al.*, 1983).

Direct neurophysiological recordings have been successfully achieved with the polyclad turbellarians, *Notoplana acticola*, *Alleoplana californica* and *Bdelloura candida*, and with the cestodarian, *Gyrocotyle fimbriata* (Solon and Koopowitz, 1982; Koopowitz, 1986; Blair and Anderson, 1993). These studies demonstrated a range of ionic channel currents and also that flatworm neurons produce classical-type action potentials. With respect to

trematodes, electrophysiological recordings from the surface of *Schistosoma mansoni* have shown that electrical activity in the tegument correlates with motor activities (Fetterer *et al.*, 1977; Pax *et al.*, 1981; Mellin *et al.*, 1983). Indeed, the nervous system of *Schistosoma* is believed to be responsible for the modulation/control of muscle action rather than its generation; a similar situation may exist in *Fasciola*.

Work on the pharmacology and neuromuscular activity of *Fasciola* was pioneered by Chance and Mansour (1949). They employed kymograph recording techniques to monitor the motor responses of flukes to selected small molecule transmitters and drugs. Since then, there have been few detailed examinations of *Fasciola* neuromuscular function, the majority concentrating instead on the blood fluke, *Schistosoma mansoni*. However, one important early finding from the work on *Fasciola* was that decerebrated specimens (brain removed) continued to display spontaneous rhythmical motor activity, indicating that contractility in the worm is not triggered in the brain, but most likely arises endogenously in the peripheral neuromusculature (Chance and Mansour, 1953).

Actions of classical transmitters

Acetylcholine

Although there is no unequivocal evidence for the presence of an inhibitory neurotransmitter *per se* in trematodes, it is generally believed that acetylcholine (ACh) functions as such. Indeed, it was thought that ACh served as a universal neuromuscular inhibitor throughout the Phylum Platyhelminthes until it was shown to have excitatory effects on isolated muscle fibres of the turbellarian, *B. candida* (Blair and Anderson, 1994). Nevertheless, ACh is still regarded as the archetypal inhibitory transmitter substance of parasitic flatworms. This situation is opposite to that seen in vertebrate systems where ACh has a myoexcitatory role.

ACh and the cholinomimetics, arecoline, carbachol and nicotine were first shown to have inhibitory effects on *Fasciola* by Chance and Mansour (1949, 1953), who demonstrated that ACh and related compounds inhibited motor activity and reduced contraction frequency and amplitude in the worm (Fig. 9.5). These studies also identified cholinergic drugs which had no discernible effects on the worm preparation, notably scopolamine (hyoscine), pilocarpine and atropine. Further evidence for the inhibitory role of endogenous ACh in adult *Fasciola* was provided from isometric force transducer recordings of worm motility (Holmes and Fairweather, 1984). In this study, ACh, carbachol and nicotine were found to lower the amplitude and frequency of muscle contraction, while the cholinergic antagonists, atropine and mecamylamine, induced excitatory effects on the motility. Excitatory effects, recorded as an increase in amplitude and frequency of contraction, were also noted following exposure to the neuromuscular blocking agents, *d*-tubocurarine and decamethonium. Assuming similar actions to those at mammalian cholinergic receptors, an excitatory role for *d*-tubocurarine would seem consistent with it having an inhibitory action at the neuromuscular junction; however,

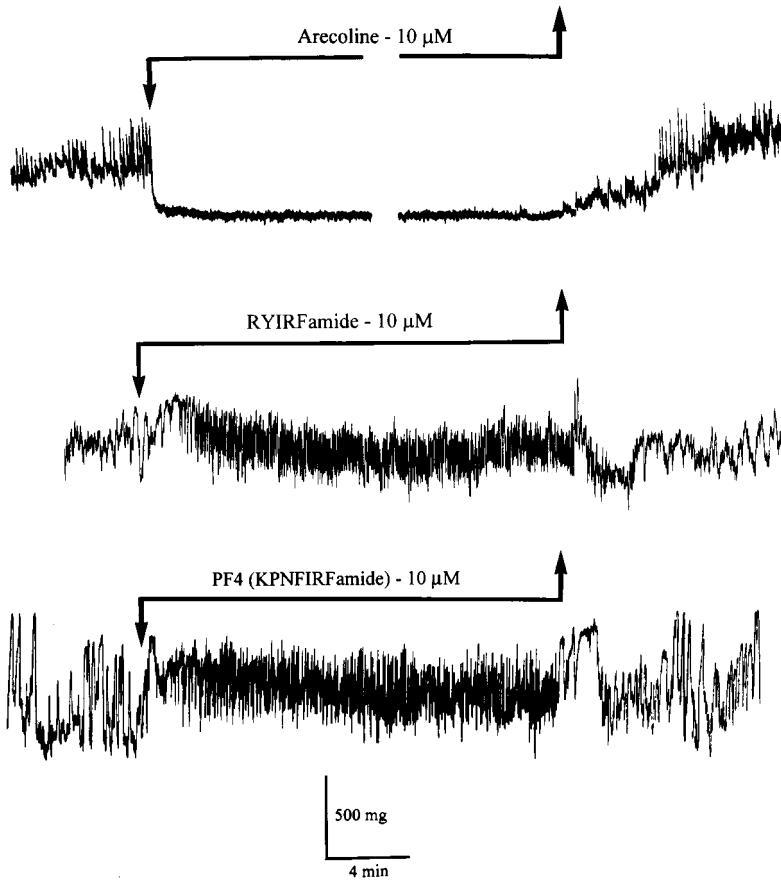

Fig. 9.5. Motility recordings of muscle activity in juvenile *Fasciola hepatica*, showing the effects of: the muscarinic agonist, arecoline; the flatworm neuropeptide, RYIRFamide; and the nematode neuropeptide, KPNFIRFamide (PF4). Note that the arecoline inhibited the spontaneous rhythmicity of the worm and induced flaccid paralysis, whereas both of the neuropeptides had excitatory effects. In each case, the test compound was added to the perfusion chamber at the point marked by the downward arrow, and was washed out at the point indicated by the upward arrow. The gap in the trace for arecoline represents 8 min.

decamethonium precipitates a depolarization block at the mammalian neuromuscular junction and therefore would not be expected to have an initial excitatory effect on the worm.

The ACh receptors of mammals are characterized as muscarinic or nicotinic, based on agonist profiles and their mode of action; muscarinic ACh receptors are G-protein linked, whereas nicotinic ACh receptors are ligand-gated ion channels. Data from experiments on *Fasciola* have revealed that native cholinergic receptors differ from those previously identified in mammals. Notwithstanding these differences, the stimulatory effects of cholinergic

antagonists and neuromuscular blocking agents on worm motility are believed to indicate a tonic release of ACh in the worm (Holmes and Fairweather, 1984; S.C. Sukhdeo *et al*., 1986). A re-examination of many of the data on the effects of cholinergic compounds on *Fasciola*, using more quantitative analytical procedures, has supported many of the previous findings for cholinergic drug effects on the worm (S.C. Sukhdeo *et al*., 1986). Nicotine was identified as the most effective cholinergic agonist, being more potent than either carbachol or ACh. Interestingly, a range of muscarinic receptor agonists (muscarine, pilocarpine, methacholine and bethanechol) and antagonists (e.g. scopolamine) were without effect. Taken alone, this evidence suggests the presence of a nicotinic-type ACh receptor in *Fasciola*.

Although *Fasciola* may possess a nicotinic-type ACh receptor, it can clearly be distinguished from its vertebrate counterpart by the effect it precipitates, i.e. relaxation. Furthermore, the muscarinic receptor antagonist, atropine, had stimulatory effects on worm motility and blocked nicotine-induced excitation, indicating that the native *Fasciola* receptor/s cannot be clearly delineated as either nicotinic or muscarinic, and would seem therefore to have a unique pharmacology. Further differences are evident by the finding that α-bungarotoxin does not affect muscle activity in *Fasciola* (S.C. Sukhdeo *et al*., 1986). The fact that *d*-tubocurarine has been found by some workers (Holmes and Fairweather, 1984) to stimulate worm motility but found to be without effect by others (Chance and Mansour, 1949; S.C. Sukhdeo *et al*., 1986) further complicates the situation.

Apart from the direct actions of cholinergic drugs on *Fasciola*, the indirect effects of cholinesterase (ChE) inhibitors have also been documented. ChE activity is abundant in *Fasciola* (see section on Cholinergic and aminergic components) and is likely to be responsible for the rapid hydrolysis of ACh following its release from presynaptic sites in the worm. Not surprisingly, eserine (physostigmine) has been found to enhance the inhibitory actions of ACh on flukes (Chance and Mansour, 1953; Holmes and Fairweather, 1984; S.C. Sukhdeo *et al*., 1986).

5-Hydroxytryptamine

Just as ACh is believed to be a candidate inhibitory neurotransmitter in parasitic platyhelminths, 5-HT is generally considered to be an endogenous excitatory neurotransmitter. A number of studies have demonstrated that 5-HT and its analogues induce increased contraction frequency, amplitude and baseline tension of *Fasciola* somatic muscle (Mansour, 1957, 1984; Beernink *et al*., 1963; Abrahams *et al*., 1976; Holmes and Fairweather, 1984). When added to flukes *in vitro*, the excitatory effects of 5-HT are evident within a few minutes of drug addition, and can persist for up to 24 h (Holmes and Fairweather, 1984). As with ACh, the responses to 5-HT are also apparent on deganglionated worms, indicating that its actions are probably on peripheral neuromusculature; this does not preclude the occurrence of 5-HT receptors in the cerebral ganglia of the worm.

The actions of 5-HT on the metabolism of *Fasciola* have also been investigated. There is now strong evidence that the indoleamine stimulates

carbohydrate metabolism in the worm, with a consequent increase in lactic acid production and in glycogenolysis (see Mansour, 1979). 5-HT has also been found to activate glycogen phosphorylase, protein kinase, adenylate cyclase (and therefore increase levels of adenosine 3′,5′-monophosphate [cAMP]) and phosphofructokinase. The effects of 5-HT on glycolysis in the worm are also demonstrable in cell-free extracts, suggesting they are independent of increases in worm motility (see Mansour, 1979), making it difficult to determine which mechanisms are specifically linked to the stimulation of motility parameters.

The serotoninergic agonists, lysergic acid diethylamide (LSD) and tryptamine, have been shown to stimulate motor activity of both intact and deganglionated preparations (Mansour, 1957). However, although the effects of LSD were much more potent than those of 5-HT, the effects of tryptamine were much less potent. The only detailed study of the pharmacology of the *Fasciola* 5-HT receptor investigated the effects of a range of 5-HT receptor-selective compounds on a muscle-strip preparation of the worm (Tembe *et al.*, 1993). Some 19 serotoninergic compounds were tested, of which 10 had excitatory actions. The most potent agonists were 5-fluorotryptamine and 5-carboxyamidotryptamine, the former being a non-selective 5-HT receptor agonist and the latter a selective agonist for the 5-HT type 1 (5-HT_1) receptor; both were more effective than 5-HT. Other effective agonists include 7-methyoxytryptamine, 5-methyoxytryptamine and *N*-methyltryptamine, all of which are non-selective 5-HT receptor agonists. Weakly active compounds included the non-selective agonists, 5-hydroxytryptophan and tryptamine, and the 5-HT_1 agonists, 8-hydroxy-2-(di-*N*-propylaminotetralin) and 1–3-trifluoromethyl phenylpiperazine; inactive compounds included non-selective agonists (4-hydroxytryptamine and 5,7-dihydroxytryptamine), 5-HT_1 agonists (chlorophenylpiperazine [mCPP], 5-methoxy-tetrahydropyridin-yl-indole [RU 24969] and sumatriptan) and 5-HT_2 agonists (α-methyl-5-HT, quipazine and chloro-piperazinylpyrazine [MK 212]). Interestingly, all of the tryptaminergic compounds which were tested had excitatory effects on the *Fasciola* muscle-strip preparations, except α-methyl-5-HT (5-HT_2 agonist) and 5,7-dihydroxy-tryptamine (non-selective agonist). These data demonstrate that although the native *Fasciola* 5-HT receptor cannot be classified as either a 5-HT_1 or a 5-HT_2 receptor subtype, in terms of agonist interaction, it is more like the 5-HT_1 receptor. It is noteworthy that the effector mechanism for the four subtypes of 5-HT_1 receptors identified in vertebrates is cAMP, which has also been implicated in the 5-HT effects on *Fasciola*.

Vertebrate receptor antagonists are generally less effective than agonists at invertebrate receptors. This is also true for 5-HT receptors, since the antagonists, ketanserin, spiperone (also a dopamine and noradrenaline receptor antagonist) and SCH 23390 have been shown to have no effects on the 5-HT response of *Fasciola* muscle strips (Tembe *et al.*, 1993). However, the 5-HT receptor antagonist, cinanserin, showed agonist effects at high concentrations. The 5-HT uptake inhibitor, fluoxetine, caused a slow inhibition of contraction amplitude and frequency, leading to flaccid paralysis of flukes within 1 h of treatment (100 µM). At higher concentrations, it

induced an immediate cessation of contractility and an increase in muscle tone which then fluctuated (Holmes and Fairweather, 1984). These authors suggest that this was due either to an initial increase in the levels of 5-HT at the neuromuscular junction, followed by eventual depletion, or to the non-specific interaction of fluoxetine with calcium movement in the fluke.

Dopamine

Even though dopamine has been shown to be more abundant in *Fasciola* than 5-HT, it has received much less attention. A few studies have examined the effects of dopamine on *Fasciola* muscle preparations (Mansour, 1957; Holmes and Fairweather, 1984) and have revealed similar excitatory actions to those of 5-HT. The dopaminergic antagonist, dihydroergotamine, was also shown to reverse the stimulatory effects of dopamine on the worm (Holmes and Fairweather, 1984).

Adrenergic compounds

Noradrenaline (NAdr) and adrenaline (Adr) were found to have inhibitory effects on the motility of *Fasciola* muscle strips, by inducing a gradual reduction in the amplitude of muscle contractions (Holmes and Fairweather, 1984). These actions were reversed by the adrenoreceptor (β_1 and β_2 types) antagonist, propranolol. A range of other NAdr-depleting agents and monoamine oxidase inhibitors was tested on the motility of the muscle-strip preparation and produced a variety of complex effects, some of which took several hours to develop (Holmes and Fairweather, 1984). Guanethidine (10 mM), which inhibits NAdr release and subsequently causes its depletion in mammals, had limited excitatory actions on the fluke, suggesting a tonic release of NAdr in the worm. Chloroamphetamine (induces the release of NAdr and DA from neurons) and reserpine (inhibits vesicular uptake of NAdr) both suppressed spontaneous rhythmicity in the fluke; the former also caused an increase in muscle tension. Desipramine and nortriptyline, both of which block the uptake of NAdr, inhibit the spontaneous contractility of flukes and induce a subsequent increase in muscle tension. Interestingly, the inhibitory effects of NAdr and Adr on *Fasciola* are in direct contrast to their excitatory actions on schistosomes. In the light of these differences, further more detailed studies of the effects of adrenergic compounds on trematodes is warranted. Clearly, the mechanisms of action of these drugs on *Fasciola* are unknown and are likely to remain so prior to membrane-level receptor characterization.

Actions of regulatory peptides

Native neuropeptides

Numerous factors are responsible for the little information there is on neuropeptide function in *Fasciola*, the most critical being the absence of structural data on an endogenous neuropeptide. Moreover, neuropeptides may not only function as neurotransmitters or neuromodulators *per se*, but they may also have trophic or hormonal effects, and these are more difficult

to evaluate. To date, no trematode neuropeptide has been completely sequenced, and the only physiological studies carried out on *Fasciola* have employed neuropeptides originating from other helminths.

The first evidence for the activity of helminth neuropeptides on *Fasciola* was by Marks *et al.* (1996), using juvenile worms (28–32 days old). Using *intact* worms, none of the currently known flatworm neuropeptides (see Table 9.2) showed any discernible effects on motility. However, when the lateral margins of the worm were removed, excitatory activity was evident for a number of platyhelminth neuropeptides, including GYIRFamide, RYIRFamide (Fig. 9.5), YIRFamide, GNFFRFamide and YAIIGRPRFamide (the C-terminal nonapeptide of *Moniezia expansa* NPF). These data suggest that even small neuropeptides fail to cross the tegument of the worm, and that their action on the neuromusculature is only apparent following tegument damage or removal. All of the peptides examined had similar excitatory effects on contraction frequency and amplitude, the only variable being their potency. In this respect, RYIRFamide was the most potent of the peptides tested, having statistically significant effects at 1 nM. The order of potency for the peptides was RYIRFamide > GYIRFamide = YIRFamide > GNFFRFamide = FAIIGRPRFamide. The fact that all of the peptides had similar actions on the motility parameters examined indicates that they may be operating via a single endogenous neuropeptide receptor. However, the localization of this receptor (or receptors) is unknown, and it may reside in the nervous system or musculature of the worm. Interestingly, peptides with a C-terminal YIRFamide motif were also the most potent of the peptides tested on isolated muscle fibres of *S. mansoni* (Day *et al.*, 1994), suggesting there may be at least some receptor homology between trematode and turbellarian neuropeptide receptors.

The effects of a number of nematode neuropeptides on *Fasciola* contractility have also been examined (Marks *et al.*, 1997). Thus, the nematode FaRPs, KNEFIRFamide (AF1), KHEYLRFamide (AF2), SDPNFLRFamide (PF1), SADPNFLRFamide (PF2), KSAYMRFamide (PF3) and KPNFIRFamide (PF4; Fig. 9.5) each induced increased contractility in a *Fasciola* muscle preparation. As with the flatworm FaRPs, the effects of these nematode peptides were indistinguishable in all parameters except potency. PF4 was the most potent of the peptides tested, suggesting perhaps that this may be due to the N-terminal protection afforded by the prolyl residue in position 2 of this peptide (Kubiak *et al.*, 1996). The above results clearly demonstrate cross-phyla activity of helminth neuropeptides and emphasize their potential as novel drug leads.

Other peptides

Apart from the role which endogenous neuropeptides likely play in the neuromuscular function of *Fasciola*, host regulatory peptides have also been found to influence the motility of the worm, including activity of the oral and ventral suckers (M.V.K. Sukhdeo and S.C. Sukhdeo, 1989). For example, caerulein (pEQDYTGWMDFamide) and motilin inhibit the frequency and amplitude of longitudinal muscle contractions and that of sucker musculature;

however, cholecystokinin (CCK) stimulated only ventral sucker activity. Although these responses may be due to endogenous peptide receptors in the worm, M.V.K. Sukhdeo and S.C. Sukhdeo (1989) propose that they could also represent fixed-action patterns triggered by host factors. Thus, CCK is known to stimulate bile-duct activity and any increase in ventral sucker activity could help maintain the worm against the resulting increased flow of bile secretion. The antagonist action of motilin on both bile-duct muscle and that of the worm's suckers would also support this hypothesis.

Future Developments

Although it is widely believed that the neuromuscular systems of helminth parasites, including *Fasciola*, are likely to provide targets for effective chemotherapies, the paucity of knowledge of the basic biology of the systems continues to frustrate hopes of future therapeutic exploitation. Nevertheless, available information demonstrates that neuromuscular receptors in helminth parasites have a unique pharmacology, often differing considerably from those in vertebrates. Also, none of the neuropeptides identified thus far in helminths is homologous to previously characterized host equivalents. These facts bode well for eventual targeting of helminth neuromuscular receptors with novel anthelmintics.

An important next step in the understanding of the neuromuscular system of *Fasciola* will be a detailed biochemical and molecular characterization of endogenous neuroactive components, including classical signalling molecules and neuropeptides, and their neurons. Neuroactive molecules will continue to be identified using immunological and microscopic techniques in conjunction with specific antisera; identified ligands may then be derivatized or radiolabelled to enable the localization of native receptors, thus paving the way for meaningful physiological experimentation. Numerous proteins are likely to be involved in the endogenous transmitter systems, including synthetic and degradative enzymes as well as re-uptake mechanisms, all of which represent potential drug targets.

In the case of neuropeptides, several molecular approaches will increasingly be adopted for the identification of encoding genes, and their characterization may identify other novel neuropeptides which are co-encoded. Neuroactive ligand characterization would allow the affinity purification and subsequent characterization of some endogenous receptors. Alternatively, polymerase chain reaction technologies may enable the identification of full-length genes for native receptor or channel proteins. The initiation of these procedures may be possible by using conserved regions of known neuroreceptors or channel proteins from other invertebrate groups, as templates for the design of degenerate oligonucleotide probes. In this regard, the *Caenorhabditis elegans* genome sequencing project is providing numerous putative receptor and channel protein candidates which may have homologues in platyhelminths. The sequences of encoding genes for *Fasciola* neuropeptides and receptor proteins will also allow *in situ* hybridization procedures and *in situ* PCR techniques to amplify and detect sites of gene expression.

Another exciting proposition would be the incorporation of expression cloning procedures to identify the endogenous *Fasciola* receptor proteins which interact with known flatworm neuropeptides. *Xenopus laevis* oocytes have proved useful in the expression of invertebrate proteins, including G-coupled receptors and channels, and are likely to allow physiological characterization of such potential targets. The challenge would then be for structural chemists to design pharmacophores from constrained analogue information, and for the pharmaceutical industries to incorporate target proteins into high-throughput screens.

Other promising developments include the establishment of isolated muscle fibre assays to enable the postsynaptic effects of transmitters and neuropeptides to be characterized (Blair *et al*., 1991; Day *et al*., 1994; Johnston *et al*., 1996). This procedure was originally developed using *S. mansoni* and should be relatively easily adapted for *Fasciola*, facilitating membrane level receptor/channel characterization through voltage-clamp techniques. As mentioned earlier, direct physiological analysis of the small, inaccessible neurons of flatworms poses immense technical difficulties to the scientist, but new methods of non-invasive imaging of living material using powerful voltage-sensitive fluorescent probes, optoelectronics and high speed cameras, are likely to yield new opportunities for advancing our knowledge of neuronal activity in trematodes. While it will take many years of skilled and dedicated research to better understand the mechanisms that control and integrate the neuromuscular system of flatworm parasites, such as *Fasciola*, the complete characterization of selected neuromuscular channel proteins and receptors would seem a realistic and readily achievable goal.

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10 Immunology of *Fasciola hepatica* Infection

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Introduction

The immune system of animals evolved to defend against a wide range of infectious organisms including viruses, bacteria, fungi, protozoa and helminths. The defence mechanisms can be divided into two separate systems: (i) a rapidly responding 'hardwired' natural or innate immune system; and (ii) a slower but specific and highly adaptable acquired immune system (Fearon and Locksley, 1996). The innate system comprises defence strategies such the complement system, macrophages and other non-specific defence cells such as natural killer (NK) cells and γδ T cells. This system is important in the early recognition of microbes and will then provide instructions that allow the acquired immune system to make the appropriate response towards their elimination. The specific acquired immune system is mediated by both B and T lymphocytes which augment and direct the various elements of the innate responses.

The innate immune system

The skin, surface mucous layers, gastric acid, body temperature or certain tissue characteristics may provide natural physiological barriers to infection by some agents, but they are generally ineffective against helminth infections. For example, cercariae of schistosomes penetrate the skin quite easily by secreting elastase-like proteinases that degrade dermal and subdermal matrix proteins (Dalton and Brindley, 1997). Also, rather than killing the metacercariae of *F. hepatica*, the gastric juice aids in the excystment process of the parasite.

The complement system is the major soluble protein component of the innate immune system consisting of approximately 20 plasma proteins that act in an enzymatic cascade. Activation of this cascade results in the production of molecules capable of directly lysing cells by attacking and forming pores in the membranes, inducing inflammatory responses and opsonizing

Fasciolosis (ed. J.P. Dalton)

targets for phagocytosis by granulocytes and macrophages. The complement cascade can be triggered through the classical pathway by the binding of antibodies or collectin (a mannose-binding protein in the plasma) to the surface of microbes. In addition, the antibody-independent, or alternative, pathway can be activated when components of the system interact directly with carbohydrate-rich particles (lacking sialic acid). Both pathways converge at a crucial step where the component C3 is converted to its activated form C3b (Roitt *et al.*, 1993).

The cellular components of the innate immune system can also recognize carbohydrate structures on microbes via cell-surface receptors. Macrophages possess receptors for lipopolysaccharides, a major component of the outer membrane of Gram-negative bacteria. Stimulation of this receptor induces the macrophage to synthesize and secrete chemical signals or cytokines, such as interleukin-1 (IL-1), IL-6, IL-12, IL-18 and tumour necrosis factor (TNF) which subsequently stimulate the growth of T-helper cells that orchestrate a type-1 immune response (see below). Dendritic cells, which reside mainly in lymph nodes (but transiently in non-lymphoid organs) possess mannose receptors like those found on macrophages, and can recognize and internalize microbial glycoconjugates. Other cells, such as natural killer (NK) cells, bear lectin-like receptors on their surface that can target cells for cytolysis (Fearon and Locksley, 1996).

The specific acquired immune system

The specific immune system is mediated by two main populations of lymphocytes, B lymphocytes (B cells) and T lymphocytes (T cells). In contrast to the innate immune system, the acquired immune system displays specificity, diversity, memory and discrimination between self and non-self.

B cells arise from haematopoietic cells in the bone marrow. Resting B cells circulate in the blood and migrate across high endothelial venules to sites of entrapped antigen in secondary lymphoid organs such as lymph nodes, the spleen, tonsils and Peyer's patches (Clark and Ledbetter, 1994). They possess an immunoglobulin-like receptor on their surface which allows them to bind to a small site, or epitope, on an antigen. Following binding the antigen–receptor complex is internalized and the antigen is processed by proteolytic cleavage in the endosomes. Small peptides derived from the antigen mix with host major histocompatibility complex (MHC) class II molecules in the endosome and those that interact with this molecule's binding site are then brought to and 'presented' on the surface of the cell. MHC class II molecules are a highly polymorphic family of dimeric proteins represented by the HLA antigens and H2 antigens in humans and mice, respectively.

The structural combination of peptide and MHC class II on the B cell surface is recognized by activated T cells, or more specifically T-helper (Th) cells, which secrete protein mediators, or cytokines, that induce B cells to migrate into B-cell follicles. Here they interact with follicular dendritic cells (FDC) which recognize their antibody–antigen immune complexes and

stimulate them to proliferate and differentiate. This interaction leads to the formation of a germinal centre or 'dark' zone which is relatively devoid of T cells. The maturing B cells then move into 'light zones', which contain abundant FDCs and activated T cells that induce them to differentiate into: (i) plasma cells, which secrete >2000 antibody or immunoglobulin (Ig) molecules per second that are capable of binding to the antigenic epitope that originally bound to the B cell receptor; and (ii) memory cells, which retain the capacity to recognize the antigen and to proliferate into further memory and plasma cells, and hence accelerate responses on future exposure to the antigen (Clark and Ledbetter, 1994).

There are five classes of Ig in humans: IgM, IgA, IgE, IgG and IgD which differ in structure, number of binding sites and function. IgM is composed of five units of the basic antibody structure and hence has ten binding sites. This Ig is produced very early in primary infections since its multiple binding sites, although exhibiting low specificity, provide it with an advantage for entrapping infectious organisms. IgM is also very effective in activating complement by the classical pathway. During the maturation of the immune response, B cells are induced by FDCs and T cells to 'switch' immunogloblin class from IgM to IgG; hence IgGs are usually produced subsequent to IgM during the course of an infection. In humans the IgG class is composed of four subclasses, IgG1, IgG2, IgG3 and IgG4. IgG1 and IgG3 are particularly effective at activating complement, in mediating phagocytosis and antibody-mediated cytotoxicity (ADCC) reactions. The precise function of IgG4 is not clear, as this Ig is inefficient at activating complement and in binding to receptors on granulocytes and macrophages. In helminth infections, and allergic diseases, IgG4 may play an immunomodulatory role as it interferes with complement activation by IgG1 and blocks the activity of IgE. IgE is present at low concentrations in serum, but is found attached to cells which bear IgE receptors. High-affinity Fcε receptors for IgE are found on mast cells; when mast-cell-bound IgE interacts with antigen it triggers cell degranulation and the release of cell mediators that result in immediate (Type I) hypersensitivity reactions. Eosinophils, macrophages and platelets are also known to possess receptors for IgE, and all are considered important for mediating IgE-dependent killing of helminth parasites (Butterworth, 1993; Hagan, 1993). IgA possesses an additional peptide (J chain) that allows it to be secreted at mucosal surfaces and hence may be important in mediating mucosal immunity in the intestinal and respiratory tract.

Ruminants, including sheep and cattle, have been shown to possess IgM, IgG, IgA and IgE immunoglobulin classes that are homologous with the respective classes in other species and are defined on the basis of antigenicity and electrophorectic mobility (Musoke *et al.*, 1986) (Table 10.1). Within the bovine IgG class, there are two subclasses, IgG1 and IgG2, with the latter sometimes being subclassified into IgG2a and IgG2b isotypes (Butler, 1983). Most workers agree that IgG2b is a relatively minor component with little functional significance. There are two allotypes of IgG2a (A1 and A2) which could potentially differ in their ability to mediate protective immune responses, although some studies have indicated that this is not a major factor

Table 10.1. Properties of bovine immunoglobulins[a].

Property	IgM	IgG1	IgG2[a]	IgA	IgE
Heavy chain	μ	γ	ψ2	α	ε
Concentration (mg ml^{-1})					
Serum	0.6–5.0	6.0–15.0	5.0–13.0	0.05–1.0	
Colostrum	3.0–12.0	30.0–75.0	2.0–4.0	2.0–15.0	
Lachrymal gland	0.04	0.34	0.08	2.45	
MW $\times 10^3$					
Intact	900	146–163	146–150		
Heavy chain	75–78	55–60	55–60		
Light chain	22.5	22.5	22.5		
Carbohydrate (%)	10–12	2.8–3.1	2.8–3.0	6.0–10.0	
Binding to protein A	—	—	+	—	

[a] See Musoke *et al.* (1986).

affecting responses to pathogens (Kacskovics *et al.*, 1995). Precise measurement of IgG2 in cattle is complicated by the fact that some polyclonal and monoclonal antibodies prepared against bovine IgG2 are reactive only with IgG2a (A1) (Butler *et al.*, 1994). Ruminants differ from other species in that IgG1, not IgA, is the major immunoglobulin present in colostrum and milk, although IgA predominates in nasal and lachrymal secretions (Musoke *et al.*, 1986). While some early reports suggested that bovine IgG1 was more efficient at fixing complement than IgG2, these differences are minimal in homologous bovine systems (McGuire *et al.*, 1979). However, IgG2 differs from IgG1 in its ability to bind to protein A and mediate phagocytosis by neutrophils (Musoke *et al.*, 1986). Production of ruminant IgG2a is stimulated by IFN-γ and augmented by IL-2 and is thus associated with type 1 immune responses; bovine IgG1 antibodies, on the other hand, are associated with type 2 responses (Table 10.2; see also below) (Estes *et al.*, 1994).

Although T cells, like B cells, are derived from the haematopoetic cells of the bone marrow they first undergo a maturation step in the thymus before migrating to the spleen and lymph nodes. T cells bear receptors (TCR) on their surface which recognize antigen peptides (T cell epitopes) in association with MHC molecules. Two main populations of T cells exist – T cytotoxic

Table 10.2. Immunoglobulins associated with type 1 and type 2 immune responses in different species.

Species	Type 1 response	Type 2 response
Human	IgG1	IgG4, IgE, IgA
Mouse	IgG2a, IgG3	IgG1, IgE, IgA
Rat	IgG2	IgG1, IgE, IgA
Sheep	IgG2	IgG1, IgE, IgA
Cattle	IgG2	IgG1, IgE, IgA

(Tc) and T-helper (Th) cells – which can be distinguished on the basis of the cell-marker molecules CD8 and CD4, respectively. $CD4^+$ Th cells, as mentioned above, are MHC class II restricted and help B cells to proliferate and to secrete antibody. $CD8^+$ Tc cells, on the other hand, effect the killing of virus- or bacteria- or protozoa-infected host cells, tissue or cell transplants and tumour cells through a direct cell–cell interaction and the release of the toxic, pore-forming molecule perforin, and of oxygen radicals. Antigens derived from the intracellular infectious agent are processed within the endosomes of the infected cell and presented in association with MHC class I molecules, present on the surface of all host cells. Recognition of the peptide/MHC class I complex by TCR on $CD8^+$ Tc intiates the killing event. In general, $CD8^+$ Tc are unable to mediate extracellular killing of eukaryotic parasites, including helminths.

The Th cell dichotomy

In the mid-1980s, Mossman and Coffman observed that murine Th clones could be differentiated into two distinct populations according to the profile of cytokines that they produced (Mossman *et al.*, 1986; Mossman and Coffman, 1989; Abbas *et al.*, 1996). The two populations, which arise from a common precursor (Th0 cells), are designated Th1- and Th2-type because they show not only phenotypic differences but also functional differences. Both cell types produce colony stimulating factors, IL-3 and granulocyte–macrophage colony stimulating factor (GM-CSF). Th1 cells promote type 1 immune responses by specifically producing TNF β (lymphotoxin), IL-2, IL-12 and IFN-γ. These cytokines stimulate the production of IgG2a (IgG1 in humans) and IgG3 by B cells which can activate complement by the classical pathway and promote phagocytosis of microbes via binding to the Fc receptor on macrophages (Table 10.2). In addition, IFN-γ can increase the microbicidal and cytocidal activity of macrophages by inducing nitric oxide synthase and hence their ability to produce nitric oxide. This very effective, antibody-independent type of killing by activated macrophages is associated with the cell-mediated immunity to intracellular organisms such as certain viruses, bacteria and protozoa, although it can also be effective against extracellular organisms such as helminths (James *et al.*, 1982). However, the killing is non-specific and host cells and tissues in the vicinity of the reaction may also be damaged; hence macrophage activation by type 1 responses are often associated with the pathological conditions (delayed Type II hypersensitivity reaction) observed in chronic infections.

Th2 cell subsets, on the other hand, promote type 2 immune responses by producing the cytokines IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. These cytokines provide help for B-cell proliferation and the secretion of IgA, IgG1 (IgG4 in humans) and IgE (Table 10.2). In addition, they also mediate the production and activation of mast cells and eosinophils. Type 2 responses are of particular importance in the control of helminth infections. Eosinophils, bound to the surface of the parasite via antibody, particularly IgE, can become activated and release highly toxic cationic compounds such as the

major basic protein (MBP) eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO). Degranulation of mast cells via the stimulation of surface-bound IgE induces immediate (Type I) hypersensitivity reactions.

Interaction between the innate and acquired immune systems

Activation of the cellular components of the innate immune system by glycoconjugates of microbes (via the cell surface mannose and lectin receptors) is believed to be a first step in their recognition and elimination. In response to this stimulation macrophages release cytokines IL-12, IL-18, IFN-γ and TNF-α which can cause the differentiation of Th0 cells to Th1 cells. Macrophages, which also possess MHC class II antigens on their surface, can internalize, process and present microbial antigens directly to T cells, inducing them to release IL-2, which, in combination with IL-15 from the activated macrophages, stimulates NK cells to produce IFN-γ. IFN-γ can then augment the production of IL-12 by its capacity to induce IL-12 receptors on T cells and NK cells. Thus, an autocrine positive feedback system is set in train that amplifies the levels of IFN-γ that will activate macrophages, and IL-12 and IL-18 that activate NK and Th1 cells and ultimately leads to a type 1 immune response (Fearon and Locksley, 1996; Bohn *et al.*, 1998).

The induction of a type 2 immune response involves the early production of the cytokine IL-4. The source of the early production of this cytokine is less clear, but it may be macrophages and a restricted set of T cells that are stimulated via surface receptors distinct from the lectin-like receptors (Fearon and Locksley, 1996). Moreover, while the antigens responsible for inducing the type 1 response appear to be microbial glycoconjugates, the characteristics of the type-2-inducing antigens are not so well defined.

The role of γδ T cells in immune responses in general, and to helminths in particular, remains to be elucidated. These cells, which are $CD4^-/CD8^-$, express TCR-I, consisting of γδ heterodimers rather than the αβ heterodimers of $CD4^+$ and $CD8^+$ TCR II cells. There is some evidence that γδ T cells function as part of the first-line non-specific 'immunosurveillance' system (Janeway *et al.*, 1988; Ferrick *et al.*, 1995; Saito *et al.*, 1998) or downregulate certain immunopathological responses (McMenamin *et al.*, 1994). γδ T cells comprise a relatively greater component of the immune system in ruminants than in most other species (Hein and Mackay, 1991). In cattle, various subsets of γδ cells have been demonstrated which form 60–75% of the peripheral blood lymphocyte (PBL) population in neonatal calves, although this declines to about 10% in adult cattle (Wyatt *et al.*, 1994). There are also subpopulations of γδ cells which predominate at epithelial surfaces including skin, mammary gland and gut (Wyatt *et al.*, 1994). γδ T cells have been shown in some systems to downregulate antigen-specific lymphocyte responses (Howard *et al.*, 1989; Saito *et al.*, 1998).

Type 1 and type 2 immune responses tend to counter-regulate one another negatively through the actions of the cytokines that are unique to each response. For example, the type 1 cytokine IFN-γ switches off type 2

responses, and conversely IL-4, IL-10 and IL-13 can inhibit the effects of IFN-γ and the development of type 1 responses. Such counter-regulation tends to invoke a polarization of the immune response to one or other type. A hypothetical scheme of the generation of type 1 and type 2 responses is shown in Fig. 10.1.

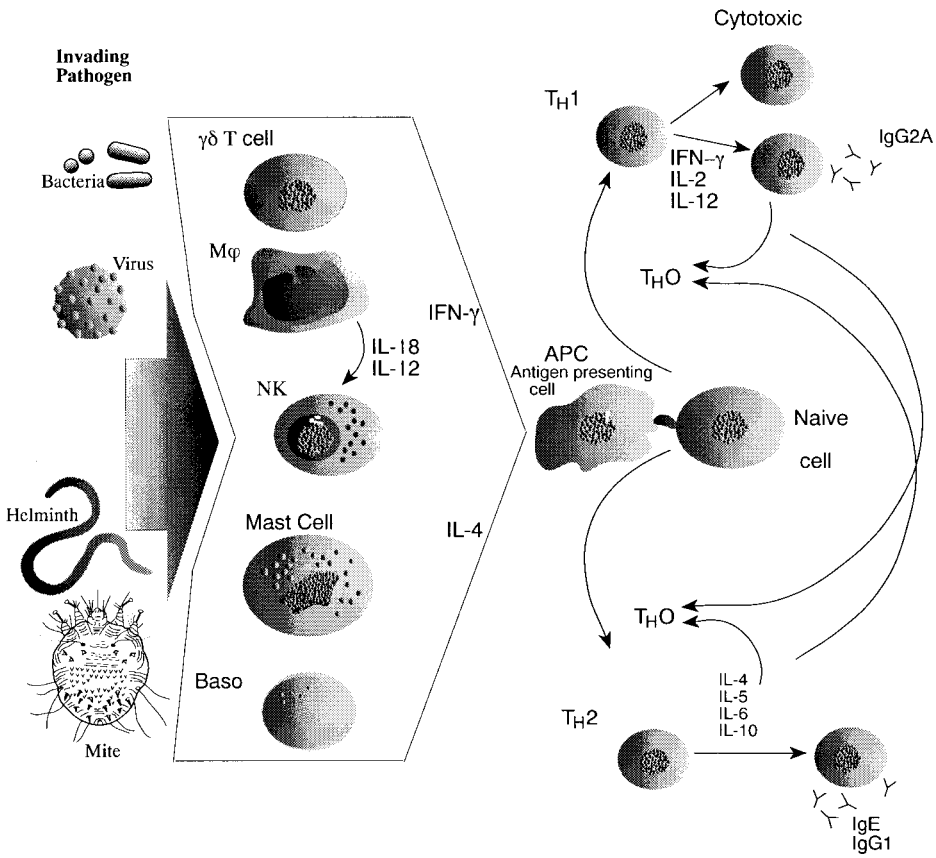

Fig. 10.1. Diagrammatic representation of the components of the innate and acquired immune systems that are involved in the generation of a type 1 or type 2 immune response. Microbes and parasites first interact with cells of the innate immune system including γδ T cells, macrophages (Mφ), natural killer cells (NK), mast cells and basophils (Baso). Cytokines released by these cells (IL-12, IL-18, IFN-γ and IL-4) provide instructions that allow the T and B cells of the adaptive acquired immune system to make the appropriate response. IFN-γ is important in inducing type 1 immune responses which are characterized by the production of the cytokines IFN-γ, IL-2, IL-12 and IL-18. On the other hand, IL-4 is important in driving type 2 immune responses which are characterized by the cytokines IL-4, IL-5, IL-6 and IL-10. Type 1 and type 2 immune responses tend to counter-regulate one another negatively through the actions of the cytokines that are unique to each response; such counter-regulation tends to invoke a polarization of the immune response to one or other type.

Immune Responses to Helminths

It is well recognized that helminths induce type 2 immune responses, and that these responses are more important than type 1 responses in effecting the expulsion of helminth parasites (Sher and Coffman, 1992; Urban *et al.*, 1992; Svetic *et al.*, 1993). For example, resistance to the intestinal nematodes *Trichuris muris* and *Trichinella spiralis* in mice can be compromised by the neutralization of IL-4 following administration of anti-IL-4 or anti-IL-4 receptor antibodies. Moreover, IL-4 knockout mice do not resist these infections (Bancroft *et al.*, 1998). A positive correlation between specific IgG2a (AI) levels and larval output in calves infected with the lungworm, *Dictyocaulus viviparus*, was found by Scott *et al.* (1996) and indicated that those calves in which the type 2 response was more pronounced were better able to control the infection.

In helminth infections there is a preferential class-switching of the antibody response towards the production of both specific and non-specific IgE (Mansour *et al.*, 1990; Hagan, 1993; Maizels *et al.*, 1993; Schallig *et al.*, 1995). The cross-linking of high-affinity Fcε receptors for IgE on mast-cell membranes by antigen results in the release of vasoactive amines and other mediators of inflammation, along with the recruitment and activation of eosinophils which in turn release mediators, such as IL-5, which can further activate eosinophils. The subsequent immediate (Type I) hypersensitivity reactions and/or ADCC reactions involving IgE, and IgG, are believed to be the principal mechanisms by which helminth infections are cleared. Indeed it is believed that immune responses involving IgE evolved as a means of protection against helminths. Only in other hypersensitivity states, such as asthma and atopy, are IgE responses, eosinophilia and mastocytosis also observed.

Nevertheless, there is much discussion regarding the relationship or balance of the type 1 and type 2 responses in immune-mediated pathology manifested in chronic disease. In general, helminth infections of humans do not cause high mortality rates, but they do cause high morbidity and tend to persist in their hosts for lengthy periods (Maizels *et al.*, 1993). Chronicity and susceptibility to secondary infection is the norm in helminth infections of animals, including fasciolosis. It could be argued that type 2 immune responses are induced by the parasite for its own benefit, i.e. to enable it to remain relatively unharmed in the host. For the host, however, its a two-edged sword that strikes a balance between worm expulsion and pathology. For example, a study of patients with lymphatic filariasis caused by *Brugia malayi* showed that those with asymptomatic filaraemia had a hyporesponsiveness manifested in a decline of their type 1 response but still retained a strong type 2 response. However, patients with chronic pathology (elephantiasis) had both a strong type 1 and type 2 response. In the latter case, it is suggested that the type 2 responses cause the expulsion and killing of worms which in turn leads to the induction of the type 1 responses that are responsible for the pathological sequelae observed in elephantiasis (Maizels *et al.*, 1993). Another important example is provided by infections caused by the trematodes of the genus

Schistosoma. Type 0 or type 1 responses which occur during the prepatent phase of infection are produced until eggs released by the female worm become entrapped in the liver tissues, and a switch to a type 2 response occurs (Pearce *et al*., 1991; Pearce and Sher, 1991; Butterworth, 1993; Sabin and Pearce, 1995). While a type 2 response has been shown to be involved in the immune-mediated granulomatous response to entrapped eggs, a reaction that causes the major pathogenesis associated with schistosomiasis including portal hypertension and fibrosis, it also plays an essential host-protective role. For example, infected SCID mice are incapable of generating a granulomatous response and die of severe hepatitis as a result of liver entrapped eggs (Amiri *et al*., 1992, 1994).

A further feature of the Th2-type response to helminth infections (and to allergens) in humans involves the relationship of IgE and IgG4 antibodies. Both isotypes are stimulated by the same cytokine, IL-4, which is produced mainly by Th0 cells and Th2 cells. While the precise function of IgG4 is unclear, this antibody blocks IgE-mediated Type I hypersensitivity reactions perhaps by competing with the IgE for the same epitope on parasite antigens. For example, the slow development of resistance in humans to *S. haematobium* has been correlated with an early production of IgG4 blocking antibody followed by the slow build-up of specific IgE antibodies (Hagan *et al.*, 1991). Although both isotypes are associated with a type 2 response, it was suggested that changes observed in the IgE and IgG4 profiles during infection may reflect the involvement of different lymphocyte populations. A recent study suggests that the production of IgE and IgG4 by B cells can be differentially regulated by IL-10 released from T cells, mast cells and macrophages, which can act either alone or in concert with IL-4 (Jeannin *et al.*, 1998).

Immune Evasion/Modulation Strategies of *Fasciola hepatica*

Like many other helminths, liver flukes can survive in their hosts for very long periods. Accordingly, these parasites must possess some means of evading the persistent attack from the host's immune system. While the final residence of the parasites is in the immunologically safe environment of the bile ducts, they must first 'run the gauntlet' in the face of the immune system as they migrate through the intestinal wall and liver tissue on their way to this site.

In both filarial and schistosome infections adult worms are, in general, more resistant to immune effector mechanisms than the larval stages suggesting that they have developed better means of counteracting these, such as enhanced expression of antioxidants or immune evasion strategies (secretion of antibody-cleaving enzymes and/or anti-inflammatory agents). Although this phenomemon, known as 'concomitant immunity', is considered a general feature of helminth infections (Maizels *et al.*, 1993) it may not pertain to liver fluke infections. While it has been shown that adult liver flukes in rats (Goose and MacGregor, 1973) and cattle (Clery *et al.*, 1996) were unaffected by challenge infections, this resistance to immune effector mechanisms may

simply be explained by the inaccessiblilty of the parasites in the bile ducts rather than the expression of some novel means of survival. Antigens and eggs liberated by adult flukes are carried into the intestines with the bile juices which limits the extent of immune stimulation and immunopathology induced by these. Consequently, antibody titres, measured in mice, rats, sheep and cattle, were observed to decline, albeit slowly, after the parasites had entered the bile ducts (Hanna, 1980a; Meussen and Brandon, 1994; Clery *et al.*, 1996). Measurements of immunoglobulin levels in the bile ducts of infected cattle showed that they are approximately 12 times lower than that measured in serum and are predominantly of the IgA isotype, confirming that the bile ducts are an immunologically privileged site (Hughes *et al.*, 1981).

It is natural to presume that most of the interactions between parasite and host will take place at the parasite surface, and therefore it is not surprising that the liver fluke surface plays a vital role in protection against immune attack. There is no evidence to suggest that liver flukes absorb host macromolecules on to their surface to 'mask' themselves from the immune system as has been demonstrated for the schistosomes (Smithers and Terry, 1969). Indeed, early experiments by Hughes and Harness (1973a, b), carried out in a simlar fashion to those performed by Smithers and Terry (1969), showed that rats immunized against mouse or hamster antigens did not reject parasites transplanted from these hosts. Furthermore, the liver fluke surface differs markedly from the two-lipid bilayer surface of the schistosomes in that it is a single surface membrane covered by a 40 nm thick polyanionic 'fuzzy' glycocalyx composed of glycoproteins with projecting side chains of oligosaccharides and gangliosides terminating in sialic acids (Threadgold, 1976).

The surface glycocalyx may contribute to immune evasion in three ways. First, the composition of the glycocalyx changes during the development of the parasite in its host, possibly to prepare the parasite for its changing environments, and thus presents the immune system with a changing target. The glycocalyx components are synthesized and packaged in vesicles within the tegumental cells which carry them to the surface to be incorporated into the glycocalyx. The glycocalyx of the newly excysted juvenile (NEJ) is derived from T0-type tegumental cells which tranform into T1-type tegumental cells soon after the parasites enter the liver tissue. Prior to entry into the bile ducts T2-type tegumental cells differentiate in the parenchyma, make contact with the tegumental syncytium and take over the function of glycoclayx synthesis (see Chapter 3 of this volume). This changing glycocalyx composition is reflected in changes in the humoral immune responses of the host. Antibodies reactive with the T0- and T1-derived components (which are antigenically related) peak between 3 and 5 weeks after infection while, following their decline, anti-T2 antibodies begin to appear. These anti-T2 antibodies then decline after the parasite enters the bile duct (Hanna, 1980a). Second, the glycocalyx is continuously sloughed off and replaced by the secretory vesicles. In the juvenile flukes this continuous turnover replaces the glycocalyx approximately every 3 h (Hanna, 1980b,c). Thus, it has been proposed that antibody-bound immune effector cells, such as eosinophils and neutrophils,

do not make a sufficiently tight contact with the parasite to allow degranulation and damage to the surface but are shed with the glycocalyx (Duffus and Franks, 1980; Hanna, 1980b). While it was demonstrated that the major basic protein isolated from bovine eosinophils is toxic *in vitro* to NEJ at micromolar concentrations (Duffus and Franks, 1980, 1981) they were resistant to antibody-mediated eosinophil damage (Duffus and Franks, 1980). Glycocalyx turnover slows down once the flukes have entered the bile duct since the parasites are no longer under such vigorous immune attack. Third, shed products of the glycocalyx may simply 'mop up' circulating anti-fluke antibodies preventing their participation in potentially lethal ADCC reactions (Duffus and Franks, 1980).

Using surface radiolabelling techniques Dalton and Joyce (1987) showed that the profile of surface glycoproteins/proteins of NEJ, immature flukes (liver stage) and mature (bile duct stage) flukes differed. The most marked changes appeared to occur between the NEJ and the later stages in that far fewer glycoproteins/proteins were detected on the NEJ surface. Tkalcevic *et al.* (1995, 1996) also observed stage-specific antigen expression in flukes taken from infected rats at various times after infection. In addition, these authors showed that three monoclonal antibodies, which were generated using B cells derived from the mesenteric lymph nodes (MLN) of recently challenged rats and represented the predominant polyclonal response, were reactive with immunogens whose expression was restricted to the first 2 days of infection. One of these antibodies reacted with a carbohydrate moiety on an array of proteins that made up the NEJ glycocalyx. Therefore, it appears that NEJ rapidly switch their antigenic surface make-up. Traversing the mucosal wall may be a difficult task and may necessitate a quick change of disguise to avoid immune attack on a weakened parasite.

The changing antigenic profile of the developing parasite while it migrates through distinct anatomical regions of the body results in the stimulation of independent immune responses in the lymph nodes that drain these separate compartments. Meeusen and Brandon (1994) showed that antibodies secreted from cells from the mesenteric lymph nodes (MLN), hepatic lymph nodes (HLN) and spleen (SPL) of rats infected for 1 month were reactive with a distinct pattern of parasite antigens. Immune responses in the HLN did not occur if the infection was cured by drug treatment 10 days after infection. Furthermore, stimulation of the different lymphoid compartments resulted in the production of different isotypic responses – for example, IgE responses were significantly higher in the HLN compared to MLN and SPL, while IgA responses were highest in the MLN – suggesting a unique regulation of the cytokines secreted by T cells in each of these microenvironments. Meeusen and Brandon (1994) suggested that by migrating from one tissue to another (intestine to liver), which are predisposed to the generation of different types of immune responses, the liver flukes may be protected from contending with a single immune effector mechanism that would otherwise become increasingly efficient as the parasite migrates.

NEJ are highly resistant to destruction by complement. Deposition of the complement component C3 on the surface glycocalyx cannot be demonstrated

in vitro or *in vivo* (Duffus and Franks, 1980; Davies and Goose, 1981). The presence of terminal sialic acids on the glycocalyx components may prevent the activation of complement activity by the alternative complement pathway (Baeza *et al.*, 1994a). In addition, antibody shedding from the surface may prevent the activation of the classical pathway. However, when NEJ were incubated in immune serum no consumption of complement components was observed (Montgomery *et al.*, 1986) suggesting that the parasite possesses a means of blocking the classical complement pathway.

Immunohistological studies of sheep livers during a primary fluke infection showed that the migratory tunnels in the parenchyma become infiltrated with granulocytes (eosinophils and neutrophils), macrophages and T and B lymphocytes (Meeusen *et al.*, 1995). While young flukes were sometimes present in these tunnels, leucocyte infiltration was never observed around them. Following a challenge infection the cellular immune response is markedly increased but was observed only around the portal tracts and lesion sites, not around the flukes. Furthermore, challenge flukes were never observed near the liver capsule where flukes of the primary infection were found. Therefore, liver flukes may be sufficiently motile to 'leave behind' the immune response – while flukes can burrow through the liver unhindered, specifically recruited leucocytes must leave the blood capillaries into the portal tract area and transverse the extracellular matrix in order to intercept the parasite (Meeusen *et al.*, 1995). Challenge flukes may simply avoid areas of vigorous cellular responses generated by the primary infection and migrate more quickly through the untroubled areas. This idea may be supported by the observation that flukes of a challenge infection in sheep migrate more rapidly into the bile ducts than flukes of a primary infection (Sinclair, 1970, 1971; Harness *et al.*, 1977; Chauvin *et al.*, 1995; Meeusen *et al.*, 1995).

Liver flukes may also possess a mechanism to disable the short-range offensives of immune effector cells such as the toxic reactive oxygen products of the respiratory burst of leucocytes (eosinophils and neutrophils) and macrophages or reactive nitrogen intermediates generated by macrophages. Studies showing undamaged flukes within host liver surrounded by immune effector cells would support this idea (Smith *et al.*, 1993a; Meeusen *et al.*, 1995). Furthermore, several studies have reported the inability of rat and bovine eosinophils to effect killing of NEJ *in vitro* (Doy *et al.*, 1980; Duffus and Franks, 1980; Doy and Hughes, 1982; Glauert *et al.*, 1985). Recently, Piedrafieta (1995) demonstrated that compared to schistosomula of *Schistosoma mansoni*, liver fluke NEJ were highly resistant to killing by reactive nitrogen intermediates generated by lipopolysaccharide (LPS)-stimulated rat peritoneal cleavage cells (predominantly macrophages) and by chemically generated reactive oxygen intermediates. Oxidant scavenging enzymes such as superoxide dismutase (SOD), glutathione peroxidase and glutathione *S*-transferase (GST) may be involved in the inactivation of reactive oxygen species (Brophy *et al.*, 1990; Creaney *et al.*, 1995). Piedrafieta (1995) noted that the specific activities of SOD and glutathione peroxidase were ten-fold higher in extracts of NEJ compared to those reported for schistosomula of *S. mansoni* which may explain the difference in the susceptibilty of these

larvae to reactive oxygen intermediates. Although antioxidants have not been localized in the parasite tissues, both SOD and GST were detected in the excretory/secretory products of adult liver flukes (C. Carmona, Montevideo, 1996, personal communication; Hillyer *et al.*, 1992). A novel antioxidant also found in the excretory/secreted products of adult liver flukes was recently identified by McGonigle *et al.* (1997, 1998). This antioxidant, which is a member of the peroxidoxin family, may be involved in the inactivation of hydroxyl radicals generated by the action of SOD and in this manner may substitute for a catalase which is not expressed by liver flukes (McGonigle *et al.*, 1997, 1998).

Liver flukes may secrete factors that suppress or modulate the host immune responses. Sandeman and Howell (1981) suggested that the lack of an anamnestic antibody response in sheep following a challenge infection may indicate that liver flukes release immunosuppressive factors. Zimmermann *et al.* (1983) observed a reduction in the proliferative responses of lymphocytes from infected sheep to concanavalin A after 4 weeks of infection. A similar non-responsiveness of lymphocytes to Con A was observed in cattle after 5 weeks of infection (McCole, Doherty, Baird, Davis, McGill and Torgerson, personal communication) suggesting that the development of an immunodepression occurs during the migratory stages of the fluke in the liver. In addition, Chauvin and Boulard (1996) reported that T lymphocytes are found only at the periphery of leucocyte infiltration sites in infected sheep livers and proposed that flukes may depress local inflammatory and immune responses to facilitate their passage through the liver parenchyma. Reduced early inflammatory responses were also observed in rats by Baeza *et al.* (1994b,c).

The molecules responsible for immune modulation have not been characterized. However, glycoconjugates released from the sloughed glycocalyx and/or phosphorylcholine-enriched antigens secreted by the parasite may interfere with antigen processing by macrophages or suppress T lymphocyte responses (Sloan *et al.*, 1991). A Kunitz-type (Fh-KTM) serine proteinase inhibitor expressed in the gut, parenchyma and tegument of adult *F. hepatica* was reported by Bozas *et al.* (1995) and may inhibit the activity of elastases released by neutrophils or interfere with cytokine production by lymphocytes. Furthermore, cysteine proteinases (cathepsin B and/or cathepsin L) and other proteinases which are secreted by developing flukes may modulate immune responses by cleaving CD-23 low affinity receptors for IgE molecules on the surface of activated B lymphocytes, eosinophils, follicular dendritic cells, neutrophils, macrophages and platelets (Hewitt *et al.*, 1995). Whatever the putative immunodulatory molecules are, they would be most likely found in the secretions of flukes. Milbourne and Howell (1990, 1993) observed a systemic eosinophilia in rats following injection of excretory/secretory products and suggested that an IL-5-like substance is produced by flukes or by T lymphocytes following stimulation by fluke antigens. In addition, Cervi *et al.* (1996) found that ES products of adult flukes could suppress delayed type hypersensitivity (DTH) responses in rats to parasite and non-parasite antigens, and Jefferies *et al.* (1996) showed that ES products can inhibit the superoxide output of phorbol myristate acetate (PMA)-stimulated human and sheep neutrophils.

Goose (1978) reported that medium in which liver flukes were cultured was toxic to splenocytes. In addition, they demonstrated that these secretory/excretory (ES) products could prevent the *in vitro* killing of NEJ by peritoneal inflammatory cells in the presence of immune serum by preventing the adherence of the effector cells to the parasites. Subsequently, Chapman and Mitchell (1982a) demonstrated that immature liver flukes secrete a papain- or cathepsin-B-like cysteine proteinase that was capable of cleaving host immunoglobulin in a manner that led to the separation of the Fab from the Fc regions. They suggested that, *in vivo*, these enzymes may prevent the attachment of immune effector cells, such as eosinophils and macrophages to the parasite. Dalton and Heffernan (1989) showed that immature and adult flukes secrete two distinct cysteine proteinase activities which they proposed were involved in tissue penetration and feeding as well as immune evasion. Subsequent biochemical studies demonstrated that the liberated enzymes were cathepsin L proteinases, termed cathepsin L1 and cathepsin L2, which could specifically cleave immunoglobulins within the hinge region, thus separating the Fab and Fc portions (Smith *et al.*, 1993a,b; Dowd *et al.*, 1994). The enzymes cleaved all classes of human IgG and the precise site of cleavage was mapped to peptide bonds in the upper- and middle-hinge region (Berasain, Carmona, Frangione, Dalton and Goni, unpublished data). Further studies demonstrated that purified cathepsin L could prevent the antibody-mediated attachment of eosinophils to NEJ (Carmona *et al.*, 1993) and indicated that the mechanism by which fluke ES products prevented the killing of NEJ by immune effector cells, as originally observed by Goose (1978), was by the cleavage of the antibody bridge between parasite and effector cell. In contrast to the findings of Goose (1978), Carmona *et al.* (1993) did not find ES products or purified cathepsin L proteinases to be lymphotoxic. While antibody-cleaving activity is secreted by all stages of the parasite that exist in the mammalian host, the NEJ, immature and mature parasites secrete a distinct set of proteinases which may have some relevance to their changing environment (Carmona *et al.*, 1993). Recently, a cathepsin-B-like enzyme, also capable of cleaving host immmunoglobulin, has been described in NEJ (Wilson *et al.*, 1998; E. Meussen, Melbourne, 1996, personal communication).

It is not known to what extent each of the immune evasion strategies outlined above contributes to the ultimate protection of liver flukes from host immune attack as they migrate through the tissues. A deeper understanding, however, of each of these evasion mechanisms will be crucial to the discovery of means of combating the parasite. Moreover, certain hosts do possess some immunity to infection/reinfection indicating that the parasite is not altogether invincible.

Immunology of *Fasciola hepatica* Infections

Natural resistance

While many mammalian species can be infected with *Fasciola*, there is a wide variation in their degree of susceptibility to infection, and in their ability

to acquire resistance to reinfection. Sheep often die from acute fasciolosis, while some infections can last as long as 11 years (Pantelouris, 1965). However, there have been some reports of differing levels of susceptibility of sheep with different genetic backgrounds to liver fluke infection (Boyce *et al*., 1987). Most notably, Javanese thin-tailed sheep have been found to be highly resistant to infection with *F. gigantica* (Wiedosari and Copeman, 1990; Roberts *et al.*, 1997; see also Chapter 15 of this volume). In contrast with sheep, cattle rarely die from liver fluke disease and exhibit a 'self-cure' between 9 and 26 months after infection. This self-cure is most likely related to the calcification and thickening of the bile duct walls that is observed in chronically infected cattle, a phenomenon not observed in sheep (see Chapter 6 of this volume).

Fluke infections do not usually develop to maturity in pigs or horses (see Chapter 12 of this volume). Some studies have equated the order in which species are susceptible to infection with ability of the host to control migration in the liver by fibrosis; hence the relative resistance is in the order of pigs, horses, cattle and sheep (Soulsby, 1982). It is interesting to compare the relative ability of these various animal species to generate fibrotic reactions against *F. hepatica* infections to their ability to tackle another fluke, *Fascioloides magna*. In the natural deer host, *F. magna* parasites become entrapped in the liver within a fibrotic cyst which connects to the bile duct and through which eggs are passed (Soulsby, 1982). In infections of cattle and pigs fibrotic encapsulation of parasites also occurs, but the cysts have much thicker walls that do not connect to the bile duct and infections do not become patent. In contrast, encapsulation of parasites does not occur in sheep, leaving the flukes to wander in the liver tissue causing extensive damage until death supervenes.

While rats can withstand quite heavy infections of *F. hepatica* metacercariae (as many as 50), mice exhibit poor resistance and die within 4 weeks after an infection of more than two parasites. Hughes *et al.* (1976) suggested that a 'self-cure' reaction occurs in rats after a period of 7–8 months of infection, whereas studies by Boray (1969) showed that flukes will remain in the bile ducts for a long as the natural life of this host.

Immunity in laboratory rodents

Studies on mice

Because mice succumb to very low doses of infection, the collection of statistically significant data requires the use of large numbers of animals; hence, there are very few reports on immunity in mice and these tend to contradict each other. Early work by Lang and colleagues (Lang, 1967, 1968; Lang *et al*., 1967) indicated that mice that had been sensitized by two liver fluke infections acquire a significant degree of resistance to reinfection. The nature of the infiltrative lymphocytes around the challenged flukes in the liver tissue, together with the pathology observed, indicated that the protective responses were delayed-type hypersensitivity reactions. Later, these researchers also reported that 8-, 12-, 14-, 16- and 18-day-old juvenile flukes

implanted into the peritoneum of mice could also induce significant levels of protection to an oral challenge infection (Lang and Dronen, 1972; Lang, 1974). The implanted flukes migrated through the liver tissue and entered the bile ducts. Since implanted flukes of 20 or 24 days old did not elicit any significant protection, the authors concluded that the duration of the migration period through the hepatic tissue, of at least 10 to 11 days, rather than fluke age was important for the induction of protective responses. When adult flukes where implanted into the peritoneum of sensitized mice, eosinophils, neutrophils and lymphocytes attached to their surface within 4 h. After 18 h these cells had degranulated and infiltrated the surface tegument of the parasites (Bennett *et al.*, 1980).

Harness *et al.* (1973, 1975, 1976) also described a resistance to challenge in mice and reported that the level of resistance differed between strains. In contrast, challenge experiments performed in several strains of mice (BALB/c, CBA/H, C57/BL and C3H/He) by Chapman and Mitchell (1982b) indicated that acquired immunity to reinfection does not develop in this host. Even large infections of 10 or 30 metacercariae, which were abbreviated by drug treatment, failed to induce any significant resistance to reinfection.

Recently, an investigation into the immune responses of mice to liver fluke infection revealed a variation among different strains (Fig. 10.2). BALB/c mice elicited a type 2 response which was characterized by high levels of the cytokines IL-4 and IL-5, low levels of the type 1 cytokine IL-2 and no significant levels of IFN-γ. 129SV/EV mice also showed type 2 responses but these differed from those of BALB/c mice in that they produced lower levels of IL-4, and higher levels of IL-5 and IL-2. The response of C57/BL mice, on the other hand, was less polarized and was characterized with a low level of IL-4 and a significant production of IFN-γ. Infection in all mice elicited IgG1 antibodies and no IgG2a which is synonymous with type 2 responses (Fig. 10.3). Preliminary analysis indicated that strains presenting a more classical type 2 response (129SV/EV and BALB/c) were more susceptible to infection compared to those mice that produced IFN-γ (C57/BL), and suggests that type 1 responses may confer some immunological resistance to infection in mice (O'Neill, Dalton and Mills, unpublished data).

Studies in rats

Early experiments showed that rats could develop high levels of protection against a challenge infection with liver fluke metacercariae, and since then this model has become very useful in the study of the mechanisms of immunity to *F. hepatica*. A low-dose oral infection of metacercariae (as low as one parasite), which does not result in significant liver pathology, elicited as high as 76% protection against a challenge infection (Goose and MacGregor, 1973; Hayes *et al.*, 1973). With higher primary doses protection levels of 92.5% against challenge infection have also been achieved (Hayes *et al.*, 1972). To determine the developmental stage of the parasite that is responsible for inducing protective responses, Rajasekariah and Howell (1978) sensitized rats by subcutaneously implanting metacercariae, 4-week-

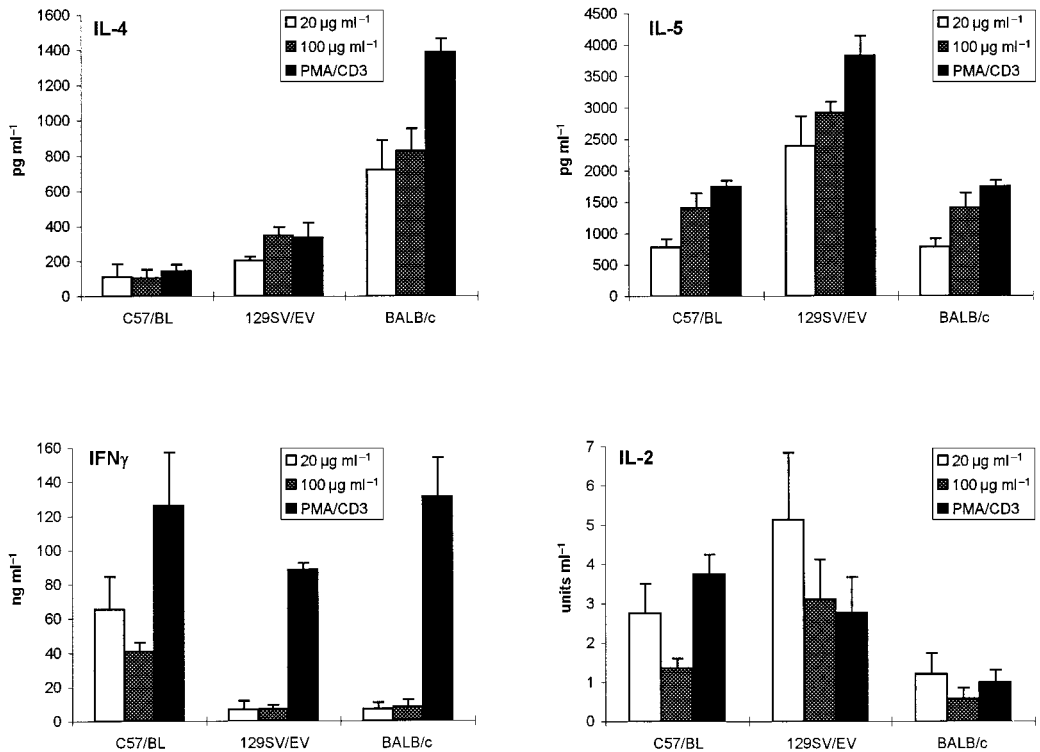

Fig. 10.2. Cytokine analysis of mice infected with *Fasciola hepatica*. Spleen cells were obtained 21 days after infection of: (i) C57/BL, (ii) BALB/c and (iii) 129SV/EV mice and challenged in culture with 20 μg ml^{-1} (white bars) or 100 μg ml^{-1} (stippled bar) of adult liver fluke homogenate. Cells were also challenged with the mitogen phorbal myristate acetate (PMA)/anti-CD3 (black bars) for positive controls.

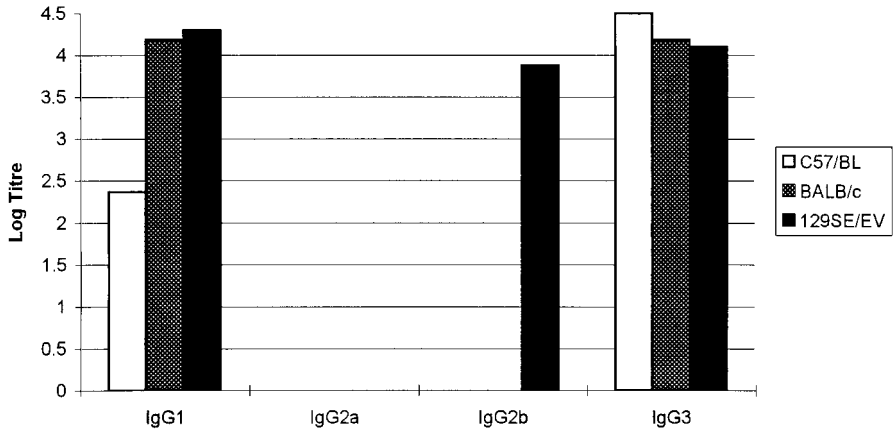

Fig. 10.3. Immunoglobulin responses of C57/BL, BALB/c and 129SE/EV mice 21 days after infection with *Fasciola hepatica*.

old juvenile flukes, adult flukes or eggs and subsequently challenging them with 30 metacercariae. Adult parasites were the only stage that did not induce significant protection to the challenge.

Although a fluke infection in rats generated a strong resistence to subsequent infection, the immune responses were not highly protective against the primary infection. Moreover, no correlation was observed between antibody titres and fluke numbers recovered from singly infected rats (Keegan and Trudgett, 1992; Poitou *et al.*, 1992, 1993). These observations may suggest that rats do not elicit an immune response quickly enough to eliminate the parasite in primary infections. Additionally, the immune evasion strategies of the invading parasites may well be able to cope with the slow-developing immune responses of the host in the primary infection, but parasites in challenge infections may be overwhelmed by them.

The transfer of serum from infected rats, cattle and sheep to rats conferred a passive protection and the levels of protection correlated with the volume of serum transferred (Dargie *et al.*, 1973; Armour and Dargie, 1974). However, while the serum of rats infected from 7 to 8 weeks was capable of transferring this protection, the same volume of serum obtained from rats infected for 25 weeks gave no protection. The authors suggested that antibodies generated by the juvenile flukes may be responsible for the passive protection. However, the difference in the ability of these two serum preparations to transfer resistance may also be explained by differences in antibody titres. Antibodies appear within 2 weeks following infection in rats, titres reach a maximum between 3 and 5 weeks, remain high for another 7 weeks and then gradually decline after the parasite enters the bile ducts (Keegan and Trudgett, 1992; Poitou *et al.*, 1992). Therefore, serum obtained from rats that were infected for 25 weeks may have had a low antibody titre. Nevertheless, while it is clear that protection against infection can be

transferred with serum, the antibody(s) isotype responsible for this passive protection remains to be identified.

The target against which resistance to reinfection in rats is directed appears to be the juvenile flukes. Experiments by Hayes *et al.* (1974), which involved the transfer of passive resistance with serum, indicated that parasites of less than 4 days old are the most susceptible. On the other hand, studies by Chapman and Mitchell (1982b) suggest that stages of up to 14 days old are most vulnerable. Since the protective response against these juvenile stages can be generated by immunization with antigens expressed by later developmental stages (Rajasekariah and Howell, 1978; Oldham and Hughes, 1982; Oldham, 1983), it follows that the molecular targets for resistance involve antigens that are not necessarily stage specific (although juvenile-specific antigens may also be important).

Several early research reports that investigated the fate of flukes in infected and reinfected rats suggested that the gut wall may act as an effective barrier against the entry of *F. hepatica* newly excysted juveniles (NEJ) into the peritoneal cavity, the effector mechanisms being secretory antibody (IgA) and non-specific inflammatory responses (Hayes and Mitrovic, 1977; Rajasekariah and Howell, 1977; Doy *et al.*, 1978; Doy and Hughes, 1982). Cell-mediated immune killing of intraperitoneally injected NEJs involving eosinophils, neutrophils and lymphocytes was described by Davies and Goose (1981). Doy *et al.* (1978) observed that the livers of previously infected rats were not damaged by an oral challenge infection, whereas the livers of those that were administered an intraperitoneal challenge exhibited extensive damage. Subequent studies provided further evidence for gut mucosal immunity; athymic nude rats (Nu/Nu) resisted an oral challenge of metacercariae to the same level as did their heterozygous (Nu/+) litter mates. However, in comparison to the Nu/+ rats, the Nu/Nu rats showed little resistance to an intraperitoneal challenge (Doy and Hughes, 1982). Therefore, the authors suggested that two mechanisms of resistance may operate in rats: a T-cell-independent mechanism effective at the intestinal wall and a mechanism requiring a functional thymus which is active at the peritoneum or liver parenchyma. However, recent reports indicate that a unique subset of γδ T cells that develop in intestinal cryptopatches, and do not pass through the thymus, may be involved in intestinal muscosal immunity (Saito *et al.*, 1998).

A more recent study carried out by Meeusen and Brandon (1994) supports the idea of two sites of immune attrition in rats. Rats cured of a 10-day-old infection by flukicide treatment resisted an oral but not an intraperitoneal challenge. However, if the rats were not drug-treated, and the primary infection allowed to develop, resistance to both orally and intraperitoneally administered flukes was observed. Resistance at the level of the liver must require parasites to develop for a period of time in the hepatic tissues. The report of Meeusen and Brandon (1994) also brought to light that observations regarding immune responses obtained from peripheral blood sampling do not necessarily reflect the responses occurring at the local lymph nodes. Antibody-secreting cell (ASC) probes derived from lymphocytes of mesenteric lymph nodes (MLN), hepatic lymph nodes (HLN) and from the

spleen (SLN) revealed that during the normal migration of the parasite from intestine to bile ducts only the HLN are stimulated. This stimulation occurs only after the parasites pass into the liver. However, following challenge infection stimulation of both MLN and HLN was observed. In the flukicide-treated rats antibody secretion was not observed in the MLN, HLN and SLN in spite of the fact that serum antibodies were detected. Oral challenge resulted in stimulation of MLN but not HLN, whereas intraperitoneal challenge did not stimulate MLN but did stimulate HLN. Therefore, responses in the MLN and HLN appear to be responsible for the protection observed at the level of the gut and liver, respectively. Moreover, the antibody isotype secreted by the different lymph nodes were IgA in MLN, IgE in HLN and IgM in SLN. Of additional interest was the observation that protective responses in either MLN or HLN correlated with the reactivity of ASC probes with a >200 kDa NEJ molecule in immunoblotting experiments.

An analysis of the antibody isotypes in serum of infected rats showed that IgM, IgE, IgG1 and IgG2a were all elevated compared to serum obtained from control, uninfected rats (Pfister *et al.*, 1983; Poitou *et al.*, 1992, 1993; Van Milligen *et al.*, 1998). The IgE responses were biphasic, peaking at 5 and 9 weeks after infection. While IgG1 titres increased up to 5 weeks after infection and then remained high, IgG2a titres rose gradually over a 10-week period. Furthermore, an increase in neutrophils and eosinophils was also observed. In rats, the Th2 cytokine, IL-4, stimulates the production of IgG2a and IgE whereas the Th1 cytokine, IFN-γ, induces the production of IgG1 (Table 10.2). These data suggest the involvement of both type 1 and type 2 responses in liver fluke infection of rats, with possibly a preferential induction of type 2 responses (Poitou *et al.*, 1993; Pfister, 1994).

As in other helminth infections, eosinophilia is characteristic of liver fluke infections. The number of eosinophils in the lamina propria of infected rats was markedly increased following infection and this increased further following a challenge infection (Doy *et al.*, 1978; Van Milligen *et al.*, 1998). An increase in peripheral and bone marrow eosinophils was observed in both rats and mice by Milbourne and Howell (1990) following infection. Keegan and Trudgett (1992) also noted an eosinophilia in the peripheral blood of infected rats and, in addition, showed that the livers become infiltrated with eosinophils, neutrophils and lymphocytes. Parasites implanted into the peritoneum of rats and mice also become infiltrated with eosinophils, neutrophils and lymphocytes (Rajasekariah and Howell, 1977; Bennett *et al.*, 1980; Kelly *et al.*, 1980; Davies and Goose, 1981; Doy and Hughes, 1982). Moreover, Van Milligen *et al.* (1998) showed that NEJs quickly become coated by IgG1 and IgG2a and surrounded by eosinophils as they migrate through the submucosa of immune rats. Collectively, these data indicate that eosinophils and neutrophils play a role in antibody (IgE, IgG1 and IgG2a)-mediated immune protection against flukes at both the gut and peritoneum/liver. While direct evidence for participation of these cells in the killing of liver flukes *in vivo* or *in vitro* is still lacking, recent studies indicate that rat peritoneal-lavage cells are able to mediate killing of NEJ *in vitro* by the production of nitric oxide (Spithill *et al.*, 1997).

Milbourne and Howell (1990) reported that the eosinophilic response to infection in rats appeared more rapidly compared with that observed in infected mice. In addition, eosinophilic responses to i.v. injected excretory/secretory antigens were less pronounced in mice than in rats. Smith *et al.* (1992) calculated that the production of free radicals by rat peritoneal leucocytes is 3.5 times more per cell and 30 times higher per animal compared with murine leucocytes. Differential cell counting revealed that the proportion of eosinophils in the peritoneal cells of rats following a primary and challenge infection were 32% and 45%, respectively, whereas eosinophil counts in peritoneal cells of both primary infected and challenged mice were only 5%. Both studies suggest a difference in the capability of rats and mice to mount an eosinophilic response following a fluke infection and reinfection.

O'Malley *et al.* (1993) used voltage-clamped sections of colon from infected and uninfected rats in an Ussing chamber to examine immediate Type I hypersensitivity reactions to fluke antigens at the host gut wall. Antigens in homogenates of adult flukes induced a change in the short circuit current in the tissue of infected but not uninfected animals. Anti-rat IgE antibodies had a similar effect and desensitized the tissue to further challenge with antigen. Further experiments indicated that the change in short circuit current was in part mediated by the secretion of chloride ions which, *in vivo*, would result in an osmotic pull of water into the lumen and a secretory diarrhoea that would flush parasites of a challenge infection from the intestine. Parasite antigens may interact with cells that bind IgE, such as mast cells, causing them to degranulate and release mediators which would cause this chloride secretion. Mepyramine, a histamine H1 receptor antagonist, did not affect the antigen-induced changes in short circuit current suggesting that histamine is not the mediator involved. However, piroxicam, a cyclo-oxygenase inhibitor, significantly attenuated the response and implies the involvement of locally synthesized eicosanoids (Baird and O'Malley, 1993; O'Malley *et al.*, 1993). These data may explain why in earlier studies immunity in rats was observed within 24 h after the challenge infection (Hayes and Mitrovic, 1977; Hayes, 1978). Recent results of Fadiel (1996) have shown that, unlike rat intestines, the intestines of mice do not exhibit an increase in mast cells and eosinophils during infection, and that parasite antigens do not cause a short circuit current when added to mouse intestinal sections in voltage clamp experiments.

Immunity in ruminants

Experiments in sheep

All experimental data suggest that sheep develop little or no acquired immunological resistance to *F. hepatica* (Haroun and Hillyer, 1986). Consequently, sheep are very prone to the pathological sequelae caused by the migrating and adult parasites, and often die from acute fasciolosis. Several early studies reported that the number of worms recovered after a challenge infection of previously infected and naive sheep were the same (Ross, 1967; Sinclair, 1962, 1970). However, some studies did observe effects on the

challenge infection, such as reduced egg production (Sinclair, 1962), retarded migration (Sinclair, 1970) and retarded fluke development (Sinclair, 1971; Rushton, 1977). Even high doses of metacercariae (1000) followed by anthelmintic treatment did not elicit protective immune responses against challenge infections when measured in terms of fluke burdens, although the sensitized sheep did live longer and exhibited a delay in the onset of anaemia (Boray, 1967)

Sheep, nevertheless, do produce antibody responses to liver fluke infection, predominantly of the IgG1 isotype, which peak at about 5 to 6 weeks after a primary infection (Movesijan *et al.*, 1975; Hanna, 1980a; Sexton *et al.*, 1994; Chauvin *et al.*, 1995). Moreover, ovine antibodies that were passively transferred to rats conferred resistance against infection (Mitchell *et al.*, 1981; Boyce *et al.*, 1986). Therefore, the lack of acquired immunity in sheep may be somehow related to insufficient cellular responses. In this respect, Sandeman and Howell (1981) noted that there was a lack of secondary antibody responses in challenged sheep. Furthermore, two research groups have demonstrated a suppression of the mitogen (Con A) responsiveness of peripheral blood lymphocytes (PBL) from *F. hepatica* infected sheep (Zimmerman *et al.*, 1983; Chauvin *et al.*, 1995). Studies on the local immunological and inflammatory reactions in the liver of infected sheep show that in the early stages of infection there is an infiltration of eosinophils and $CD4^+$ T cells, but that by 4 months after infection there is a more marked infiltration of $CD8^+$ and $\gamma\delta$ TCR^+ cells (Meussen *et al.*, 1995; Chauvin and Boulard, 1996). These studies suggest that type 2 responses regulate the local immune responses in the acute/hepatic stage of infection but that the regulation of responses at the chronic stages is quite different.

Experiments in cattle

A number of reports in the 1960s and 1970s documented acquired protective immunity in cattle. The level of this acquired protection to reinfection was often very high, ranging from 60% to 84% (Ross, 1966, 1968; Boray, 1967; Doyle, 1971). Resistance to reinfection did not require the continuing presence of the parasites of the primary infection as infection followed by anthelmintic treatment also elicited a protective immunity (Boray, 1967; Kendall *et al.*, 1978). While these studies led to the general consensus that cattle do acquire immunity to reinfection, some reports implied that this resistance may not be totally immunologically based. Both Ross (1967) and Boray (1967) suggested that hepatic fibrosis resulting from the primary infection may be an important factor contributing to resistance to challenge infections. Doyle (1971, 1973a) also pointed out that the duration of the primary infection, and hence the extent of liver fibrosis, correlated with the level of resistance. The development of fibrotic lesions in cattle liver may also contribute to resistance in primary infections and may explain why these hosts rarely die of acute fasciolosis (Ross, 1965a). For example, infections of 200 to 1300 metacercariae result in the establishment of approximately 30% of the parasites in the bile duct, whereas only approximately 3% of the parasites in an infection dose of 2500 to 15,000 metacercariae make it to the bile ducts (Ross, 1965a, b).

Recently, McCole *et al.* (1998a) showed that fluke antigen induced electrogenic chloride secretion when added to voltage-clamped colonic mucosae from infected cattle. They suggested that *in vivo* chloride-led fluid secretion mediated by components released by eosinophils or mast cells may be involved in the expulsion of flukes of a challenge infection. However, studies by Anderson *et al.* (1978) and Doy and Hughes (1984a) indicate a lack of mucosal immunity in cattle since the number of immature flukes recovered from the peritoneal cavity of previously infected and naive calves at both 4 and 14 days after a challenge infection was the same. A resistance to infection that prevents or retards the entry by the flukes was suggested to operate at the level of the liver capsule. Metaplastic changes in the mesothelial cells of the liver capsule of infected cattle, changing them from a cuboidal to a highly active columnar state, which may impair the penetration of this tissue by flukes, were described by Doy and Hughes (1984b).

Most of the early studies describing acquired protective immunity in cattle involved the administration of a large single challenge dose (≥ 500 metacercariae). Recent experiments by Clery *et al.* (1996) demonstrated that cows harbouring a chronic *F. hepatica* infection can be easily superinfected by a trickle challenge of 50 metacercariae per day for 10 days. This infection protocol was used because it simulated the type of challenge encountered by cattle in natural field conditions. These authors also pointed out that the high prevalence of chronic liver fluke infections in cattle (and sheep) and the continuing susceptibility of these to repeated challenge infections in the field reveals the ineffectiveness of their immune responses against the parasite.

The antibody responses in cattle (and sheep) show a marked predominance of the IgG1 isotype over IgG2 (Doyle, 1973a, b; Flakstad and Eriksen, 1974; Movesijan *et al.*, 1975; Duffus and Franks, 1981; Clery *et al.*, 1996) which is consistent with the responses of ruminants to other helminth infections (Mansour *et al.*, 1990; Schallig *et al.*, 1995). The IgG1 titres peak from 8 to 10 weeks after infection, and then decline slowly (Fig. 10.4A). Reagents for the specific detection of bovine IgE are not yet widely available and hence there are no indications of the profile of this isotype response during infection; however, reagenic (IgE-like) antibodies have been detected in the serum of infected cattle (Doyle, 1973b). Kendall *et al.* (1978) observed an increase in fluke-specific serum antibody following secondary infection in calves, but found no correlation between antibody precipitin titres and fluke burdens in the animals. Moreover, while proliferative responses of PBLs to stimulation with fluke antigen followed a similar pattern as the IgG1 antibody response, Oldham (1985) and Oldham and Williams (1985) also found no correlation between these responses and fluke burden. On the contrary, Clery *et al.* (1996) and Mulcahy *et al.* (1998) have recently demonstrated a positive correlation between both PBL responsiveness and specific anti-fluke IgG1 titres with fluke burden in experimentally infected cattle, indicating that the immune responses generated in cattle do not protect them against infection (Fig. 10.4B).

Definitive analysis of the type of cellular immune responses in cattle awaits the development of reliable assays for the measurement of a range of

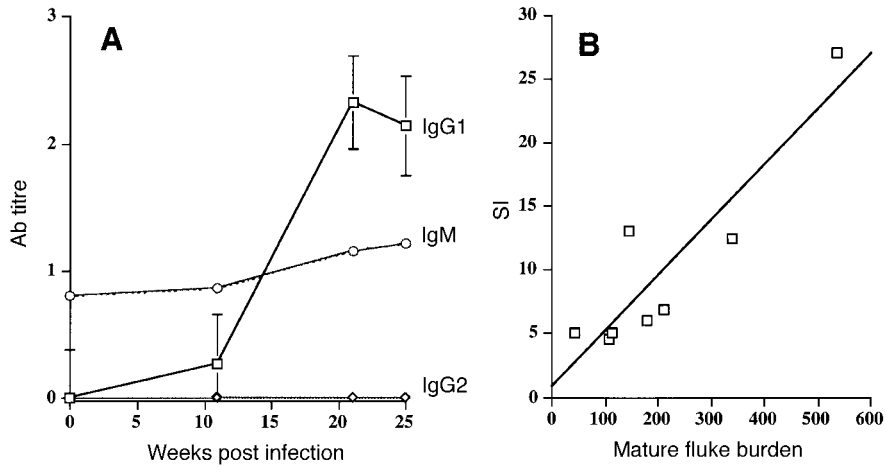

Fig. 10.4. A. Immunoglobulin responses of cattle (Holstein–Friesian) during the course of an experimental infection with *Fasciola hepatica*. (Adapted from Mulcahy *et al.*, 1998.) B. Correlation of lymphocyte responses and adult fluke burden in cattle 13 weeks after an experimental infection. SI, stimulation index. (Adapted from Clery *et al.*, 1996.)

bovine cytokines. However, some cellular and cytokine-based information is already documented in the literature. The studies of Brown *et al.* (1994) suggested that $CD4^+$ cells are more important in the immune responses of chronically infected cattle; the authors could isolate Th2 and Th0 cell clones, but not Th1 cell clones, from these animals. On the other hand, recent studies by McCole *et al.* (1988b) demonstrated the involvement of both $CD4^+$ and $CD8^+$ cells in the peripheral blood lymphocyte (PBL) responses in acutely infected cattle. Furthermore, depletion of the γδ subset from PBLs from fluke-infected cattle did not have a marked effect on their proliferation in response to fluke antigen (McCole *et al.*, 1998b). Production of the type 1 cytokine IFN-γ can be detected in peripheral blood from weeks 2 to 5 post-infection, after which production subsequently declines (Clery and Mulcahy, 1998). Furthermore, mRNA for the type 2 cytokine IL-4 was detected in hepatic lymph nodes of cattle throughout the course of a *F. hepatica* infection. The levels of this cytokine increased several fold higher in the hepatic lymph nodes as compared with prescapular lymph nodes as infection progressed (Clery, 1996; Clery and Mulcahy, 1998). These limited data suggest that early in infection the immune response of cattle is not polarized but as the infection progresses uncommitted Th0 cells are stimulated to differentiate into Th2 cells which leads to a predominating type 2 response.

High levels of protection against infection (72%) with liver fluke could be achieved in cattle by immunization with a vaccine cocktail containing cathepsin L proteinases and fluke haemoglobin formulated in Freund's adjuvant (Dalton *et al.*, 1996; Mulcahy *et al.*, 1998). An analysis of the antibody

responses of the vaccinated cattle following the challenge infection revealed that they elicit both IgG1 and IgG2, the titres of which were >eightfold and >200-fold higher, respectively, than in the non-vaccinated controls. Since the IgG2 isotype in cattle is associated with the type 1 cytokine IFN-γ, it would appear that the generation of the protective responses by this vaccine involved the induction of type 1 responses.

Immune responses in humans

Fasciola hepatica does not usually cause mortality in human hosts. Humans are not considered to be a very susceptible host and most migrating fluke become entrapped in the liver parenchyma (however, some flukes can reach, and become mature in, the bile duct; see Chapter 12 of this volume). Abdominal pain, fever and sickness are symptomatic of the acute stages of the disease, and clinical manifestations include systemic eosinophilia and elevated levels of IgM, IgG and IgE. Specific anti-parasite IgE was detected in 48% of patients infected with liver fluke (Chen and Mott, 1990). Recently, O'Neill *et al.* (1998) analysed the immunoglobulin responses of liver-fluke-infected humans to excretory/secretory antigens and to a fluke cysteine proteinase, cathepsin L1, and demonstrated that the predominant isotypes elicited by infection were IgG1 and IgG4. Moreover, a positive relationship between the level of infection (expressed as eggs/gram of faeces) and the titres of IgG4 was observed. Collectively, the data suggest that, as with other helminth infections, *F. hepatica* evokes a type 2 response to infection in humans. At present, there is no evidence to suggest that this response results in an immunological resistence to infection.

Concluding Remarks

The immunology of parasitic infections has contributed a great deal to fundamental concepts of adaptive immunity. The Th1/Th2 dichotomy, for example, is perhaps most clearly illustrated by comparison of intracellular protozoan infections with extracellular helminth infections. While our understanding of liver fluke immunology is still deficient in many important respects, the tools will soon be available for a renewed burst of activity in this field. To date, limited data suggest that *F. hepatica* generates predominantly type 2 responses in animals and humans, which is consistent with the responses observed against other helminths. Important questions which should be asked and answered over the coming years include:

- What is the immunological basis for susceptibility and resistance to fasciolosis?
- What are the signals which trigger type 2 responses in liver fluke infection?
- Are type 2 immune responses inherently non-protective, or are they rendered deficient by parasite immunomodulatory strategies?
- What patterns of lymphocyte subset and accessory cell involvement occur, systemically and locally, in liver fluke infection?

- What are the detailed patterns of cytokine production during infection in each host?
- What effect does blockage of individual components of the response (for example by anti-cytokine or anti-lymphocyte subset monoclonal antibodies) have on the course of infection?

Despite many yawning gaps which remain to be filled in order for us to have a real understanding of the immunology of fasciolosis, considerable progress has been made towards the development of immunoprophylactic strategies and there have been many reports on the identification of putative protective antigens including excretory/secretory antigens (Irving and Howell, 1982), T0/T1 surface antigens (Hanna *et al.*, 1988), glycoproteins (Dalton *et al.*, 1985), fatty-acid binding proteins (Hillyer, 1985; Tendler *et al.*, 1996), glutathione *S*-transferase (Sexton *et al.*, 1994; Morrison *et al.*, 1996), cysteine proteinases (Dalton and Heffernan, 1989; Carmona *et al.*, 1993; Wijffels *et al.*, 1994; Dalton *et al.*, 1996; Wilson *et al.*, 1998) and other molecules (Poitou *et al.*, 1993; Meeusen and Brandon, 1994) (see Chapter 11 of this volume).

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11 Development of Vaccines Against *Fasciola hepatica*

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Introduction

Infection of domestic ruminants with *Fasciola hepatica* (temperate liver fluke) and *F. gigantica* (tropical liver fluke) causes significant economic loss estimated at over US$2000 million per annum to the agricultural sector worldwide with over 600 million animals infected (Boray, 1985; Hillyer and Apt, 1997). In addition, fasciolosis is now recognized as an emerging human disease: WHO has recently estimated that 2.4 million people are infected with *Fasciola*, and a further 180 million are at risk of infection (Anon., 1995). High prevalence of human fasciolosis has been reported in Bolivia and Peru where fasciolosis is regarded as an important human health problem (Maurice, 1994; Anon., 1995; Esteban *et al.*, 1997; Hillyer and Apt, 1997; O'Neill *et al.*, 1997). In tropical regions, fasciolosis is considered the single most important helminth infection of cattle (Fabiyi, 1987) with prevalence rates of 30–90% in Africa (Schillhorn van Veen, 1980; Schillhorn van Veen *et al.*, 1980), 25–100% in India (Sharma *et al.*, 1989; Roy and Tandon, 1992), 27–91% in Iran (Sahba *et al.*, 1972), up to 85% in north-east Thailand (Pholpark and Srikitjakarn, 1989) and 25–90% in Indonesia (Edney and Muchlis, 1962; Soesetya, 1975). In developed countries the incidence of *F. hepatica* ranges up to 77% (Wilson *et al.*, 1982; Dargie, 1986). Although triclabendazole is an effective drug at controlling disease caused by both species of *Fasciola* (Boray *et al.*, 1983; Estuningsih *et al.*, 1990; Suhardono *et al.*, 1991), the cost of treatment with this drug is a barrier to its wide adoption by rural producers in developing countries. Furthermore, resistance to triclabendazole has been reported in sheep infected with *F. hepatica* (Overend and Bowen, 1995) suggesting that the effectiveness of this drug may eventually be compromised by the selection of resistant parasites in the field. The development of a vaccine will provide producers with an alternative, environmentally friendly, cost effective and sustainable strategy for the control of fasciolosis.

Fasciolosis (ed. J.P. Dalton)

Several studies in different laboratories have shown that *F. hepatica* infection can induce host immune responses which are effective at killing this parasite and conferring protection against fasciolosis (reviewed in Rickard and Howell, 1982; Haroun and Hillyer, 1986; Hughes, 1987). Passive transfer of immune sera and adoptive transfer of immune cells have been shown to confer protection against *F. hepatica* in recipient sheep, cattle and rats (Dargie *et al*., 1974; Rickard and Howell, 1982; Haroun and Hillyer, 1986). There is good evidence that cattle can be protected against *Fasciola* infection by vaccination using irradiated metacercariae or parasite extracts (Bitakaramire, 1973; Nansen, 1975; Hall and Lang, 1978; Rickard and Howell, 1982; Haroun and Hillyer, 1986; Haroun *et al*., 1988). These observations suggest that antigens of *F. hepatica* can induce protective immune responses in cattle and imply that vaccination with defined antigens is, theoretically, an achievable goal. In contrast, numerous studies have shown that sheep do not acquire resistance to a secondary *F. hepatica* infection following primary exposure (Boray, 1969; Boyce *et al*., 1987; Chauvin *et al*., 1995). These apparently contradictory results in cattle and sheep suggest that either the immune response induced in cattle is qualitatively or quantitatively different to that induced in sheep (i.e. the cattle response is missing or expressed to a lesser degree in sheep) or that *F. hepatica* can resist or subvert the sheep immune response in some way. The fact that Sudanese, Indonesian and Merino sheep do acquire resistance against *F. gigantica* (A'Gadir *et al*., 1987; Roberts *et al*., 1996, 1997) shows that sheep are competent to mount effective immune responses against *Fasciola* and indirectly suggests that *F. hepatica* and *F. gigantica* differ in some fundamental biochemical trait, as discussed elsewhere (Spithill *et al*., 1997).

There have been many attempts to vaccinate laboratory animals, sheep and cattle with various liver fluke extracts, such as crude somatic antigens and excretory/secretory antigens. The results of these studies, which have been mixed or even contradictory, have been reviewed elsewhere (Rickard and Howell, 1982; Haroun and Hillyer, 1986; Hughes, 1987). However, the focus of the present review is to discuss more recent data on the development of defined vaccines against fasciolosis from the use of irradiated attenuated vaccines to the strategies being used to develop new subunit vaccines. The current state of knowledge with different candidate antigens and the prospects for the future are considered.

Vaccine Strategies and Candidate Antigens

Irradiated attenuated vaccines

High levels of resistance to homologous challenge by blood flukes of the genus *Schistosoma* can be achieved by sensitization with either irradiation-attenuated cercariae or schistosomula in a variety of host species (Taylor, 1987; Spithill, 1992). The immunization of host species against *F. hepatica* using irradiation-attenuated metacercariae was one of the earliest strategies tested in an attempt to identify host-protective antigens of *Fasciola* (Table 11.1). Protection with

irradiation-attenuated vaccines has been successfully induced against fasciolosis in cattle and rats but generally not in mice, rabbits or sheep. Vaccination of cattle against *F. hepatica* with 3 to 3.5 krad γ-irradiated metacercariae has been highly successful (Dargie *et al*., 1974; Nansen, 1975; Acosta *et al*., 1989) but dependent upon the vaccine protocol employed (Table 11.1). For example, a 70% reduction in fluke burden was observed in cattle vaccinated twice, 4 weeks apart, with 3.5 krad irradiated metacercariae and challenged 8 weeks later whereas only a 30% reduction was observed if the challenge was given 4 weeks after the second vaccination dose (Dargie *et al.*, 1974). Sensitization of rats with metacercariae attenuated with 2.5–3 krad of X-irradiation resulted in a reduction of approximately 50% in challenge fluke burden (Thorpe and Broome, 1962; Corba *et al*., 1971; Armour and Dargie, 1974). Even greater protective levels, in excess of 80%, were achieved with metacercariae attenuated with 5 and 7.5 krad of X-rays. However, as seen in cattle, protection was dependent upon the time of challenge after vaccination, and 10 krad-irradiated metacercariae did not induce protection (Thorpe and Broome, 1962). Resistance was immunologically mediated as lymphoid cells transferred from vaccinated rats conferred 100% protection in recipient rats to subsequent challenge (Corba *et al*., 1971). The fact that protection in cattle and rats appears to depend on the time post-vaccination when the challenge is administered suggests two possible interpretations: first, that the protective immune response is transiently induced (i.e. there is a window of protection following vaccination); or second, that the immune response develops with time post-vaccination (i.e. there is maturation of the protective response).

Sheep (and cattle) can be highly protected against *F. gigantica* by immunization with irradiated metacercariae (Bitakaramire, 1973; A'Gadir *et al*., 1987). However, similar attempts to stimulate significant resistance to *F. hepatica* in sheep have not been successful (Boray, 1967; Dargie *et al*., 1974; Campbell *et al*., 1978; Creaney *et al*., 1995a; see also Table 11.1). Sheep do not acquire resistance to reinfection following initial infection with either non-irradiated or irradiated metacercariae (Boray, 1969; Boyce *et al*., 1987; Chauvin *et al*., 1995). Yet, intriguingly, adoptive transfer of lymphoid cells from sheep vaccinated with 3 krad-irradiated metacercariae conferred resistance to naive sheep following subsequent *F. hepatica* challenge (Dargie *et al*., 1974). In addition, passive transfer of serum from such sensitized sheep also protected rats from *F. hepatica* challenge (Dargie *et al*., 1974). In an attempt to optimize the experimental design of vaccination of sheep against *F. hepatica* using irradiated metacercariae, Creaney *et al*. (1995a) examined a range of irradiation attenuation doses and of sensitizing infections in a checkerboard experimental vaccine design. No significant reduction of fluke burden was seen in any group (Table 11.1). Fluke viability, however, was severely affected by doses of γ-irradiation of 3 krad or greater and no mature flukes were recovered from control sheep given metacercariae attenuated with 7 krad or greater. From the experiments of Creaney *et al*. (1995a) and others using either *Fasciola* sp. or *Schistosoma* sp., it would appear that irradiation doses around 3 krad represent a threshold beyond which normal development of juvenile trematodes cannot proceed.

Table 11.1. Protection against *F. hepatica* by vaccination with irradiated metacercariae.

	Sensitizing schedule					Challenge infection			
Host species	Irradiation dose (krad)	Type	No. of mc	Boosted	Interval (weeks)	No. of mc	Week post sensitization	Reduction in fluke burden (%)	Reference
Rat	1	X-ray	40	No		20	7	+70	Thorpe and Broome (1962)
	2.5	X-ray	40	No				46	
	5	X-ray	40	No				4	
	7.5	X-ray	40	No				0	
	10	X-ray	40	No				+50	
Rat	1	X-ray	40	No		20	11	+300	Thorpe and Broome (1962)
	2.5	X-ray	40	No				45	
	5	X-ray	40	No				85	
	7.5	X-ray	40	No				80	
	10	X-ray	40	No				25	
Rat	2.5	γ-ray	20	×3	1	20	11	80	Corba *et al.* (1971)
Rat	3	γ-ray	20	×3	1	20	10	56	Armour and Dargie (1974)
Rat	3.8	γ-ray	50	No		3 adult fluke	6	0	Hughes *et al.* (1981)
Mice	3 and 4	X-ray	40	×2	3	10	10	0	Hughes (1963[a])
Mice	3	X-ray	40	No		10	10	0	Dawes (1964)
Mice	3.8	γ-ray	20	No		100	3	36; day 2 pc	Harness *et al.* (1976)
	3.8	γ-ray	20	×2	1	100	3	40; day 2 pc	
Mice	3.8	γ-ray	20			100	3	38; day 2 pc	Harness *et al.* (1977)
	3.8	γ-ray	20			100	3	0; day 14 pc	
Rabbits	4	γ-ray	40	×2	3		10	0	Hughes (1963[a])
Cattle	20	X-ray	3000	×3	6	5000	3	ns	Boray (1967)
Cattle	3	γ-ray	1500	×3	4	Field trial	4	71	Nansen (1975)

Cattle	3.5	γ-ray	1000	×2	4	1000	8	70	Dargie *et al.* (1974)
	3.5	γ-ray	1000	×2	4	1000	4	30	
Cattle	3	γ-ray	500	No		500	6	72.5	Acosta *et al.* (1989)
Cattle[a]	3	γ-ray	500	No		500	6	83.5	
Sheep	3	X-ray						0	Hughes (1963[a])
Sheep	20	X-ray	400	×3	6	4000		ns	Boray (1967)
Sheep	3	γ-ray	100	×6		750		ns(66)	Dargie *et al.* (1974)
Sheep	2.5	γ-ray	100	×2	6	500	6	ns	Campbell *et al.* (1978)
	2.5	γ-ray	1000	×2	6	500	6	ns	
Sheep	3	γ-ray	500	No		260	10	ns	Creaney *et al.* (1995a)
	10	γ-ray	500	No		260	10	ns	
	40	γ-ray	500	No		260	10	ns	
	3	γ-ray	2000	No		260	10	ns	
	10	γ-ray	2000	No		260	10	ns	
	40	γ-ray	2000	No		260	10	ns	
	7	γ-ray	2000	×1	4	300	4	ns	
	10	γ-ray	2000	×1	4	300	4	ns	
	15	γ-ray	2000	×1	4	300	4	ns	

[a] Hughes, PhD Thesis (1963) cited in Dawes (1964); pc, postchallenge; ns, not significant

Despite the lack of significant protection, sera from sheep vaccinated with irradiated metacercariae were used in ELISA and Western immunoblot analyses to identify *F. hepatica* antigens that were uniquely or more strongly recognized when compared to sera from sheep receiving a challenge infection only (Creaney *et al.*, 1995a). All sheep given irradiated metacercariae developed a strong humoral immune response to somatic *F. hepatica* antigens. Surprisingly, only sera from sheep vaccinated with 7 krad-irradiated metacercariae recognized two candidate liver fluke antigens, *F. hepatica* GST and cathepsin L proteases. The effect of irradiation of metacercariae on the expression of carbohydrates and the cathepsin B secreted by irradiated newly excysted juvenile *F. hepatica* (NEJ) (Wilson *et al.*, 1998) was examined: this revealed a reduction in tissue expression by irradiated NEJ of cathepsin B, changes in the parasite surface and gut expression of concanavalin A- and wheat germ agglutinin-specific sugars and a reduction in proteolytic cleavage of immunoglobulin by NEJ excretory–secretory (ES) products (Creaney *et al.*, 1996). Biosynthetic labelling experiments, however, indicated that ES material continued to be actively synthesized post-irradiation by NEJ cultured *in vitro* and suggest that the secreted cathepsin B may have been inactivated or conformationally modified by irradiation (Creaney *et al.*, 1996). The irradiation-induced alterations to carbohydrate and protease expression observed in NEJ may be detrimental to parasite invasion of the host tissues and may also contribute to protective responses generated within these hosts following administration of irradiation-attenuated metacercariae of *Fasciola* sp.

Mechanism(s) of protection induced by irradiated vaccines

It has been suggested that protection of cattle and rats against *F. hepatica* by sensitization with irradiation-attenuated metacercariae may be due to the establishment of a small, but normal, population of fluke from the irradiated sensitizing infection in the liver which causes a fibrosis in the liver and that resistance is, therefore, induced by a physiological barrier due to tissue fibrosis rather than immunological means (Boray, 1969; Rickard and Howell, 1982; Hughes, 1987). However, in the experiments of Bitakaramire (1973), where the 3 krad-irradiated sensitizing infection induced a 98% reduction in *F. gigantica* burdens in cattle, there was no liver damage induced by the vaccine and no adult fluke were recovered. Indeed, metacercariae prevented from maturing by high levels of irradiation stimulated greater resistance against *F. gigantica* in cattle than that induced by minimally irradiated cysts (Younis *et al.*, 1986). Similar findings were observed with irradiated schistosome vaccines (Bickle *et al.*, 1979). These results suggest that physical interference with intrahepatic migration of the challenge fluke population by hepatic fibrosis and calcification is not solely responsible for development of resistance. Indeed, successful passive and adoptive transfer experiments have demonstrated that immune-based mechanisms are operative in the acquisition of resistance to *F. hepatica* in rats and cattle (Corba *et al.*, 1971; Armour and Dargie, 1974; Dargie *et al.*, 1974). The high levels of protection observed in cattle with irradiated vaccines suggest that careful analysis of the bovine immune response to irradiated *F. hepatica* may identify candidate vaccine

antigens, as have been previously characterized for *S. mansoni* using rodent and rabbit sera.

Although sheep have been successfully vaccinated against *F. gigantica* and *S. mattheei* using irradiated homologous metacercariae (Taylor, 1987), it appears unlikely that sheep will be successfully protected against *F. hepatica* using this approach. Whether this is due to an intrinsic inability of the sheep to respond in an effective manner to key parasite antigens presented by the irradiated parasite, or whether the immune response induced is ineffective against *F. hepatica*, is not known. Several recent observations appear to suggest that survival of *F. hepatica* in sheep may result from a combination of variation in host responsiveness and parasite resistance to immune killing (discussed in Spithill *et al.*, 1997). The fact that sheep are significantly protected in some experiments with the GST vaccine (see below) shows that sheep *can* mount effective immune responses against *F. hepatica*, albeit the effects of these responses are not always reproducible in different groups of animals. Such variability in vaccine efficacy between experiments may result from variation in immune responses between outbred animals, creating a window of escape for *F. hepatica* larvae if the immune response fails to reach some critical threshold below which the parasite can survive.

Defined antigens

Fatty acid binding proteins (FABP)

Fatty acid binding proteins are a large family of proteins involved in the binding and transportation of a variety of hydrophobic ligands, such as oleate, palmitate and a variety of bile acids. The best characterized class of these proteins is the cytoplasmic FABP family. Within this family there exist a variety of FABPs of differing tissue specificities (for vertebrates) and a number of FABPs have been isolated from invertebrates. The most striking feature of the cytoplasmic FABP family is the conservation of size; all known members range between 14 and 16 kDa in mass and 127–133 amino acids in length (Veerkamp *et al.*, 1991). The crystallization of several members of the family has revealed a significant structural conservation within the FABP family (Jones *et al.*, 1988).

FABPs were the first defined, purified antigen fraction to be tested as a vaccine against fasciolosis. The recognition of this antigen as protective came from experiments performed in the laboratory of George Hillyer who, in the mid-1970s, identified a set of *Fasciola* proteins which could be purified by virtue of their cross-reactivity to antisera raised to soluble *S. mansoni* proteins (Hillyer *et al.*, 1977). A subset of such proteins (termed $Fh_{SmIII(M)}$) was isolated and shown to be able to protect mice (Hillyer, 1985) and calves (Hillyer *et al.*, 1987) against challenge by *F. hepatica* (reducing worm burdens by 69–78% and 55%, respectively) when incorporated in Freund's adjuvant (FCA) (Table 11.2). Additionally, cross-trematode protection was also demonstrated. Thus, mice vaccinated with $Fh_{SmIII(M)}$ were protected up to 81% from infection by *S. mansoni* cercariae (Hillyer, 1979). This result confirmed the conservation of protective epitopes between *Fasciola* and *Schistosoma* antigens.

Table 11.2. Vaccine antigens and formulations tested against *F. hepatica*.

Antigen	Source	Host	Schedule	Adjuvant	Result	References
$Fh_{SmIII(M)}$ (FABP)	Purified *S. mansoni* cross-reactive proteins	Mice	5 μg × 2	FCA/FIA	69–78% reduction in worms	Hillyer (1985)
		Cattle	500 μg × 2	FCA/FIA	55% reduction in worms	Hillyer *et al.* (1987)
rSm14	Recombinant protein	Mice	10 μg × 2	FCA/none	100% reduction in worms	Tendler *et al.* (1996)
rFh15	Recombinant protein	Rabbits	40 μg × 2	FCA/FIA	11–17% reduction in worms (not significant)	Muro *et al.* (1997)
FABP3	Adult fluke	Cattle	100 μg × 2	DEAE	No reduction in worms	Bozas *et al.* (pers. comm.)
GST	Adult fluke	Sheep	See Table 11.3	FCA/FIA	57% reduction in worms	Sexton *et al.* (1990)
		Sheep	See Table 11.3	FCA/FIA	6–65% reduction in worms	Sexton *et al.* (pers. comm.)
		Sheep	See Table 11.3	Quil A/SM	26% reduction in worms	Sexton *et al.* (pers. comm.)
				Other adjuvants	0–41% reduction in worms	Sexton *et al.* (pers. comm.)
		Cattle	400 μg × 1[a] 200 μg × 1[b] (or 2 × 400 μg)	Quil A/SM	Mean 43% reduction in worms (range 19–69%)	Morrison *et al.* (1996)
			400 μg × 1[a] 200 μg × 1[b]	MF59	41% reduction in worms 38% reduction in worms	Morrison *et al.* (1996) Morrison *et al.* (pers. comm.)
			400 μg × 1[a] 200 μg × 1[b]	PLG/SM	59% reduction in worms	Morrison *et al.* (1996)
			400 μg × 1[a] 200 μg × 1[b]	NAGO	65%, 9% reduction in worms	Morrison *et al.* (pers. comm.)
			400 μg × 1[a] 200 μg × 1[b]	Other adjuvants	0–37% reduction in worms (not significant)	Morrison *et al.* (1996)

CatL	Adult E/S	Sheep	120 μg × 1 90 μg × 1	FCA/FIA	No reduction in worms, 69.4% reduction in egg output	Wijffels *et al.* (1994b)
CatL1	Adult E/S	Cattle	10–500 μg × 3	FCA/FIA	38.2–69.5% reduction in worms	Dalton *et al.* (1996)
CatL1	Adult E/S	Cattle	200 μg × 3	FCA/FIA	42.5% reduction in worms, 40–65% egg viability (controls 96–100%)	Dalton *et al.* (1996) Dalton *et al.* (1996)
Haemoglobin (Hb)	Adult E/S	Cattle	200 μg × 3	FCA/FIA	43.8% reduction in worms, 30–75% egg viability	Dalton *et al.* (1996)
CatL1 + Hb	Adult E/S	Cattle	200 μg × 3	FCA/FIA	51.9% reduction in worms, 0–80% egg viability	Dalton *et al.* (1996)
CatL2 + Hb	Adult E/S	Cattle	200 μg × 3	FCA/FIA	72.4% reduction in worms, 0–7% egg viability	Dalton *et al.* (1996)
Paramyosin	Adult fluke	Sheep	100 μg × 2	FCA/FIA	45% reduction worms, 94% reduction FEC	Morrison *et al.* (pers. comm.)
			100 μg × 2	DEAE Dex/SM	No reduction in worms, 57% reduction FEC	Morrison *et al.* (pers. comm.)
		Cattle	100 μg × 2	QA/SM	47% reduction in worms (P<0.05) 55% reduction FEC	Morrison *et al.* (pers. comm.)
KTM	Adult fluke	Sheep Cattle	100 μg × 2 100 μg × 2	FCA/FIA Quil A	No reduction in worms No reduction in worms	Bozas *et al.* (pers. comm.)

[a] First dose; [b] second dose.

Further purification of the $Fh_{SmIII(M)}$ fraction yielded a purified, homogeneous protein of apparent molecular mass 12 kDa (Hillyer *et al.*, 1988a). Antibodies to this protein were present in serum from animals infected with either *F. hepatica* or *S. mansoni* (Hillyer *et al.*, 1988b). Moreover, vaccination with this protein was also able to protect mice against *S. mansoni* infection (up to 77%). It was therefore proposed that this 12 kDa protein was able to protect animals against infection by at least two trematode species and is a cross-reactive, cross-protective antigen. The identity of the protective 12 kDa component was resolved when a *F. hepatica* cDNA library was screened with antisera to the protein and the isolated cDNA clones were examined (Rodriguez-Perez *et al.*, 1992). These cDNAs were found to encode a homologue of the cytoplasmic FABP family, with maximum identity (44%) to a recently identified FABP clone (Sm14) isolated from *S. mansoni* (Moser *et al.*, 1991). The *Fasciola* cDNA encodes a predicted protein of 14.7 kDa (termed Fh15), which is similar in size to that of the *Schistosoma* homologue (Sm14). Debate still exists as to whether Fh15 does encode the Fh12 protein, although it is apparent that they are at least immunologically related, since there are several FABP isoforms present in *Fasciola*.

Recently, Muro *et al.* (1997) tested the efficacy of recombinant Fh15 FABP as a vaccine in rabbits but the reductions in worm burdens were not significant and less than those elicited by the native molecule (Table 11.2). It may be that the recombinant protein is subtly different to the native one, such that on vaccination the epitopes which are recognized are not those which are presented by the native protein, and hence do not yield a protective response. It is also possible that the recombinant FABP is not the member of the family which is the specific target of a protective response.

In terms of vaccination, it should be borne in mind that *Fasciola* (and probably *Schistosoma*) have several different FABP isoforms. A second *Fasciola* FABP was deposited in the protein databases in 1994 (R.M Chicz; Pir database, accession number A44638). This protein has 72% identity to that described by Rodriguez-Perez *et al.* (1992). In addition, Bozas and Spithill (1996) identified 12 kDa proteins from *F. hepatica* which showed peptide sequence homology to FABP. One peptide showed identity with a region of the protein identified by Chicz while the other peptide, also showing similarity to the FABP family, matched with neither that protein nor the Fh12 (=Fh15) identified by Rodriguez-Perez *et al.* (1992) and represents a third homologue (FABP3).

This FABP3 protein has been purified by reverse phase HPLC (with concomitant denaturation of antigen) and tested as a vaccine against fasciolosis in cattle (E. Bozas *et al.*, Attwood, 1995, personal communication). Cattle were vaccinated with two doses of 100 µg of FABP in DEAE adjuvant and challenged 4 weeks after the second dose: no protection was observed which may be due to the use of denatured protein or a different adjuvant to the FCA used by Hillyer and colleagues (Table 11.2). As discussed below, the induction of protective responses to *Fasciola* antigens is dependent on the choice of adjuvant, implying that particular immune responses are necessary for protection against *Fasciola*. It is clear that *Fasciola* has at least three

members of the cytoplasmic FABP family, which may have different functions, substrate specificity and immunogenicity. Recently, an FABP fraction from *F. gigantica* has been tested as a vaccine in cattle and a low but significant 31% protection was observed using FCA as adjuvant (Estuningsih *et al*., 1997; see also Chapter 15 of this volume for details of this experiment).

Recently, attention has turned to the *S. mansoni* FABP, Sm14, as a vaccine candidate against fasciolosis and schistosomiasis. Tendler *et al.* (1996) tested the efficacy of recombinant Sm14 (produced in *E. coli*) to protect mice against trematode challenge. Rabbits and mice were immunized with rSm14 in FCA and challenged with up to 1000 cercariae of *S. mansoni*. Protection of rabbits was 89% (compared with 93% for a soluble extract) and protection of mice ranged from 37% to 66%. It was also shown that the presence of FCA, while generally stimulating immunity, was not required and high levels of protection could be achieved in mice by vaccination with the recombinant protein alone. An intriguing observation was the fact that Sm14 protected mice 100% against challenge with *F. hepatica*. However, it should be noted that mice are not a natural host for *F. hepatica* and are killed by an infection of several parasites. Thus, the challenge in these experiments of Tendler *et al.* (1996) was three metacercariae which may bias the results in favour of the vaccine. The efficacy of Sm14 as a vaccine should now be evaluated in ruminants.

FABP is one of the most promising vaccine candidates against fasciolosis and has the additional benefit of also being a candidate for vaccination against schistosomiasis. *Schistosoma* (and probably other trematodes) cannot synthesize long chain fatty acids *de novo* (Meyer *et al*., 1970) and therefore presumably rely on the transport of fatty acid precursors from host serum for fatty acid supply. Vaccination against the FABPs may interfere with the process of fatty acid uptake and thus be a promising avenue of attack. Future development of the Sm14 and Fh12/15 antigens is awaited with interest.

GST

The glutathione *S*-transferases (GST, EC 2.5.1.18) comprise a family of isoenzymes involved in the cellular detoxification of a broad range of chemical substrates. Neutralization of the substrates, through the conjugation of glutathione, renders the product more water soluble, less toxic and more readily excreted from the host. Virtually all species examined exhibit multiple forms of GST that are characterized by distinct but often overlapping substrate specificities. Cytosolic GSTs of mammalian cells are functionally dimeric enzymes composed of identical or non-identical subunits of 24–29 kDa. Six classes of cytosolic GSTs have been characterized (alpha, mu, pi, theta, sigma and kappa) that generally share high sequence identity (approximately 70%) within a class with only low sequence identity between classes (approximately 30%) (Mannervik *et al*., 1985; Meyer *et al*., 1991; Ji *et al*., 1995; Pemble *et al*., 1996).

Phase 1 detoxification enzymes in mammals, cytochrome P-450 and cytochrome b_5, have not been identified in helminth parasites (Precious and Barrett, 1989a, b). In contrast, GSTs are highly abundant and present in all

helminth parasites characterized to date (Brophy *et al*., 1990a; Brophy and Pritchard, 1994). Such high levels of GST infer an important role for these enzymes in helminth homeostasis and survival and this may be related to the 'naked tegument' of helminth parasites and their potential exposure to a wide range of xenobiotics (Brophy and Pritchard, 1994). Possibly of greater physiological significance is the demonstration of enzymatic activity of helminth GSTs with the secondary products of lipid peroxidation (O'Leary and Tracy, 1988; Brophy and Barrett, 1990; Brophy *et al*., 1990b; Salvatore *et al*., 1995). Apart from reactions from their endogenous metabolism, GSTs of helminth parasites may protect against exogenous free radical damage as a result of immune effector mechanisms from the host directed at the parasite (Brophy and Pritchard, 1994).

Purification of GST from adult *F. hepatica* by glutathione affinity chromatography yielded two closely migrating species at approximately 23–26.5 kDa as defined by SDS–PAGE (Howell *et al*., 1988; Brophy *et al*., 1990b; Wijffels *et al*., 1992). Early studies using starch gel electrophoresis and chromatofocusing identified a minimum of five isoenzymes within this mixture (Howell *et al*., 1988; Brophy *et al*., 1990b). Studies within our own laboratory demonstrated a higher level of heterogeneity with eight charged species separated by 2-D gel analysis in conjunction with Western blotting (Wijffels *et al*., 1992; Sexton, 1994).

Recombinant *F. hepatica* GST cDNA clones, encoding four of these isoenzymes, have been identified by screening an adult fluke cDNA expression library with rabbit antiserum to the native GST (Panaccio *et al*., 1992). These four cDNAs, designated rGST51, rGST47, rGST7 and rGST1, share 59–89% identity at the nucleotide sequence level and 71–89% identity at the amino acid level. Another GST with 98% identity to GST51 was cloned by Muro *et al*. (1993), showing that GST sequences from a US and UK parasite are very similar. Interestingly, N-terminal sequencing of native GST, together with the translated amino acid sequence predicted by the four cDNAs, found that the GSTs share the greatest homology with only a single class of mammalian GST, the mu-class (Panaccio *et al*., 1992; Wijffels *et al*., 1992). A biochemical analysis of the four recombinant GST (rGST) clones revealed that they exhibited overlapping but unique substrate specificities with differing sensitivities to inhibitors (Salvatore *et al*., 1995). Presumably, expression of multiple mu-class GSTs in *F. hepatica* allows the generation of a range of substrate specificities necessary for survival in the host. Brophy *et al*. (1990b) provided biochemical evidence in *F. hepatica* for an alpha-class like GST which would have been excluded from the above study due to a low affinity for the GSH–agarose matrix.

Immunocytochemical studies using rabbit antibodies against the native GST mixture localized GST widely throughout the tissues of the adult parasite: within the intestine, the parenchymal cells, the tegument and the adjacent muscle cells (Howell *et al*., 1988; Wijffels *et al*., 1992). Interestingly, a significant proportion of the GST in the intestine was found to be associated with the lamellae surface, apparently attached to the glycocalyx (Wijffels *et al*., 1992). Another study using a more defined set of reagents revealed a further level of specificity. Antisera were generated to synthetic

peptides of regions unique to each of the four rGST proteins as predicted by the cDNA sequences. Of these, the antiserum to rGST1 was specific, localizing rGST1 to the parenchyma of the adult fluke but not to the lamellae of the intestinal caeca. In contrast, the antiserum to rGST51 (this antiserum cross-reacted with rGST47 and rGST7) localized GST to both the parenchyma as well as the lamellae of the intestinal caeca (Creaney *et al*., 1995b). This finding represents the first evidence of tissue-specific expression of a GST isoenzyme in trematodes.

Interestingly, a similar analysis with these antisera on NEJ fluke localized GST in the parenchymal cytoplasm, cytoplasmic extensions of the parenchymal cells in the subtegumental area, the excretory ducts but not in the intestinal epithelium (Creaney *et al*., 1995b). This altered distribution within the intestine of the juvenile as compared to the adult may relate to the different morphology and functions of the gut that exist between the different life stages. The juvenile fluke gut contains few lamellae and appears to have a secretory function which contrasts with the dual secretory/absorptive role in the adult gut (Bennett and Threadgold, 1973). Therefore, a GST, that is not rGST1, is associated with the gut lamellae of the adult fluke which may have a role in the absorptive function of the adult fluke (Creaney *et al*., 1995b).

GSTs have proved to represent an interesting class of vaccine candidate. GSTs in helminth parasites first came to prominence following the initial identification of a GST as the dominant antigen recognized by sera from a strain of mice genetically resistant to infection with *S. japonicum* (reviewed in Brophy and Pritchard, 1994). Although Sj26 (a 26 kDa GST from *S. japonicum*) proved a poor vaccine candidate in mice, stimulating on average 30% reduction in worm burden against an homologous challenge infection (Mitchell *et al*., 1988), the corresponding 28 kDa GST from *S. mansoni* (Sm28) has consistently stimulated high levels of protection in a range of animal models (summarized in Capron *et al*., 1994; Brophy and Pritchard, 1994). In these parasites, the GSTs have been ascribed roles in the solubilization of molecules such as haem and, as previously mentioned, in detoxifying products of lipid peroxidation.

An early vaccine study had described the failure of GST in FCA to protect rats from infection with *F. hepatica* (Howell *et al*., 1988). In contrast, sheep which received multiple vaccinations with native *F. hepatica* GST in FCA showed a 57% reduction in worm burden, marking the first demonstration of protection in sheep against *F. hepatica* using a defined antigen (Sexton *et al*., 1990) (Tables 11.2 and 11.3). Since 1990, we have performed a further six trials, comprising a total of 11 vaccine groups, to assess the reproducibility of GST as a vaccine in sheep using FCA and other adjuvants. As shown in Table 11.3, protection levels reached as high as 65%, with mean efficacy of 29%, but it was not possible to consistently induce a protective response despite using comparable vaccination protocols (J. Sexton, Attwood, 1995, personal communication; Sexton *et al*., in preparation). Vaccination with GST in an alternative range of adjuvants (such as QuilA/Squalene Montanide [QA/SM], SM, DEAE, MF59, PLG microspheres) was unsuccessful in stimulating a significant reduction in worm burdens in sheep (Table 11.3).

Table 11.3. Vaccination results in sheep with GST of *F. hepatica*.

Trial no.	Group no.	Adjuvant	No. of vaccinations	% Reduction in worm burden	% Reduction in FEC	FEC/worm	Reference
1		Freund's	14	57[a]	−35	+55[e]	Sexton *et al.* (1990)
2		Freund's	2	6	−54	−57[f]	Sexton *et al.* (pers. comm.)
3	a	Freund's	2	15	−30	−19	Sexton *et al.* (pers. comm.)
	b	Freund's	3	45[a]	−29	+4	
	c	Freund's	2	40[b]	−60	−35	
	d	Freund's	2	33[c]	−46	−22	
	e	Freund's	3	16	+21	+72	
	f	Freund's	6	19	−11	+29	
4		Freund's	2	11	−63	−67	Sexton *et al.* (pers. comm.)
5		Freund's	2	19	+170	+181[a]	Sexton *et al.* (pers. comm.)
6		Freund's	2	65[d]	−94	−68	Sexton *et al.* (pers. comm.)
7	a	DEAE/Sm	2	36	n.d.	n.d.	Sexton *et al.* (pers. comm.)
	b	QuilA/SM	2	26	n.d.	n.d.	
	c	DEAE/ISA50	2	41	n.d.	n.d.	
	d	MF59	2	0	n.d.	n.d.	
	e	PLG/SM	2	27	n.d.	n.d.	
8	a	NAGO	2	37	−52	−35	Sexton *et al.* (pers. comm.)
	b	NAGO/MF59	2	27	−22	−32	
	c	Algammulin	2	27	−20	−32	
	d	ISA206	2	25	−50	−41	

FEC, effect on FEC; FEC/worm, eggs/g faeces/worm; n.d., not determined.
[a] $P<0.001$; [b] $P<0.002$; [c] $P<0.004$; [d] $P<0.003$; [e] $P<0.075$; [f] $P<0.0125$.

Vaccine trials were also performed in cattle assessing the efficacy of GST in several adjuvants. Morrison *et al.* (1996) showed that GST formulated in two adjuvants, QA/SM and MF59, stimulated high levels of protection from a challenge infection whereas vaccination with GST in FCA was ineffective in one trial (Table 11.2). Over a total of four separate vaccination trials with eight groups of animals, vaccination of cattle with GST in QA/SM, challenged at various times after boosting, stimulated a mean reduction in worm burden of 43% (19%; 26%; 39%; 45%; 49%; 49%; 52%; 69%) (Table 11.2). Thus, the differences observed between sheep and cattle in response to the GST vaccine suggest that the protective immune mechanisms induced by vaccination of cattle with GST in QA/SM are either not stimulated in sheep or the response is less effective allowing the parasite to escape immune attrition. A detailed comparison of immune effector responses in cattle and sheep vaccinated with GST in QA/SM would be informative.

Interestingly, vaccination of a range of species with GST from *S. japonicum* and *S. mansoni* dramatically affected egg production of the homologous challenge parasites. Vaccination of cattle with GST from *S. bovis* led to a 35–84% reduction in mean faecal egg counts with little effect on adult worm burdens (Bushara *et al*., 1993). A similar anti-fecundity effect on the challenge parasite was observed in monkeys vaccinated with recombinant Sm28 (Boulanger *et al*., 1995), mice and pigs vaccinated with recombinant Sj26 (Liu *et al*., 1995a, b) and sheep vaccinated with a mixture of native Sj26 and Sj28 GST (Xu *et al*., 1995). Contrasting results were observed in the six trials we performed in sheep to assess the vaccination potential of GST of *F. hepatica* in FCA. Both a statistically significant positive (2/11 groups) and negative (1/11 groups) effect on fecundity was observed (Table 11.3) (Sexton *et al*., in preparation). No consistent pattern was identified between the different trials and thus it was difficult to infer an effect on fecundity due to vaccination with *F. hepatica* GST in FCA.

In an analysis of the sera collected during the first three sheep trials (using FCA) (Table 11.3) anti-GST antibodies of both the IgG1 and IgG2 isotypes were stimulated, with low but detectable levels of IgA (Sexton, 1994; Sexton *et al*., 1994; Sexton *et al*., in preparation). Using overlapping peptides predicted from the amino acid sequence encoded by the four GST cDNAs, the antibody responses in the same sera to linear peptide epitopes were defined and spatially represented on an homology 3-D model of rGST51 (Sexton *et al*., 1994). No correlation was identified between an antibody response to a linear peptide and the level of protection induced in sheep by vaccination with GST. However, antibodies reactive against conformational epitopes would not be identified in this experiment.

Recently, Rossjohn *et al.* (1997) have solved the crystal structure of rGST47 allowing a more accurate assessment of the relevance of epitopes defined on linear peptides. The results show that the two dominant epitopes shared by the different recombinant GSTs (epitopes A and B), i.e. those which elicited the highest antibody titres in sheep, were found to reside on one face of rGST47 – the face that participates in dimer formation and therefore not exposed to solvent. Thus, although these antibodies are induced

they would not be expected to bind to the native GST dimer and interfere with GST activity. Consistent with this conclusion is the observation that these sera were shown not to inhibit the activity of native GST using CDNB as substrate (Sexton *et al.*, 1994). These observations raise the interesting prospect that antibodies to epitopes A and B may interfere with the process of GST dimerization if the antibodies were taken up by cells of *F. hepatica in vivo* since native GST exists as a dimer (Brophy *et al.*, 1990b; Wijffels *et al.*, 1992). Interference with GST dimerization would also interfere with the formation of the ligandin site which lies in a hydrophobic pocket at the dimer interface of the Sj26 GST and binds praziquantel (McTigue *et al.*, 1995). Since GSTs of *F. hepatica* may also contain a ligandin site, any interference with dimer formation would impact on the ability of GST to bind hydrophobic ligands (Rossjohn *et al.*, 1997).

The mode of action of the immune response against GST which leads to parasite elimination remains to be determined. There appear to be at least two possibilities: (i) an antibody response directed to the active or ligandin site of GST neutralizes or reduces GST activity in the parasite by steric hindrance at substrate binding sites: this results in tissue damage in the fluke resulting from the exogenous action of reactive oxygen/nitric oxides released by the host inflammatory response on to the parasite; (ii) GST is acting as an abundant antigen released by the fluke which induces an inflammatory immune response which kills the parasite. To date, evidence from our studies appears to favour the second hypothesis. In cattle, there is little evidence for a dominant role for antibody in immunity in the GST vaccine model. High levels of protection were stimulated in cattle using QA/SM, MF59 and PLG microspheres as adjuvants, the latter stimulating negligible antibody responses (Morrison *et al.*, 1996). We have also performed two trials using neuraminidase/galactose oxidase (NAGO) as adjuvant (Zheng *et al.*, 1992) and observed high levels of protection in cattle in one trial but a second trial failed to confirm this result: animals in the first trial did not exhibit anti-GST antibodies in ELISA assays (Table 11.2) (C.A. Morrison *et al.*, St Aubin, 1995, personal communication). Although many of the sera from cattle trials neutralized GST enzyme activity, using the universal substrate CDNB, no correlation was identified between protection levels and the level of inhibition of each serum against enzyme activity *in vitro* (Morrison *et al.*, 1996). However, a great number of compounds that interact with GST bind at a non-substrate binding site (the ligandin site) which lies in a hydrophobic pocket at the dimer interface (Wilce and Parker, 1994; Rossjohn *et al.*, 1997). A full assessment of the role of antibodies in immunity in GST-vaccinated animals should also address the role of substrates which bind at the ligandin site.

The second hypothesis suggests that GST is released by living flukes *in vivo*. Adult flukes contain about 200 µg GST/worm (Wijffels *et al.*, 1992) and low amounts of GST have been detected in the culture supernatant of flukes incubated *in vitro*, suggesting that GST may be secreted by living parasites (J. Sexton, Attwood, 1994, personal communication). Rabbits immunized with adult fluke ES extract develop low levels of antibody to GST implying that

GST is released to some extent by adult parasites *in vitro* (Hillyer *et al*., 1992). Another possibility to be considered is that GST may be presented to the immune system from parasites which fail to establish in a natural infection. In naive sheep and cattle, only a proportion (11–53%) of the *F. hepatica* metacercariae establish as adult flukes with recoveries of 16–53% in sheep and 11–21% in cattle (Boray, 1969; Boyce *et al*., 1987; Morrison *et al*., 1996; Spithill *et al*., 1997). This poses the question of the fate of the other 47–89% of metacercariae which do not establish. Hsu (1986) showed that, in mice, only 8% of *F. hepatica* metacercariae failed to migrate out of the gut and into the tissues. This observation suggests that the majority of the parasites which do not develop to adult fluke die in the tissues of the host as bystander parasites. If the same is true in ruminants, it is possible that there is an immune response to GST released by the bystander parasites and that this response to GST recalls the effector response which kills the invading larvae. This hypothesis implies that the inflammatory effector arm may be mediating parasite elimination via a bystander-induced response which is non-specific at the effector phase. In support of this, we have recently found that killing of NEJ of *F. hepatica* by rat macrophages involves antibody-dependent nitric-oxide-mediated immune responses (Piedrafita, 1995; Spithill *et al*., 1997).

Cathepsin L

Fasciola contains an abundance of proteolytic enzymes, in particular in the excretory–secretory (ES) material, which can be easily collected from the adult parasite *in vitro*. This was first demonstrated by Thorsell and Bjorkman (1965), testing the effects of secreted material on the digestion of a gelatine substrate. Early experiments demonstrated that the proteolytic activity in ES material was of the cysteine protease family (see for example Chapman and Mitchell, 1982). The cysteine proteases comprise a large family with a number of classes and cathepsin L and B in particular have been studied in relation to parasite invasion, feeding, immune evasion and vaccine potential (reviewed in Knox, 1994). In *Fasciola*, the cathepsin L proteases have been proposed to play a number of functional roles including promoting tissue penetration (Howell, 1966; Smith *et al*., 1993a; McGinty *et al*., 1993; Beresain *et al*., 1997), nutrient acquisition (Halton, 1967; Dalton and Heffernan, 1989; Rege *et al*., 1989; Yamasaki *et al*., 1992; Smith *et al*., 1993a; Dowd *et al*., 1994) and egg production (Wijffels *et al*., 1994b; Dalton *et al*., 1996; Spithill and Morrison, 1997). In addition, cathepsin L proteases have been implicated in immune evasion by cleavage of the Fc regions of antibodies (Chapman and Mitchell, 1982; Dalton and Heffernan, 1989; Smith *et al*., 1993a,b, 1994), by prevention of the antibody-mediated attachment of immune effector cells to newly excysted juvenile flukes (Goose, 1978; Carmona *et al*., 1993; Smith *et al*., 1993a,b, 1994) and by the generation of fibrin clots which could prevent access to the fluke surface by immune effector cells (Dowd *et al*., 1995).

The work by Dalton and Heffernan (1989), Yamasaki *et al*. (1989), Smith *et al*. (1993b), McGinty *et al*. (1993) and Wijffels *et al*. (1994a,b) demonstrated that the secreted cysteine proteases released by adult *F. hepatica* are predominantly of apparent molecular mass 27–28 kDa. Screening of adult *F.*

hepatica cDNA libraries with antisera raised to these proteins (or in one case screening with PCR fragments) confirmed that the proteases were of the cathepsin L class and predicted a secreted protein of generally 326 amino acids, comprising a 17 amino acid signal sequence, 90 amino acid activation sequence and 219 amino acid mature protease (Yamasaki and Aoki, 1993; Heussler and Dobbelaere, 1994; Wijffels *et al*., 1994a; Roche *et al*., 1997; Dowd *et al*., 1997; M. Panaccio and T.W. Spithill, Attwood, 1995, personal communication). It is apparent that *F. hepatica* contains multiple genes encoding cathepsin L, as evidenced by the isolation of multiple PCR fragments encoding cathepsin L (Heussler and Dobbelaere, 1994) and at least three complete (but differing) cDNA sequences (Yamasaki, Spithill and Dalton laboratories). In contrast, NEJ of *F. hepatica* secrete a cysteine protease with sequence homology to cathepsin B which may play a role in tissue invasion, glycocalyx turnover or excystment of metacercariae (Wilson *et al*., 1998).

Cathepsin L proteases derived from the ES material of *Fasciola* have been tested as vaccines against the parasite. The utility of cathepsin L as a vaccine was first demonstrated in sheep using FCA (Wijffels *et al*., 1994b; see also Table 11.2). It was found, somewhat surprisingly at the time, that there was no reduction in the worm burden of vaccinated animals, but that the faecal egg counts were significantly reduced (69.7%) such that there were 2.7 eggs g^{-1} $worm^{-1}$ in vaccinates compared to 9.2 eggs g^{-1} $worm^{-1}$ in the control, unvaccinated animals. The mechanism by which this reduction in fecundity occurs was not apparent, as there was no correlation between FEC and the antibody titre against cathepsin L generated in the animals.

More recently, the efficacy of cathepsin L as vaccine against fasciolosis in cattle has been examined (Dalton *et al*., 1996) (Table 11.2). Two previously isolated secreted homologues, cathepsin L1 (Smith *et al*., 1993b) and cathepsin L2 (Dowd *et al*., 1994), were tested alone and in concert with *Fasciola* haemoglobin (see below). Both cathepsin preparations elicited protection against infection; the worm burdens were reduced up to 69% (mean 53.7%) with cathepsin L1. Cathepsin L2 was not tested alone but induced a 72% reduction in worm burdens when immunized with haemoglobin (see below). Cathepsin L1 was also demonstrated to reduce fluke fecundity, in that only 40–65% of eggs produced were viable. Eggs collected from the cathepsin L1/haemoglobin group showed 0–80% viability and those from the cathepsin L2/haemoglobin group showed 0–7% viability. These results confirm that cathepsin Ls are effective targets of protective immunity in cattle but the mechanism of immunity remains to be determined.

The anti-fecundity results observed in cattle and sheep trials with cathepsin L can be interpreted in two ways: (i) the reduction in egg production is a secondary consequence on parasite egg metabolism due to a primary effect on parasite feeding by inhibition of activity of the secreted cathepsin L; and (ii) egg production in *F. hepatica* requires the activity of a second tissue-located cathepsin L and that vaccination against the secreted cathepsins induces cross-reacting antibodies which can interfere with egg production by neutralization of this tissue cathepsin L. This second hypothesis is supported

by the observation that the adult flukes recovered from vaccinated sheep were morphologically normal, suggesting that their development (and presumably their ability to feed) was not retarded by vaccination (Wijffels *et al.*, 1994b). In contrast, Dalton *et al.* (1996) did observe a reduction in size of flukes recovered from vaccinated cattle, suggesting that the effects of the immune response in cattle and sheep may be different.

It is noteworthy that both the eggshell proteins, and the catechol oxidase which catalyses the eggshell tanning process, are synthesized as precursor proteins in *F. hepatica* that undergo proteolytic processing during synthesis (Smyth and Halton, 1983; Rice-Ficht *et al.*, 1992). The cDNA encoding the major eggshell protein of *F. hepatica* (vitelline B) has been sequenced together with the N-terminal sequence of the mature vitelline B protein (Rice-Ficht *et al.*, 1992; Waite and Rice-Ficht, 1992). These sequences predict a cleavage site for the processing of the vitelline B precursor protein at the residues A19/R20 but the nature of the protease which performs this function is not known. Curiously, we have studied the primary substrate specificity of the major secreted cathepsin L proteases from adult flukes using the esterase assay of Whittaker *et al.* (1994) in which the synthetic substrate Z–Ala–X–O Me is cleaved (where X = any amino acid). We have shown that the secreted cathepsin Ls have a dominant P1 specificity for R>K>M (R. Good *et al.*, Attwood, 1995, personal communication). This result indirectly suggests that a cathepsin L may play a role in the processing of the vitelline B precursor protein and is consistent with a vaccine-induced effect on eggshell processing via inhibition of cathepsin L activity. In addition, a cathepsin L has been immunolocalized in the Mehlis' gland of adult flukes (Wijffels *et al.*, 1994b; M. Panaccio *et al.*, Attwood, 1994, personal communication). The Mehlis' gland is a cluster of cells known to secrete substances that catalyse the formation of the eggshell (Smyth and Halton, 1983). We speculate that the Mehlis' gland cathepsin L is the target of the immune response in vaccinated animals resulting in interference of eggshell synthesis. Cathepsin L proteinases have been identified in the reproductive organs of schistosomes and treatment of *S. mansoni*-infected mice with cathepsin L inhibitors leads to reductions in worm numbers and egg production (Wasilewski *et al.*, 1996).

From the trials performed using native cathepsin L preparations it is apparent that the protein, when administered with FCA, can induce an anti-fecundity effect in either sheep or cattle. Additionally, there is an anti-worm effect using this immunogen in cattle. An antigen which can produce a significant reduction in egg production and/or viability is an attractive vaccine component, reducing the number of eggs shed to pasture and hence reducing available metacercariae for subsequent ingestion by ruminants.

Haemoglobin

Dalton *et al.* (1996) have reported the use of cathepsin L1 and L2 in concert with fluke haemoglobin in vaccine trials in cattle (Table 11.2). Haemoglobin had been previously isolated from *F. hepatica* ES material (McGonigle and Dalton, 1995) and shown to be a large haem-containing protein, with absorption spectra indicative of a haemoglobin, although N-terminal sequence of

the protein did not reveal any homology with haemoglobin sequences in the sequence databases. The protein was recognized in an ELISA assay using sera taken as early as 1 week after experimental infection of cattle with 500 metacercariae.

In vaccination trials, haemoglobin in FCA elicited a 43.8% reduction in fluke burden, which in concert with cathepsin L1 increased to 52%; this level of protection was greater than that observed with cathepsin L1 alone (42.5%) (Dalton *et al*., 1996; see also Table 11.2). However, combining haemoglobin and cathepsin L2 increased protection to 72%, a value which, if reproducible, may represent a commercially viable vaccine. More importantly, the reductions in fluke burdens were paralleled by a marked reduction in egg viability. Vaccination with all antigen preparations resulted in a decrease in egg viability; however, in the case of the haemoglobin/cathepsin L2 combination, this was most dramatic. Three animals had no eggs in the gall bladders, one yielded eggs with a viability of 7% and three further animals yielded eggs which only underwent partial embryonation. This represents a greater than 98% anti-fecundity effect. As mentioned above, an anti-fecundity component to a vaccine is very attractive and, combined with the anti-worm activity, may over time result in near sterile pastures after vaccination, as seen with anthelmintic treatment in sheep (Taylor *et al*., 1994).

The mechanism of immunity induced by *Fasciola* haemoglobin as a vaccine is unknown. However, as postulated by Dalton *et al.* (1996), the oxygen storage properties of haemoglobin may be vital in regions of low oxygen tension, such as the bile duct. As it is known that egg production by *Fasciola* is a process requiring oxidative metabolism (Bjorkman and Thorsell, 1963), immunological interference with haemoglobin function may compromise egg development. The striking anti-fecundity effect seen on co-vaccination with haemoglobin and cathepsin L2 is not surprising, as individually haemoglobin and cathepsin L (both in sheep and cattle) can elicit this effect, presumably in different ways.

Paramyosin

Several studies have reported the use of the subtegumental protein paramyosin of *Schistosoma* as a vaccine in mice (Pearce *et al*., 1988; Flanigan *et al*., 1989; Ramirez *et al*., 1996). In view of the ability of antigens from *Fasciola* and *Schistosoma* to cross-protect, we tested the potential of paramyosin purified from *F. hepatica* in two vaccination trials in ruminants (Table 11.2). Paramyosin of size 94 kDa was extracted from whole adult fluke homogenates, essentially using the method of Harris and Epstein (1977) originally applied to *C. elegans*.

In the sheep vaccination trial, two groups of six animals were vaccinated subcutaneously 4 weeks apart with a dose each time of 100 µg paramyosin emulsified either in FCA or SM containing 5% w/v DEAE–dextran, and challenged 2 weeks after the booster immunization. Sera were analysed by ELISA for their specific antibody content to paramyosin. Titres rose rapidly but were significantly higher in the FCA group (>1:100,000) than in the DEAE–dextran group (1:30,000). At autopsy, there was a marked reduction in

the mean fluke burden compared to controls (45%) for the FCA group although this was not statistically significant due to the considerable variation in fluke 'take'. By contrast, the DEAE–dextran group exhibited no reduction in mean fluke burden (Table 11.2). An analysis of the FEC data showed a mean reduction of 95% for the CFA group and 57% for the DEAE–dextran group.

In the cattle trial, a similar protocol was followed using only QA/SM as adjuvant. Titres of anti-paramyosin IgG which developed were much lower than in the sheep trial (1:20,000). At autopsy, mean fluke burdens in the liver were reduced by 47% and this was significant ($P<0.05$) (Table 11.2). There was additionally a significant reduction in FEC values at week 13 (55%). However, a subsequent cattle trial failed to reproduce this protective effect (C.A. Morrison *et al*., St Aubin, 1995, personal communication). Paramyosin of *F. gigantica* has also been tested in cattle but no protection was observed (Estuningsih *et al*., 1997). The potential of paramyosin as a vaccine candidate for *Fasciola* remains to be confirmed.

Kunitz-type serine proteinase inhibitor (KTM)

A Kunitz-type serine proteinase inhibitor (termed Fh-KTM) of 6751 Da belonging to the bovine pancreatic trypsin inhibitor (Kunitz) family has been isolated from *F. hepatica* (Bozas *et al*., 1995). Fh-KTM is a weak inhibitor of trypsin but the specific target enzyme of Fh-KTM within adult *F. hepatica* has yet to be identified. Immunofluorescent and immunogold localization studies on adult *F. hepatica* revealed that Fh-KTM is an abundant molecule localized to the luminal surface of the gut, the entire outer tegument which includes the tegument tissue surrounding the oral sucker and specific subcellular organelles of parenchymal cells.

The abundance of Fh-KTM and the gut and tegumental localization pattern suggested that this molecule could be used as a vaccine target since gut-associated molecules have successfully been used as vaccines against other blood-sucking parasites such as *Boophilus microplus* and *Haemonchus contortus* (Willadsen *et al*., 1995; Munn, 1997). Vaccine trials using HPLC purified Fh-KTM were carried out in both sheep and cattle (Table 11.2). Sheep were given two doses (100 µg) of the molecule in CFA followed by IFA and then challenged with 300 metacercariae; the same dose emulsified in Quil A rather that CFA/IFA was administered to cattle. Although high antibody titres were observed in sheep following vaccination, no reduction in worm burden was observed in any of the vaccinated infected animals. A similar result was observed in cattle showing that the preparation of Fh-KTM used in both of the trials was not protective with the protocol employed.

Cocktail vaccines

The results with several defined antigens discussed above raise the realistic prospect of controlling infection with *F. hepatica* by the use of cocktail vaccines. Successful parasitism by *Fasciola* will involve the expression by the fluke of multiple gene products which together must function to allow the fluke to migrate and develop in its host. For example, the juvenile and immature

fluke must penetrate the gut and liver capsule, migrate through the liver, digest tissue, evade immune responses and finally enter the bile duct to feed on host blood and tissue. Tissue invasion requires the function of proteins such as proteases, detoxifying enzymes such as GST and superoxide dismutase for protection from damaging inflammatory responses, FABP for uptake of fatty acids and haemoglobin for oxygen supply to the tissues. The successful use of the cathepsin L/haemoglobin combination is timely and validates the concept of cocktail vaccines for liver fluke (Dalton *et al.*, 1996).

The results showing that vaccination with cathepsin L induces an anti-fecundity effect in sheep and cattle (Wijffels *et al.*, 1994b; Dalton *et al.*, 1996) raise the possibility of a vaccine to reduce transmission of fluke infection on to pasture. A GST/cathepsin L/haemoglobin vaccine would be expected to reduce worm burdens by at least 70% and simultaneously reduce egg release by the remaining 30% of parasites by up to 90%, resulting in a >97% reduction in egg release on to the pasture. High reductions in pasture contamination with parasite eggs would be expected to dramatically reduce transmission of infection to ruminants (Taylor *et al.*, 1994).

Vaccine formulations

Optimal vaccine efficacy requires the identification of a formulation for delivering the vaccine antigen such that the umbrella of protection extends to the majority of animals in the herd. The choice of adjuvant, route of delivery and dosage critically determine the outcome of vaccination in different parasite systems (James, 1985; Spithill, 1992). For example, protection against *F. hepatica* with GST in both sheep and cattle varies dramatically with the adjuvant used (Tables 11.2 and 11.3). Sheep can be protected with GST delivered in FCA but not with GST delivered in several other adjuvants including QA/SM. In contrast, GST in QA/SM is the optimal formulation for cattle whereas GST in FCA was found to be ineffective (Morrison *et al.*, 1996). MF59 is an effective adjuvant with GST in cattle but not in sheep (Tables 11.2 and 11.3; Morrison *et al.*, 1996). Similarly, cathepsin L in FCA reduces worm burdens in cattle but not in sheep (Wijffels *et al.*, 1994b; Dalton *et al.*, 1996). Such variation in vaccine efficacy between species with the same formulations suggests that the immune responses induced by the vaccine in cattle and sheep are qualitatively or quantitatively different. Moreover, we have recently found that vaccination of Brahman cross cattle with GST of *F. gigantica* in QA/SM did not protect cattle against *F. gigantica* challenge (Estuningsih *et al.*, 1997). These results show that each host/parasite combination is unique and that each host/parasite relationship may require its own unique vaccine formulation, a somewhat sobering thought which will impact on the rate of development of vaccines.

The nature of the immune effector pathway induced in response to parasite infection is profoundly influenced by the cytokine response of the host (Wakelin, 1992; Abbas *et al.*, 1996). The use of cytokines as adjuvants might overcome the problems of non-specific toxicity associated with administration of crude adjuvants such as FCA and may allow a more controlled manipulation of the immune response. In sheep, rIL-1β and rIL-1α

have been used to enhance antibody production to antigens (Nash *et al*., 1993; Andrews *et al*., 1994). In cattle, rIL-1β and rIL-2 have been shown to boost antibody and cell-mediated immune responses to viral antigens (Reddy *et al*., 1989, 1992). The use of cytokines with *F. hepatica* vaccines such as GST, FABP and cathepsin L/haemoglobin should be evaluated.

Nucleic acid vaccines

A new approach to vaccine delivery is the use of naked DNA encoding protective antigens to vaccinate animals. In this strategy, plasmid DNA containing an insert encoding the antigen of interest is delivered by intramuscular or intradermal injection. The plasmid construct contains a promoter, such as the cytomegalovirus promoter, to drive expression of the encoded antigen in mammalian cells. The application of nucleic acid vaccines against parasites has recently been reported. Nucleic acid constructs have been used to protect mice against malaria (Sedegah *et al*., 1994; Doolan *et al*., 1996; Hedstrom *et al*., 1996) and *Leishmania* (Xu and Liew, 1995). Antibody responses have been induced in mice vaccinated with DNA encoding paramyosin of *S. japonicum* (Yang *et al*., 1995). We have recently observed antibody responses in mice vaccinated with a DNA vaccine encoding rGST47 of *F. hepatica* showing that nucleic acid vaccination with *Fasciola* sequences may be feasible (Smooker *et al*., Clayton, 1998, personal communication). Sheep have been vaccinated with DNA encoding the 45W *T. ovis* antigen but high antibody titres were only observed in animals given a protein vaccine boost immunization (Rothel *et al*., 1997a). Sheep have been protected against *T. ovis* infection with a protocol using a nucleic acid 45W construct followed by a boost with an adenovirus 45W construct (Rothel *et al*., 1997b). Although work in this area is still preliminary, these results suggest that nucleic acid vaccines may be a viable alternative to conventional vaccination of ruminants with protein antigens. In particular, the ease of formulation of nucleic acid vaccines and the potential of vaccinating with multiple plasmids (for example antigen and cytokine) or with multiple antigens on the same plasmid make these vaccines an attractive proposition.

Conclusions and Future Prospects

It is now clear that the control of fasciolosis by immunological intervention appears to be an achievable goal. The high levels of efficacy observed in vaccine trials in cattle using several antigens of *F. hepatica* have now validated the subunit vaccine approach. The mean level of reduction in worm burdens observed in cattle with different antigens is in the range 43–72% (Table 11.2) raising the question of whether such levels of efficacy are commercially useful. Economic loss due to fasciolosis in ruminants results from reductions in weight gain, milk production and/or wool production which are determined by the intensity of infection. In cattle, significant weight loss is only observed with fluke burdens above 30–80 (Hope Cawdery, 1984; Dargie, 1986) suggesting that a vaccine with mean efficacy as low as 43% will still reverse losses in animals infected with as few as 53–140 flukes. Fluke burdens reported in

different countries are in the range 4–14 (Roy and Tandon, 1992), 24 (McCausland *et al*., 1980), 40–140 (Malone *et al.*, 1982), 68 (Sahba *et al*., 1972) and 99 (Schillhorn van Veen *et al.*, 1980); in Glasgow (UK) only 3% of cattle livers had more than 50 flukes (Dargie, 1986). These reports indicate that the current experimental vaccine formulations are potentially viable products.

The successful development of a vaccine for *F. hepatica* will require several issues to be addressed. FCA is not a commercially acceptable adjuvant for use in the FABP and cathepsin L/haemoglobin formulations and other adjuvants are needed which will mimic the efficacy obtained with FCA. An understanding of the immune responses induced by FCA correlating with immunity in cattle will allow a rational choice of adjuvant. The production of recombinant molecules which mimic the high efficacy of native antigens is also needed. The fact that recombinant GST and cathepsin L have been produced in active form indicates that production of native-like antigens is feasible (Salvatore *et al.*, 1995; Dowd *et al.*, 1997; Roche *et al.*, 1997). However, the inability of a recombinant *F. gigantica* FABP construct to mimic the protection observed in cattle with the native FABP mixture is a note of warning (Estuningsih *et al*., 1997); a recombinant Fh15 vaccine was less effective in protecting rabbits than the native FABP vaccine (Muro *et al*., 1997). Encouragingly, commercial recombinant vaccines for ruminants have been developed for the cattle tick and *T. ovis* showing that the recombinant subunit vaccine strategy is feasible (Lightowlers, 1994; Willadsen *et al*., 1995).

The variability in responsiveness exhibited in outbred animals is also an issue of concern from the point of view of a commercial vaccine product. This may be overcome with the use of vaccine cocktails, comprising several antigens delivered as a single vaccine, which may have synergistic or additive protective effects. Nucleic acid vaccines may be an alternative to conventional vaccine approaches which may resolve some of these issues. Encouragingly, Doolan *et al.* (1996) have shown that vaccination of different inbred strains of mice with a cocktail of nucleic acid constructs encoding malaria antigens was able to overcome the genetically restricted non-responsiveness of some strains to individual malaria antigens. Such an approach with a nucleic acid cocktail of different liver fluke constructs (e.g. FABP, GST and cathepsin L) may result in effective coverage of most animals leading to a higher mean efficacy of herd protection with concomitant increase in productivity. DNA constructs of FABP, GST and cathepsin L are currently being prepared for testing in ruminants. The simplicity of formulation of multicomponent nucleic acid vaccines with or without a cytokine adjuvant may yet lead us to commercially viable vaccines for fasciolosis.

Acknowledgements

This work was supported by the Australian Centre for International Agricultural Research (Canberra), Monash University, Agriculture Victoria and Novartis Produkte (Animal Health Sector). Jenette Creaney's PhD studies were supported by a scholarship from the Victorian Institute of Animal Science, Attwood, Australia.

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12 Human Fasciolosis

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Classification of Reports of Human Fasciolosis

Human infection by *Fasciola hepatica* has always been considered a disease of secondary importance (Malek, 1980; Boray, 1982). The WHO review by Chen and Mott (1990) was the first paper to highlight the importance of human fasciolosis as a public health issue. This was due to the high number of human cases recorded in the 1970–1990 period (2594 infected persons from 42 different countries covering all continents) (Chen and Mott, 1990). Our conception of human fasciolosis has drastically changed because of recent surveys of human endemics in some regions, with either low or high prevalences and intensities. Reports estimate that as many as 2.4 million (Rim *et al*., 1994) or even 17 million people (Hopkins, 1992) are infected with liver fluke worldwide.

Epidemiological and transmission characteristics of fasciolosis give rise to a typical patchy distribution of the disease, foci being related to the local distribution of intermediate snail host populations as well as to the physiographic and climatic conditions. These facts make it appropriate to refer to given physiographic and climatic homogeneous areas, rather than to individual countries.

Present knowledge has allowed us to establish a new classification of epidemiological areas presenting human fasciolosis (Mas-Coma *et al*., 1998b):

- *Imported cases*: human subjects diagnosed in a zone lacking *F. hepatica* even at animal level, who were infected in another area presenting *F. hepatica* transmission.
- *Autochthonous, isolated, non-constant cases*: human subjects having acquired the infection in an area they inhabit and which presents animal fasciolosis; these human cases only appear sporadically.
- *Endemics*: three types of human endemic situations can be distinguished using coprological diagnostic data from several regions (serological tests reveal somewhat higher prevalences):

Fasciolosis (ed. J.P. Dalton)

  - *Hypoendemic*: prevalence less than 1%; arithmetic mean intensity less than 50 eggs per gram of faeces (epg); high epg only in sporadic cases; human participation in transmission through egg shedding may be negligible; hygiene–sanitation characteristics usually include latrines and waste or sewage disposal facilities; outdoor defecation is not commonly practised.
  - *Mesoendemic*: prevalence between 1% and 10%; 5–15-year-old children may present higher prevalences (holoendemic); arithmetic mean intensity in human communities usually between 50 and 300 epg; high epg numbers can be found in individuals, although intensities over 1000 epg are rare; human subjects may participate in transmission through egg shedding; hygiene–sanitation characteristics may or may not include latrines and waste or sewage disposal facilities; outdoor defecation may be practised.
  - *Hyperendemic*: prevalence more than 10%; 5–15-year-old children may present higher prevalences (holoendemic); arithmetic mean intensity in human communities usually more than 300 epg; very high individual epg numbers are encountered, intensities over 1000 epg being relatively frequent; human subjects significantly participate in transmission through egg shedding; hygiene–sanitation characteristics do not include the use of latrines; no proper waste or sewage disposal facilities; indiscriminate defecation is commonly practised.
- *Epidemics*: there are different types of outbreaks according to the endemic/non-endemic situation of the zone:
  - *Epidemics in animal endemic areas*: outbreaks appearing in zones where previous human reports have always been isolated and sporadic; such outbreaks usually concern a very few subjects infected from the same contamination source (family or small group reports; contaminated wild, home-grown or commercially grown watercress or other metacercariae-carrying vegetables).
  - *Epidemics in human endemic areas*: outbreaks appearing in zones presenting human endemics; a larger number of subjects may be involved; usually related to previous climatic conditions having favoured both the parasite and the snail life cycles; epidemics can take place in hypoendemic, mesoendemic and hyperendemic areas.

Geographical Distribution

Human fasciolosis has been reported from countries in Europe, America, Asia, Africa and Oceania. Major reviews on human infection by *F. hepatica* have been carried out by Chen and Mott (1990) and Esteban *et al.* (1998c). The numbers of clinical cases reported and of infected persons identified during epidemiological surveys have been increasing since 1980.

Esteban *et al.* (1998c) compiled a total of 6848 human cases reported from 51 countries in all continents in the last 25 years. However, the number

of human cases is undoubtedly much greater than that reported. A global analysis shows that the expected correlation between animal and human fasciolosis only appears at a basic level. Although it is true that human infection is not rare in areas where infected domestic herbivorous mammals are present, high/low human prevalences are not related to high/low animal prevalences, respectively. High prevalences in humans are not necessarily found in areas where fasciolosis is a great veterinary problem. For instance, in South America hyperendemics and mesoendemics are found in Bolivia and Peru where the veterinary problem is less important than in countries such as Uruguay, Argentina and Chile, in which human fasciolosis is only sporadic or hypoendemic.

In Europe, a total of 2931 human cases diagnosed involved 19 countries. Cases are predominantly found in France, Portugal, Spain and the former USSR (including its Asian region). France is considered an important human endemic area, with 3297 cases being catalogued between 1950 and 1983. However, 5863 human cases were recorded from nine hospitals between 1970 and 1982 indicating that published data largely underestimate the real situation. The disease is also important in Portugal, mainly the northern part, in which 1011 cases were diagnosed in 1970–1992. In Spain, human fasciolosis is mainly distributed in northern Spain. Concerning the former Soviet Union, almost all reported cases were from the Tadzhik Republic, near the Afghanistan border. Several findings suggest that high prevalences can exist in the Samarkand region: 1–14 flukes were found in each liver at post-mortem in 81 inhabitants in 1968–1986.

In North America, human fasciolosis appears only sporadically in the United States and Canada. In Mexico, 53 cases have been reported. In Central America, fasciolosis is a human health problem in the Caribbean Islands, especially in zones of Puerto Rico and Cuba. In Cuba, an outbreak involved more than 1000 subjects in 1983. In the mainland, only Costa Rica and Guatemala appear in the records. A total of 16 human cases have been diagnosed in Guatemala. In the Dominican Republic and El Salvador, fewer than 100 cases were documented.

In South America, human fasciolosis is a serious problem in Bolivia and Peru, and probably also in Ecuador. In Bolivia, the human hyperendemic region involves only the northern Altiplano zone, with prevalences in given communities up to 72% and 100% in coprological and serological surveys, respectively. Here, a human population of around 2.5 million people is at risk. In Peru, human cases have been detected throughout the country, with mesoendemics and hyperendemics in given zones. The high human prevalences in Arequipa, Mantaro Valley, Cajamarca Valley and the Puno region are worth mentioning; estimations suggest a rural population of almost 8 million people is at risk. Despite the lack of epidemiology information, the characteristics of Ecuador suggest a situation similar to that in Peru. In Chile, human fasciolosis is hypoendemic in the Valparaiso and Viña del Mar zones, and in Regions V, VI and VII. In countries like Argentina, Uruguay, Brazil, Colombia and Venezuela, human fasciolosis appears to be focal in distribution and sporadic, with fewer than 100 cases reported.

In Asia an additional problem appears because of the overlapping distribution of *F. hepatica* and *F. gigantica*, including intermediate forms traditionally referred to as *Fasciola* sp. That is why in several papers the liver fluke species was not determined. Although Hashimoto *et al.* (1997) have recently demonstrated by means of molecular techniques that intermediate forms may be ascribed to *F. gigantica* in Japan, this is not certain for other Asian countries. In Asia, only a few cases have been described in several countries, among which Iran is worth mentioning because of the recent estimates of more than 10,000 human cases and about 6 million at risk.

Only a few human cases have been reported from African countries. In some parts of Africa overlapping *F. hepatica* and *F. gigantica* infections may occur. *F. hepatica* appears to be more restricted in northern countries like Morocco, Algeria and Tunisia, as well as in southern Zimbabwe and South Africa, and is also the species present in the highlands in Kenya and Ethiopia. *F. gigantica* is present in most of the African continent, from the Nile Delta to the Cape Provinces of South Africa. Most human cases have been reported from Egypt, where the *Fasciola* species involved remain undetermined in most reports, although sometimes *F. hepatica* is specified. An increasing number of human cases with both *Fasciola* species have been diagnosed in the Nile Delta. In rural areas prevalences vary between 2% and 17%. The population at risk is 27.7 million and the number infected is at least 830,000. In the Ivory Coast, Madagascar, Mali and Mozambique fewer than 100 cases are documented, and in Ethiopia 100–1000 cases.

Concerning Oceania, there are only 12 human reports from Australia and none from New Zealand, despite the importance of livestock production here and the high prevalences of fasciolosis in sheep and cattle.

Epidemiology and Transmission

Human *F. hepatica* infection is determined by the presence of the intermediate snail hosts, domestic herbivorous animals, climatic conditions and the dietary habits of man (Chen and Mott, 1990). However, recent studies have demonstrated that, in endemic areas, the epidemiological and transmission factors are not as simple as previously believed and that many additional factors should be considered.

Humans as the definitive host

While it is well known that *F. hepatica* adults are able to develop, mature and produce eggs, the human host was never perceived as participating in the transmission of disease. Results obtained in our laboratory indicate that human subjects can act as a viable definitive host in the life cycle of the parasite and actively participate in its transmission, at least in hyperendemic areas. Moreover, all developmental characteristics and tranmission potential of the human parasite isolates are similar to isolates from sheep and cattle.

A period of at least 3 to 4 months is necessary for the flukes to attain sexual maturity in humans (Facey and Marsden, 1960), although, as in

animals, the greater the number of flukes invading the liver, the longer the time required for the juvenile flukes to mature in the bile ducts and to initiate egg laying (Boray, 1969). It is generally believed that man is not a suitable host, most migrating flukes becoming trapped in the liver parenchyma and dying without reaching the bile ducts (Acosta-Ferreira *et al.*, 1979); nevertheless, the results of our laboratory suggest that at least in hyperendemic areas the parasite is better adapted to the human host. The duration of infection in man is unknown but it has been estimated that the parasite may survive for 5 to 12 years (Karnaukhov, 1978) and from 9 years to 13.5 years (Dan *et al.*, 1981).

Prevalences and intensities in humans

Most studies on the prevalence of human fasciolosis are not true epidemiological surveys, but only estimations from the number of cases detected in a non-surveyed zone. Examples of prevalence estimates for hypoendemic areas are: 0.34–3.1 cases/100,000 inhabitants in Basse-Normandie, France (Bourée and Thiebault, 1993; Cadel *et al.*, 1996); 0.83–1.16 cases/100,000 inhabitants in Corsica (Gil-Benito *et al.*, 1991; Gil-Benito, 1994); and 0.7% prevalence (41 cases/5861 subjects studied) in the VII Region of Chile (Apt *et al.*, 1993).

Examples of prevalences from epidemiological surveys in mesoendemic areas are: 7.3% in Nile Delta, Egypt (Farag *et al.*, 1979); 8.7% found in Cajamarca, Peru (Knobloch *et al.*, 1985); and 3.2% in the inner Porto region, Portugal (Sampaio Silva quoted in Chen and Mott, 1990).

A low hyperendemic prevalence of 10.9% was found in Corozal, Puerto Rico (Bendezú *et al.*, 1982). Higher hyperendemic prevalences were found in epidemiological surveys in localities of Peru and Bolivia. In Peru, Stork *et al.* (1973) found prevalences of up to 34.2% in the Mantaro valley and Sánchez *et al.* (1993) recorded a mean prevalence of 15.6% in the Puno region. The highest human prevalences have been reported in the Bolivian Altiplano: up to 66.7% by means of coprological techniques (Hillyer *et al.*, 1992; Esteban *et al.*, 1997a,b) and up to 53.0% by means of immunological methods (Hillyer *et al.*, 1992; Bjorland *et al.*, 1995); higher rates of 72% and 100%, respectively, have been reported by local health workers (see Mas-Coma *et al.*, 1995) and 67% by O'Neill *et al.* (1998). It is worth mentioning that the highest human prevalences have always been detected in zones of very high altitude (Hillyer *et al.*, 1992; Mas-Coma, 1996; O'Neill *et al.*, 1998).

In high prevalence areas children less than 15 years old usually present the highest rates (Farag *et al.*, 1979; Knobloch *et al.*, 1985; Apt *et al.*, 1993; Sánchez *et al.*, 1993; Esteban *et al.*, 1997a,b). In contrast, in human non-endemic areas all age groups can be affected, and children under 6 years of age present the lowest prevalences (García-Rodriguez *et al.*, 1985; Sampaio Silva quoted in Chen and Mott, 1990). Among the positive cases detected by Apt *et al.* (1993), 51.2% were children aged 8–15 years, 34.1% were 16–45-year-old persons and 14.6% were over 45. In localities of the Altiplano, Esteban *et al.* (1997a) detected prevalences of 17.5, 35.3 and 25.2% in children aged 5–8, 9–12 and 13–19, respectively, and Esteban *et al.* (1997b)

found prevalences of 71.4, 87.5, 42.9 and 40% in subjects aged less than 10, 11–20, 21–40 and 41–75, respectively.

Concerning intensity in humans, a relationship between the number of adult fluke and eggs in faeces has never been studied. Data from animals suggest that a direct relation does not necessarily exist. In humans, the results available on egg output, measured by eggs per gram of faeces (epg), yield very variable values: 1–4 epg (Bendezú *et al.*, 1982; Knobloch *et al.*, 1985) is the most common, and a case with 440 epg (Akahane *et al.*, 1975) may be considered as rare. However, the study by Sampaio Silva (quoted in Chen and Mott, 1990) provided a geometric mean (range) of 233 (25–2100) epg.

In children of the Bolivian Altiplano, eggs in stools ranged between 24 and 5064 epg, with arithmetic and geometric means of 474–1001 and 201–309 epg, respectively, which proved to be the highest known worldwide (Esteban *et al.*, 1997a,b). Despite the existence of a decrease of the prevalence and intensity from children (75%, 24–4440 epg) to adult subjects (41.7%, 144–864 epg), results showed that in a hyperendemic zone adult subjects either maintain the parasites acquired when young or can be reinfected as the consequence of inhabiting a zone with high risk of infection (Esteban *et al.*, 1997b). Interestingly, in keeping with the highest prevalence, the highest egg outputs known in humans also appear in this high altitude zone (Mas-Coma, 1996).

Animal reservoirs

Sheep, goats and cattle are considered the predominant animal reservoirs. While other animals can be infected, they are usually not very important for human disease transmission. However, Mas-Coma *et al.* (1998c) have observed that donkeys and pigs contribute to disease transmission in Bolivia. Among wild animals, Mas-Coma *et al.* (1988) have demonstrated that the peridomestic rat *Rattus rattus* may play an important role in the spread as well as in the transmission of the parasite in Corsica. Lagomorphs can also develop a role in the epidemiology of the disease, although their ecology (not in contact with humans) suggests they have no impact in the transmission of disease to humans, except in particular circumstances (Apt *et al.*, 1993).

Intermediate snail hosts

Recent studies have shown that under special circumstances given lymneids are able to adapt to extreme conditions, thus contributing to the spread of the disease. In Corsica, *L. truncatula* has preferentially adapted to reservoir habitats (permanent presence and renewal of water) instead of invasion habitats (only seasonal presence of water) as is usual in the European continent. Several atypical habitats even suggest an ecological niche widening (Oviedo *et al.*, 1992) that led to human infections in unexpected places (beaches, trails, etc.) (Gil-Benito, 1994). In the Bolivian Altiplano, *L. truncatula* is perfectly adapted to the extreme climatic conditions at 4000 m altitude, where it is even found in dirty, eutrophic water collections in some villages (Oviedo *et al.*, 1995a,b; Bargues *et al.*, 1995; Mas-Coma, unpublished

data). This large adaptation capacity of given lymnaeids makes it possible for transmission to take place in or near human habitats. The adaptation of lymnaeids to permanent water collections makes year-long transmission possible in southern Europe (Oviedo *et al.*, 1992) and South America (Oviedo *et al.*, 1995b), which explains why human infection can be acquired in all months (Gil-Benito, 1994; Mas-Coma, 1996). Computer-based Geographic Information System (GIS) methods have been proposed for the analysis of the overlapping distribution of snail and human populations (Malone and Zukowski, 1992; Hillyer and Apt, 1997; see also Chapter 5 of this volume).

Climatic conditions

Although studies have demonstrated that the parasite development is arrested below 10°C or over 30°C (Boray, 1969), recent research carried out with *F. hepatica* isolates from the Bolivian Altiplano has shown that this parasite is able to adapt to more extreme conditions. This capacity appears to be related to the human hyperendemic zones at high altitude (Mas-Coma, 1996).

Lymneids are more resistant to lower rather than to higher temperatures. They can survive through the winter, although there is little or no development and multiplication (Boray, 1969). In contrast, persistent high temperatures and dry conditions adversely influence both snail populations and parasite larval stages. The metacercariae may survive for long periods at low temperatures if the level of moisture is sufficient, but they are susceptible to desiccation and to temperatures over 25°C (Boray, 1969). On the other hand, high humidity associated with heavy rainfall and moderate temperatures may herald hyperendemicity in herbivorous animals. Thus, human infection has been more frequently observed in years with heavy rainfall (Ripert *et al.*, 1987; Pérez *et al.*, 1988; Cadel *et al.*, 1996).

Temperature and rainfall determine seasonal incidence in many countries, which may be reflected in cases of human infection. Although human infections may occur throughout the year (Rondelaud *et al.*, 1982; Farag *et al.*, 1993; Gil-Benito, 1994), monthly distribution of the fasciolosis human cases exhibits a seasonal distribution which may vary according to the areas. In Europe human infection takes place in summer and autumn and symptoms appear in winter (Bourée and Thiebault, 1993; Lejoly-Boisseau *et al.*, 1996). A prolonged, wet summer in Europe has often been followed by an outbreak of the disease (Chen and Mott, 1990). In northern Africa, the number of acute human infections appears to peak in August (Farag *et al.*, 1993). Sometimes the seasonality is related to the ingestion of infected plants, so that most human cases occurred during the watercress season, October–April (Ripert *et al.*, 1987), with a maximum from November to February (Rondelaud *et al.*, 1982).

Sources of human infection

Freshwater plant species incriminated in human infection differ according to the geographical zones as well as the human dietary habits in the different

areas. Moreover, plant species involved are not necessarily the same in subjects 'infected at the table' (through vegetables being part of the normal diet) compared to subjects 'infected in the field' (through ingestion or chewing of vegetables directly taken from nature and which are not necessarily part of the usual human diet). Certain metacercariae-carrying plants may be so important in the human diet of a given area as to be cultivated (at family or even industrial level) and commercially sold in public markets – this can explain why subjects living far away from the endemic area can become infected.

Most human reports are related to watercress ingestion. The general term watercress includes different aquatic species such as *Nasturtium officinale* (common watercress), *N. silvestris* and *Roripa amphibia* (wild watercress). Wild watercress is reported as the main source of human infection in Europe, where fasciolosis in domestic animals is highly endemic (Ripert *et al*., 1987; Rondelaud, 1991; Bourée and Thiebault, 1993), and in other regions (Wood *et al*., 1975; Bendezú *et al*., 1988). A study in France showed that home-grown watercress was the cause in 23 cases, wild watercress in eight cases, and commercially grown watercress in two cases (Anonymous, 1988). Watercress grown at home or at industrial level is related to outbreaks involving a few individuals (Gil-Benito *et al*., 1991). Encysted metacercariae were found in about 1% of lettuces from a local market in Peru (Bendezú, 1969) and on 10.5% of green vegetables sold in a Samarkand market (Sadykov, 1988).

Other aquatic vegetables reported as vehicles of human infection are *Taraxacum dens leonis* (dandelion leaves), *Valerianella olitora* (lamb's lettuce), and *Mentha viridis* (spearmint) in France (Rondelaud *et al*., 1982; Anonymous, 1988), other green leafy *Nasturtium* spp., and *Mentha* spp. in Iran (WHO, 1995), and several freshwater plants in Bolivia (Mas-Coma *et al*., 1995).

Water is often cited as the source of human infection**.** The production of a low rate (less than 10%) of floating metacercariae has sometimes been reported (Vareille-Morel *et al*., 1993). In some countries, like China, where vegetables are always cooked for eating, infection may rarely occur by ingestion of unboiled drinking water, or from the metacercariae on cutting boards and other kitchen utensils (Chen and Mott, 1990). Infection by ingestion of salads contaminated with metacercariae-carrying water used for irrigation has been also reported (Cadel *et al*., 1996). In Bolivia, Bargues *et al*. (1996) found that 13% of the metacercariae are floating, independent of the parasite isolate, which is in keeping with information on human infection in certain areas of the Americas where the people do not have a history of eating watercress (Hillyer and Apt, 1997). Bjorland *et al*. (1995) found that in the Bolivian Altiplano several edible aquatic plants such as 'berro-berro' (watercress), 'algas' (algae), 'kjosco' and 'tortora' had Lymnaeidae snails on them and suggested that these could be a source of infection. Recent experimental data suggested that humans consuming raw liver dishes prepared from fresh livers infected with immature flukes could become infected (Taira *et al*., 1997).

Risk factors

Although not yet understood, human prevalences and intensities appear to be higher in females than in males, at least in given areas. Prevalence by sex appears very similar in Spain (García-Rodriguez *et al*., 1985) and Bolivia (Bjorland *et al*., 1995; Esteban *et al*., 1997a), whereas in Chile and Egypt a higher prevalence was observed in women (73.2% and 10.3%, respectively) than in men (26.8% and 4.4%, respectively) (Farag *et al*., 1979; Apt *et al*., 1992, 1993). With regard to egg output, significant differences between boys and girls have been found in Bolivia: 24–3408 epg vs. 24–5064 epg; arithmetic mean: 329 epg vs. 664 epg; geometric mean: 150 epg vs. 295 epg (Esteban *et al*., 1997a).

The incidence of infection is significantly concentrated within family groups because the family shares the same contaminated food (Peña Sánchez *et al*., 1982; García-Rodriguez *et al*., 1985; Borie *et al*., 1990; Chandenier *et al*., 1990; Bechtel *et al*., 1992). In Egypt, Farag *et al*. (1979) found that among 25 families with at least one infected person, 20% had two members infected and another 20% had three members infected. The 49 patients identified as infected by Rondelaud *et al*. (1982) came from a total of 24 families.

The distribution of the disease is essentially rural. The professions connected with cattle- and sheep-breeding are more frequently affected than other professions (Stork *et al*., 1973; Rondelaud *et al*., 1982; Bourée and Thiebault, 1993). In Iran the risk arises from the use of animal manure as fertilizer or of wastewater effluent for irrigating the aquatic or semiaquatic vegetable crops (WHO, 1995).

Pathology

It is generally believed that man is not a suitable host, most migrating flukes becoming trapped in the liver parenchyma and dying without reaching the bile ducts. However, little information is available on the pathology of fatal human fasciolosis since death rarely occurs (Chen and Mott, 1990; Mas-Coma *et al*., 1998a). Pathogenesis depends on the number of infecting flukes, and is similar to that reported in animals (see Chapter 6 of this volume). The penetration of the duodenum or jejunum wall by metacercariae may cause focal haemorrhage and inflammation, although lesions may not be clinically evident. The fluke migration through the liver parenchyma induces the major pathological changes. Parasites digest hepatic tissue and cause extensive parenchymal destruction with intensive haemorrhagic lesions and immunological and inflammatory reactions. Migration tracks are observed in histological sections. Migratory flukes sometimes die leaving cavities filled with necrotic debris and considerable liver areas may subsequently be replaced by scar tissue (Smithers, 1982). The flukes may reach the bile ducts where they can live for several years. Less pathogenic effects may be caused in the bile ducts, although inflammation resulting in fibrosis, thickness and expansion is common (Chen and Mott, 1990). Anaemia is one of the most characteristic symptoms (Boray, 1969; Dawes and Hughes, 1970). Blood loss in the bile seems to be the most important, if not the only, factor contributing to severe anaemia.

Pathology in liver and biliary tract

The liver is usually enlarged with a smooth or uneven surface. Common macroscopic lesions are multiple soft, yellowish or greyish white nodules ranging from 2 to 30 mm in diameter, which correspond to eosinophilic abscesses. Nodules are also observed in the parietal peritoneum close to the liver and on the round liver ligament. Haemorrhagic stippling appears at the margin of the nodules. White or yellow striae, surrounded by telangiectasia, are observed on the liver capsule. Close to the nodules, ribbed or vermiform formations with colour and consistency similar to the nodules are also observed under laparoscopy. Hepatic capsular thickening of varying degrees appears, and in a few cases the entire hepatic capsule is thickened. Subcapsular lymphatic vessels are dilated. The lymph nodes near the porta hepatis may be markedly enlarged. Multiple subcapsular cavities filled with necrotic material were observed below the liver capsule in one patient who died. Several reddish purple tracks radiated from the nodules, whereas others were greyish white and fibrous. The tracks extended from the liver capsules and ended in subcapsular cavities (Chen and Mott, 1990). Most lesions are less than 20 mm below the capsules (Acosta-Ferreira *et al.*, 1979). In cases with marked involvement of the peritoneal wall and the liver surfaces, yellow and opalescent ascites was present. Apart from mild splenomegaly in two patients, no significant portal hypertension was found (Chen and Mott, 1990).

The common bile ducts are usually large and dilated and the wall is thickened on palpation. The gall bladder wall is greatly thickened and oedematous (Chen and Mott, 1990; Riedtmann *et al.*, 1995). Multiple, greyish white subserous nodules are present and adhesions of the gall bladder to adjacent structures are common (Acosta-Ferreira *et al.*, 1979). The mucosal folds of the gall bladder appear prominent. The wall of the gall bladder is thickened owing to muscular hypertrophy and perimuscular fibrosis. There is glandular epithelial hyperplasia. All layers of the wall contain patchy infiltrates with lymphocytes, plasma cells and eosinophils. Lithiasis is very frequent (Chen and Mott, 1990; Arjona *et al.*, 1995).

Migration tracks are found in the liver and other organs. The walls of the tracks in the liver often contain Charcot–Leyden crystals and eosinophils. The cavities of the tracks are filled with necrotic cellular debris, including hepatocytes, fibrin and red cells. A considerable eosinophilic infiltrate surrounds the tracks. In older lesions macrophages, lymphocytes, eosinophils and fibrous tissue are observed. Focal calcification is sometimes seen in the margin of the necrotic debris. Calcifications may form the outline of a dead fluke (Acosta-Ferreira *et al.*, 1979).

Egg granulomas have been reported (Peña Sánchez *et al.*, 1982; Park *et al.*, 1984; Chen and Mott, 1990; Sapunar *et al.*, 1992). Multinuclear giant cells surround a single egg with subsequent layers of epithelioid cells, fibrous tissue, plasma cells, lymphocytes and eosinophils. The portal triads appear dilated and oedematous with infiltrates of lymphocytes and eosinophils. Bile duct proliferation, periductal fibrosis, necrotizing arterial vasculitis and portal venous thrombosis are frequent (Chen and Mott, 1990).

Ectopic fasciolosis

Immature flukes may deviate during migration, entering other organs and causing ectopic fasciolosis. In man, the most frequent ectopic lesions are those of the gastrointestinal tract (Acosta-Ferreira *et al*., 1979; Park *et al*., 1984). Other ectopic locations reported are: subcutaneous tissue (Ozkan and Genç, 1979; Vajrasthira and Sunthornsiri, 1979; Aguirre *et al*., 1981; Park *et al*., 1984; García-Rodriguez *et al*., 1985; Chang *et al*., 1991; Prociv *et al*., 1992); heart, blood vessels, the lung and pleural cavity (Moretti *et al*., 1971; Couraud *et al*., 1975; Park *et al*., 1984; García-Rodriguez *et al*., 1985; Arias *et al*., 1986; El-Shazly *et al*., 1991); brain (Aguirre *et al*., 1981; Arias *et al*., 1986); orbit (García-Rodriguez *et al*., 1985; Arias *et al*., 1986; Cho *et al*., 1994); abdominal wall (Sato *et al*., 1975; Totev and Georgiev, 1979); appendix (Park *et al*., 1984); pancreas (Chitchang *et al*., 1982); spleen (Chen and Mott, 1990); inguinal nodes (Arjona *et al*., 1995); cervical node (Prociv *et al*., 1992); skeletal muscle (Park *et al*., 1984); epididymis (Aguirre *et al*., 1981). Such ectopic flukes never achieve maturity. The usual pathological effects of ectopic lesions are due to the migratory tracks causing tissue damage with inflammation and fibrosis. Parasites may be calcified or become incorporated in a granuloma (Facey and Marsden, 1960).

Clinical Presentations

The following clinical periods can be distinguished: incubation phase (from the ingestion of metacercariae to the appearance of the first symptoms); invasive or acute phase (fluke migration up to the bile ducts); latent phase (maturation of the parasites and start of oviposition); and obstructive or chronic phase.

Incubation phase

This phase varies considerably depending on number of metacercariae ingested and host's response. In man, the onset of this phase has not been accurately determined but has been reported as taking 'a few' days, 6 weeks, 2–3 months, or even longer (Chen and Mott, 1990; Bourée and Thiebault, 1993).

Invasive or acute phase

The symptomatology is due mainly to the mechanical destruction of the liver tissue and the abdominal peritoneum by the migrating larvae causing localized or generalized toxic and allergic reactions (Facey and Marsden, 1960) lasting 2–4 months. In endemic areas, *F. hepatica* infection is usually repetitive and the acute lesions are superimposed on chronic disease. Thus, the acute phase may be prolonged and overlap on to a latent or an obstructive phase.

The major symptoms of this phase are:

- *Fever:* this is usually the first symptom, generally low or moderate but may reach 40°C, and in heavily infected cases as high as 42°C; it may be remittent, intermittent or irregular with a higher temperature in the evening; in some cases, a low, recurrent fever lasting for as long as 4 to 18 months occurs (Chen and Mott, 1990).
- *Abdominal pain:* from mild to excruciating, sometimes vague, it may be generalized at the outset but is usually localized in the right hypochondrium or below the xyphoid.
- *Gastrointestinal disturbances:* loss of appetite, abdominal flatulence, nausea and diarrhoea are common, whereas vomiting and constipation are infrequent.
- *Urticaria:* a distinctive feature in the early stage of the fluke invasion and may be accompanied with bouts of bronchial asthma (Chen and Mott, 1990; Arjona *et al*., 1995).
- *Respiratory symptoms:* cough, dyspnoea, haemoptysis and chest pain occur occasionally, but in some cases are the first manifestation of infection (Chen and Mott, 1990; Arjona *et al*., 1995).

The following signs may appear on physical examination:

- *Hepatomegaly and splenomegaly:* the liver is usually enlarged and tender, sometimes expanding to the right iliac fossa (Facey and Marsden, 1960), but is never hard to the touch (Peña Sánchez *et al*., 1982); the degree of hepatomegaly seems to increase during the course of the disease and hepatic abscesses are detected (Abou Basha *et al*., 1989; Chen and Mott, 1990; Tchirikhtchian *et al*., 1997). Splenomegaly is not common but has been reported by different authors (Chen and Mott, 1990).
- *Ascites:* this has been reported several times; it is yellow with a high leucocyte count, eosinophils predominating; the pathogenesis is considered to be an inflammatory response to a large number of juvenile flukes penetrating the intestinal walls, irritation of the peritoneum and penetration through the liver capsule during their migration rather than hepatic failure *per se* (Chen and Mott, 1990).
- *Anaemia:* mild to moderate anaemia can be seen; pallor of the skin and mucosa is commonly associated with lassitude, dizziness, palpitation and weakness (Chen and Mott, 1990).
- *Chest signs:* on auscultation, dry or moist rales can occasionally be elicited upon coughing at the base of the right lung probably due to migration of the juvenile flukes; pleural rub with effusion and even spontaneous pneumothorax have been reported (Chen and Mott, 1990); parenchymal infiltrates resembling the Loeffler syndrome (Aliaga *et al*., 1984) and pleural effusion (Arias *et al*., 1986) are the most common radiological manifestations; pyopneumothorax has been also reported (Chen and Mott, 1990).
- *Jaundice:* this is infrequent and when it appears it is milder than that seen in the chronic phase (Chen and Mott, 1990; Kumar *et al*., 1995).

Latent phase

This phase can last for months or years. The proportion of asymptomatic subjects in this phase is unknown. They are often discovered during family screening after a patient is diagnosed (Hardman *et al.*, 1970; Apt *et al.*, 1995; Arjona *et al.*, 1995). A prominent eosinophilia may already be suggestive of infection (Gil-Benito *et al.*, 1991; Gil-Benito, 1994). These persons may have gastrointestinal complaints or one or more relapses of the acute symptoms (Chen and Mott, 1990).

Chronic or obstructive phase

This phase may develop months or years after initial infection. Adult flukes in the bile ducts cause inflammation and hyperplasia of the epithelium. Thickening and dilatation of the ducts and the gall bladder walls ensue. The resulting cholangitis and cholecystitis, combined with the large body of the flukes, are sufficient to cause mechanical obstruction of the biliary duct.

In this phase, biliary colic, epigastric pain, fatty food intolerance, nausea, jaundice, pruritus, right upper-quadrant abdominal tenderness, etc., are clinical manifestations indistinguishable from cholangitis, cholecystitis and cholelithiasis of other origins. Hepatic enlargement may be associated with an enlarged spleen or ascites (Acosta-Ferreira *et al.*, 1979; Duan *et al.*, 1986).

Bile ducts are usually distended and thickened; the diameters are 1.5–3.0 times normal size. The most frequent site of obstruction is the common bile duct (Khorsandi, 1977). A diverticulum in this duct have been observed and the head of the pancreas was enlarged and firm (Maroy *et al.*, 1987).

In case of obstruction, the gall bladder is usually enlarged and oedematous with thickening of the wall. The gall bladder may measure $12 \times 7 \times 7$ cm and the lower edge reach the umbilicus (Chen and Mott, 1990). Fibrous adhesions of the gall bladder to adjacent organs are common. Lithiasis of bile duct or gall bladder is frequent and the stones are usually small and multiple (Wong *et al.*, 1985; Chen and Mott, 1990; Arjona *et al.*, 1995). The bile duct and the gall bladder may contain blood mixed with bile (haemobilia), blood clots and fibrinous plugs (Chen and Mott, 1990).

Clinical Laboratory Signs

Laboratory findings involve haematological aspects, hepatic functions and serum immunoglobulin levels.

Haematological characteristics

In the acute phase, the leucocyte counts are usually over 10,000 mm^{-3} and up to 43,000 mm^{-3}. The eosinophil count is nearly always greater than 5% of the total leucocytes and may be as high as 83%. Anaemia is common, but usually not very severe, mostly between 7.0 and 13.5 g dl^{-1} haemoglobin. Levels as low as 2.8 and 4.0 g dl^{-1} have been reported. The erythrocyte

sedimentation rate may be high in the acute phase, reaching 165 mm in an hour, normal in the latent phase, and normal or only moderately high in the obstructive phase (Peña Sánchez *et al.*, 1982; Chen and Mott, 1990; Salem *et al.*, 1993; El-Zawawy *et al.*, 1995).

Hepatic functions

Abnormal results in liver function tests may be found:

- *Acute phase:* sometimes results include elevated serum glutamic pyruvic transaminase (GPT), glutamic oxalacetic transaminase (GOT), thymol turbidity, zinc sulphate turbidity, serum globulin, and serum bilirubin (Hardman *et al.*, 1970; Peña Sánchez *et al.*, 1982; Duan *et al.*, 1986; Riedtmann *et al.*, 1995). In other cases, tests give normal results, with the exception of alkaline phosphatase (AKP). Serum electrophoresis showed an increase of α_2- and γ-globulins (Chen and Mott, 1990). Osman *et al.* (1995) observed that serum triglycerides and very low density lipoproteins increased, while total serum cholesterol, high density lipoprotein cholesterol and low density lipoprotein cholesterol exhibited a significant decrease. These changes were due to the degenerative necrotic damage of the hepatocytes. Abnormally high levels of β-glucuronidase were also encountered (Ebied *et al.*, 1993).
- *Obstructive phase:* jaundice is a prominent feature. Serum bilirubin levels between 2.0 and 8.6 have been reported (Chen and Mott, 1990). Biliary colic is usually followed by a higher level of serum bilirubin as well as dark urine positive for bilirubin. Serum bilirubin may be normal in this phase and between attacks of biliary colic. AKP, GPT, GOT and serum globulin (mainly γ-globulin) are often elevated, while albumin is decreased (Chen and Mott, 1990).

Immunoglobulin levels

IgG, IgM and IgE levels are usually elevated (Chen and Mott, 1990; Pailler *et al.*, 1990; Youssef and Mansour, 1993). Specific IgE antibodies were detected in 48% of the patients. Total and specific IgE levels were shown to be positively correlated with the egg burden, age, clinical features and degree of eosinophilia (Sampaio Silva *et al.*, 1985). IgA levels are usually normal (Sampaio Silva *et al.*, 1985). IgG1 and IgG4 are the predominant isotypes elicited by infection (O'Neill *et al.*, 1998).

Complications and Causes of Death

One complication may be bleeding. Subcapsular haematoma and severe haemobilia have been reported. Haematemesis and melaena were associated with obstructive jaundice, epigastric pain and severe anaemia. In one case, bleeding occurred due to an ulcer in the common bile duct, and in other cases no single bleeding point was detected in the common bile ducts and/or in the gall bladders (Chen and Mott, 1990).

Biliary cirrhosis is another complication. During the course of the infection, inflammation, hyperplasia and hypertrophy of the bile duct epithelia may induce periductal fibrosis. Prolonged heavy infection rarely leads to biliary cirrhosis. The outstanding findings on physical examinations were firm untender hepatomegaly with or without jaundice and ascites. Splenomegaly was not prominent. Sclerosing cholangitis with biliary cirrhosis was reported (Chen and Mott, 1990).

Another serious complication is multiple extrahepatic venous thrombosis. In one patient who died suddenly, multiple thrombosis of the ovaric, suprahepatic, mesenteric and myocardial veins, along with massive pulmonary embolism were disclosed. During the invasion period, another patient developed a complete thrombosis of the superior vena cava (Peña Sánchez *et al*., 1982).

Only a few deaths related to fasciolosis have been reported (Peña Sánchez *et al*., 1982; Chen and Mott, 1990). Post-mortem examination of a patient who died from an acute liver condition showed *F. hepatica* to be responsible for blockage of the bile duct (Totev and Georgiev, 1979). More than 40 liver flukes were detected in a patient at autopsy (Chen and Mott, 1990). Between 1 and 14 flukes were found in each liver at post-mortem in 81 inhabitants of the Samarkand region, although deaths were not presumed to be due to fasciolosis (Sadykov, 1988).

Associations with Other Human Parasites

In animals, the clinical synergistic capacity of *F. hepatica* in concomitant infection with other pathogenic agents is well known. In man, several isolated case reports of fasciolosis associated with other parasitoses have been described. The association of *F. hepatica* with other parasites was studied by Esteban *et al*. (1997a, 1998a,b) in patients from the Bolivian Altiplano. Among the faecal samples positive to *F. hepatica*, the number of other parasite species found varied from one to eight (up to five different pathogenic protozoans and six helminths were found). However, significant positive association was only found with *Giardia intestinalis*.

Laboratory Diagnosis

The principle laboratory techniques for the diagnosis of human fasciolosis are coprological techniques and immunological tests. Other, non-invasive diagnostic techniques have been attempted but these are not generally employed except in clinical practices in hospitals; these include radiology, radioisotope scanning, ultrasound, computed tomography and magnetic resonance (see Esteban *et al*., 1998c).

Coprological techniques

Finding parasite eggs in faeces is still the main method of diagnosis. However, several important points must be considered:

- *Immature flukes:* man is generally believed to be a non-suitable host; the possibility of hepatic infections by flukes unable to attain maturity cannot be disregarded; eggs would never be found in subjects where flukes do not develop to maturity.
- *Acute phase:* in man, the incubation phase is shorter than the prepatent period and clinical findings may appear long before eggs can be found in stools; thus, coprological techniques are useful only after 3–4 months post-infection.
- *Egg output dynamics:* in man, egg output number and dynamics are unfortunately unknown. Release of eggs may be low and/or intermittent; several stool samples taken at different times of the day should be examined.
- *Eggs in transit:* people ingesting infected animal liver a short time before sample taking may show 'false' fasciolosis when eggs appear in stools; in such cases, diagnosis requires placing the patient on a liver-free diet and performing repeated follow-up stool examinations.
- *Ectopic infections:* a mature ectopic fluke has never been found, so eggs are presumably never produced.

Techniques ranging from a simple direct smear to different concentration methods have been used to diagnose chronic fasciolosis. Egg concentration has been achieved by flotation and sedimentation techniques (Esteban *et al.*, 1998c). The sedimentation technique appears to be more accurate and sensitive than flotation techniques, as most of the hyperosmotic solutions distort the eggs (Boray, 1969).

The rapid, low cost, and reproducible cellophane faecal thick-smear technique (Kato, Kato-Katz) according to Katz *et al.* (1972) has also been used (Esteban *et al.*, 1998c), although opinions differ on its sensitivity. Cup sedimentation using tap water (the simplest and cheapest) was more sensitive than formol-ether concentration, which was in turn more sensitive than the Kato-Katz thick smear according to Hillyer and Apt (1997). However, Esteban *et al.* (1997a,b) found that the Kato-Katz technique was most appropriate in large epidemiological surveys, taking into account time needed, low cost and sensitivity. All concentration techniques may be used for egg count if started from a known stool volume (Esteban *et al.*, 1998c).

Immunological techniques

Immunological techniques present the advantages of being applicable during all phases of the disease, but especially during the acute phase and in the other situations in which coprological techniques may present problems. The most recently applied tests are discussed in Chapter 13 of this volume.

Clinical Factors Important for Diagnosis

The clinical presentations in which fasciolosis should be considered in conjunction with the above laboratory tests are: history of ingestion of raw wild

or cultivated watercress or other vegetables, eosinophilia, fever of unknown origin, atypical abdominal pain, focal intrahepatic lesions, granulomatous hepatitis, serositis and meningitis with peripheral or fluid eosinophilia, family history of fasciolosis, biliary colic or cholangitis, and normal ultrasonography (Chen and Mott, 1990; Arjona *et al.*, 1995). Eosinophilia has also been successfully used for a first selection in general surveys (Gil-Benito *et al.*, 1991; Gil-Benito, 1994).

In the acute phase there are fever, pain in the right hypochondrium and abnormal laboratory findings, fundamentally eosinophilia with leucocytosis. Computed tomography scan and/or a positive serological reaction are most suggestive (Chen and Mott, 1990). In the chronic phase the clinical picture is attenuated and easily confused with other diseases. The classic pattern includes: vague gastrointestinal complaints, pain in the right hypochondrium or epigastrium, cholecystitis, cholangitis and bile duct or gall bladder stones. The liver is usually enlarged with or without pain on palpation. Ascites may appear in advanced cases. In both phases, ectopic localization of the parasite may cause a confusing clinical presentation. Clearly, febrile diseases and other parasitic infections causing eosinophilia and/or similar symptoms should be ruled out.

Treatment

Many drugs for the effective treatment of human fasciolosis are available (Esteban *et al.*, 1998c). Emetine derivatives, the classic drugs, were used widely and continue to be used today, given intramuscularly or subcutaneously: emetine at doses of 1–10 mg kg^{-1} per day for 10 days; dehydroemetine, at a usual dose of 1 mg kg^{-1} daily for 10–14 days, was even considered the therapy of choice a few decades ago. They cause a variety of toxic manifestations involving the heart, liver and digestive tract. Frequent changes are seen in the electrocardiogram. Hypotension sometimes occurs during treatment. Dehydroemetine has a shorter tissue half-life and disappears more rapidly from the heart and liver as compared with emetine. No deaths have been reported due to emetine derivative treatment.

Bithionol was considered the drug of choice (see below), applied at 30–50 mg kg^{-1} daily, divided into three oral doses on alternate days for 20–30 days, although other dose regimes have been used. In cases of resistance to emetine and praziquantel, bithionol achieved cure in dosages of 50 mg kg^{-1} daily for 10 alternate days or 40 mg kg^{-1} daily for 14–15 alternate days. Occasionally, the patients required a second course to obtain a complete cure. The side effects, including diarrhoea, anorexia, nausea, vomiting, pruritus, urticaria and abdominal pain, are usually mild, and drug withdrawal is not necessary.

Triclabendazole has been reported as very effective against both acute and chronic fasciolosis. The recommended dose is two separate regimens of 10 mg kg^{-1} of body weight. Clinical tolerability is excellent, although a transient febrile episode with reversible liver function alteration has been observed. Among 24 asymptomatic individuals with chronic fasciolosis treated

with triclabendazole at a single oral dose (10 mg kg^{-1} of body weight) after an overnight fast, 19 (79.2%) were egg-negative 2 months after treatment. Three of five cases that harboured eggs in their faeces after the first treatment were treated again and the parasitologic cure was achieved. A cure rate of 79.2% when first used and 100% after a second administration, the ease of a single oral dose, its tolerability, and the absence of side effects, make it the drug of choice today. The drug is absorbed better if administered after meals; when two courses of 10 mg kg^{-1} each were administered after meals on the same day, cure rates of 100% were obtained (Apt *et al.*, 1995).

Hexachloro-*para*-xylol was effectively used at a dose of 100–150 mg kg^{-1} body weight in four doses at 15 min intervals in Romania, at 60 mg kg^{-1} daily for 5 days in the former Soviet Union, and at 50–80 mg kg^{-1} body weight daily divided into three doses given orally for 7 consecutive days in China. The side effects include gastrointestinal complaints and dizziness.

Two oral doses of niclofolan at 2 mg kg^{-1} body weight for 3 days apart or 0.5 mg kg^{-1} twice a day for 3 days have been applied. The side effects include sweating, palpitation, nausea, diffuse upper abdominal pain, itching and jaundice with dark urine. Such a toxicity shows that its clinical use cannot be recommended.

Daily oral doses of 1.5 g of metronidazole for 13 and 28 days proved to be effective, but a smaller total dose of 4 g was reported to have failed to cure a chronic infection.

Other drugs used have been: mebendazole, in a daily dose of 4 g for 3 weeks, reported to have cured a *F. hepatica* infection in the invasive phase; albendazole, with a high rate of failure; rafoxanide, used once on a child; and prednisone at 5–10 mg daily**,** as an adjunct therapy before the administration of fasciolicidal drugs in acutely ill children.

It is worth mentioning that *Fasciola* may be the only trematode genus that has practically no response to praziquantel.

Concluding Remarks

Human infection with liver fluke disease is usually associated with animal endemics. Control measures of human fasciolosis are carried out in conjunction with the control of animal fasciolosis, by adding measures to prevent metacercariae ingestion by humans (Roberts and Suhardono, 1996; see also Chapter 4 of this volume). In areas presenting sporadic cases of human disease, diagnosis should be followed by effective treatment. Immediate identification of the infection source(s) is needed to prevent further human infections. A strict control of watercress and other metacercariae-carrying aquatic plants for human consumption is needed. Commercial growing of watercress should be carried out under completely controlled conditions. The use of vinegar and potassium permanganate is recommended for washing salad vegetables, as washing in running water is not sufficient (El-Sayad *et al.*, 1997). However, humans may also be infected by drinking natural water carrying floating metacercariae (Bargues *et al.*, 1996). In endemic areas it is important that the community should be appropriately informed about the

disease, its mode of transmission and its dangers. Since parasites and the snail hosts may adapt to particular environmental conditions and therefore change the transmission characteristics of the disease, eco-epidemiological studies must be undertaken and control measures tailor-made to each particular zone.

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13 Immunodiagnosis of Human and Animal Fasciolosis

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Introduction

Fasciolosis historically has been a disease of ruminants worldwide, and has caused serious economic losses in the animal husbandry industry. On the other hand, human fasciolosis tends to be sporadically reported. In one review only some 100 human infections were collected in a 20-year span involving cases from Egypt, Cuba, Peru, France, Portugal, Spain and the former Soviet Union (Chen and Mott, 1990). However, these numbers were considerably under-represented. For example, in the Chen and Mott (1990) review paper, the country of Bolivia was not even listed as having human infections. Yet today it is known to have one of the highest endemicity rates in the world. For example, a recent seroprevalence study found over 40% prevalence in Aymara Indians from Corapata in the Altiplano of Bolivia (Hillyer *et al*., 1992b). A second study reported on an outbreak of acute fasciolosis in Calasaya and Santa Ana, also in the Altiplano of Bolivia. Consumption of kjosco, an aquatic plant, was associated with acute fasciolosis in humans. Cross-sectional studies of 18% of the population randomly surveyed found 21% positive by a single Kato-Katz thick smear examination, all of whom were antibody positive for *Fasciola hepatica* by the Falcon™ Assay Screening Test-ELISA (FAST-ELISA). It was estimated that 49% of this population had evidence of current or previous infection. A recent report by O'Neill *et al.* (1998), which employed ELISA, confirmed the high level of human fasciolosis in this region. In children it may be even more significant as Esteban *et al.* (1997a) found almost 28% of 558 school children from four communities in the northern Bolivian Altiplano positive for *F. hepatica* in stools. Since approximately 1.8 million subsistence farmers live in the Department of La Paz in the Altiplano, a conservative estimate of 20% of humans with fasciolosis would suggest that as many as 360,000 persons are infected in this region (Bjorland *et al*., 1995). In cattle and sheep in this same region, antibody levels to *F. hepatica* are 58% and 89%, respectively (Hillyer *et al*., 1995). Even in this endemic area, hyperendemic

Fasciolosis (ed. J.P. Dalton)

subzones have been identified with 67% of the humans found infected by stool examination (Esteban *et al.*, 1997b). A large number of unpublished manuscripts and reports related to fasciolosis in Bolivia are found in Mas-Coma *et al.* (1995).

Another study on human fasciolosis in region VII of Chile which includes Curico, Talca and Linares, reported by Apt *et al.* (1993), found almost 4% positive for antibodies by ELISA (using *F. hepatica* crude worm extracts) with 0.7% of 5861 persons evaluated with confirmed fasciolosis parasitologically. The authors estimated that there were 2000 persons with fasciolosis in that region. Thus, in South America, large numbers of rural populations are exposed and infected with *F. hepatica*, the real magnitude of which still needs to be determined.

In the Sharkia Governorate of Egypt, Hassan *et al.* (1995) found 69 of 1350 (5%) school children had *F. hepatica* eggs in stools. More than twice this number had antibodies to *F. hepatica* by ELISA and did not have antibodies to schistosome egg antigens.

Diagnosis

Definitive diagnosis of infection with *F. hepatica* is usually achieved parasitologically by finding the fluke eggs in faeces. However, parasitological diagnosis alone is generally inadequate because the incubation period presenting clinical findings in human fasciolosis is from a few days to 2–3 months, yet the flukes require a period of at least 3–4 months to attain sexual maturity and release eggs (Hillyer, 1988; Chen and Mott, 1990). Thus infected humans have important clinical presentations long before eggs are found in the stools. Moreover, in many human infections, the fluke eggs are often not found in the faeces, even after multiple faecal examinations. As an example, Hillyer *et al.* (1984) had to perform multiple coprologic assays on the same patient to find one sample positive for *F. hepatica* eggs, whereas serum samples taken at similar time points as those taken for the negative stool examinations were always positive for antibodies. Lastly, people ingesting infested bovine livers often have fluke eggs in their stools reflecting 'false' fascioliosis in which case diagnosis requires placing the patient on a liver-free diet and performing repeated follow-up stool examinations.

A review of 20 cases of human fasciolosis in Spain over a 10-year period showed a wide variety of symptomatology (Arjona *et al.*, 1995). Because *F. hepatica* has a special tropism for the liver, the primary presentation in acute stage fasciolosis was abdominal pain, hepatomegaly and constitutional symptoms. However, in the chronic stage, biliary colic and cholangitis are the predominant manifestations. The clinical spectrum of fasciolosis was found to be variable, and patients may present with extrahepatic abnormalities, such as pulmonary infiltrates, pleuropericarditis, meningitis or lymphadenopathy. Therefore, a high index of suspicion is required to establish a correct diagnosis (see also Stark *et al.*, 1993). Eosinophilia is the most frequent laboratory abnormality. The computed tomography (CT) scan has become a useful technique in the diagnostic work-up. A definitive diagnosis may be

established with the observation of the parasite egg in faeces, but most cases may be diagnosed by serologic methods (Arjona *et al*., 1995). Thus, immunodiagnosis is an important procedure to help define infection as an adjunct to clinical findings. This is important in children where acute symptoms tend to be more severe and can be fatal; it is also important in areas of the world where human fasciolosis is rare, such as in the United States. Immunodiagnosis is also useful in epidemiological studies to map the presence of human and animal infections.

Immune Diagnosis

General comments

Over the past 15 years the vast majority of investigators have used an ELISA-based assay or variant, with counterelectrophoresis or indirect haemagglutination as close second, for the detection of antibodies. The ELISA, especially the FAST-ELISA, is an excellent screen test, followed by the Western immunoblot for the confirmatory test (Hillyer and Soler de Galanes, 1988, 1991). The antigenic preparations used have been primarily derived from adult worm extracts or excretion–secretion products of adult worms, or with partially purified fractions. More recently, purified native and recombinant antigens have been used, although further evaluations on their sensitivity and specificity are still needed. Furthermore, exciting studies have focused on the direct detection of fluke antigen either in serum/plasma or faeces. These may be important in estimating fluke burden, and in the early prediction of success of chemotherapy.

Overall, the need for immunodiagnostic tests for fasciolosis is much greater for humans than for their livestock. The need for testing in humans is obvious in seroepidemiology for the study of populations; there is also a real need in individual patients either in endemic areas or after visiting such areas, before specific chemotherapy can be prescribed, and to predict success or failure of this chemotherapy. The immunodiagnosis of fasciolosis has been reviewed recently by Chen and Mott (1990), Hillyer (1993) and Boray (1997).

Antigen detection

Exciting developments have recently been reported on antigen detection for the immunodiagnosis of fasciolosis and for the rapid prediction of success of chemotherapy. Antigen detection assays have been developed for their use with serum or plasma, or with stool homogenates. Langley and Hillyer (1989) used a two-site ELISA for the detection of circulating *F. hepatica* antigens in murine fasciolosis. The horseradish-peroxidase-based antigen capture assay had a sensitivity of 25 ng ml^{-1} FhES antigen and diagnosed infections at 1 week with circulating antigen levels maximal at 3 weeks of infection. Circulating excretion–secretion antigens are detectable in the serum of sheep by 2 weeks of infection (Rodríguez-Pérez and Hillyer, 1995).

Fagbemi and colleagues used two separate, elegant approaches. In the first (Guobadia and Fagbemi, 1997a, b) antigens were excised from gels and monospecific, polyclonal antibodies developed against them in rabbits. A 17 kDa antigen (see section on Western blotting, below) was detected by 1 week of infection in sheep, becoming negative 2 weeks after cure with oxyclozanide. In the second approach (Fagbemi *et al.*, 1997), an *F. gigantica*-specific monoclonal antibody was prepared against immunoaffinity-purified antigens. This antibody detected circulating antigen in cattle by 3 weeks of infection, turning negative 2 weeks after cure with oxyclozanide. In infected goats, serum antigens are similarly found quickly by the first week of infection, becoming ELISA negative by 4 weeks post-cure (Mbuh and Fagbemi, 1996).

Regarding humans, Espino *et al.* (1990) developed a sandwich ELISA to detect circulating parasite antigen in patients with fasciolosis using an anti-FhES mouse monoclonal antibody (IgG2a) to capture the circulating antigens in patient serum. They then detected the captured antigens using a peroxidase-conjugated human IgG obtained from humans with high antibody titres to *F. hepatica*. The assay had a sensitivity of 10 ng ml^{-1} and all 25 patients with fasciolosis were positive in this assay with antigen levels ranging from 14 to 82 ng ml^{-1}. No cross-reactivity was seen with sera from humans with schistosomiasis, trichinosis, filariasis or giardiasis. Espino *et al.* (1992) found eight of ten infected patients positive for antibodies to FhES, whereas all ten had circulating FhES antigens in serum. Five patients that were treated with bithionol became negative for circulating antigen 3 months post-curative therapy. Four patients treated with praziquantel were not cured, of whom three remained antigen positive and one became antigen negative in this test. Using polyclonal antisera, Hammouda *et al.* (1997) showed that persons cured after triclabendazole treatment became serum antigen negative, confirming that the detection of circulating antigen is an accurate tool for the assessment of cure.

Mikhail *et al.* (1990) used an antiserum prepared to fractions of *F. gigantica*, which did not cross-react with schistosomes in counter-electrophoresis, for the detection of fluke antigen in stool samples. All 42 patients with confirmed fasciolosis were positive in this assay, and most were positive 1 to 57 days prior to a definitive parasitologic diagnosis. In subsequent work these authors again detected *Fasciola* antigens in all of 108 stool samples of patients with fasciolosis (Youssef and Mansour, 1991; Youssef *et al.*, 1991). Parenthetically, antibodies to similar antigen fractions have been found useful for the specific diagnosis of acute fasciolosis gigantica using ELISA (Osman and Helmy, 1994).

Using a monoclonal antibody, Espino and Finlay (1994) found that the mean antigen concentration in stool eluates of humans with fascioliosis was 250 ng ml^{-1}, approximately ten times that which they found in serum. Two months after treatment with bithionol all cured patients were antigen negative in stools. This monoclonal-antibody-based assay has been adapted for antigen detection in faeces and has been commercialized in Cuba. An important study by El-Bahi *et al.* (1992) showed that a stable 26 kDa diagnostic antigen could be detected in bile and faeces of cattle with fasciolosis.

An antigen competition assay was recently developed by Leclipteux *et al.* (1998) for the detection of liver fluke infection in cattle. The assay involved adding an IgA monoclonal antibody prepared against an excretory–secretory (ES) antigen to microtitre wells which then captured antigen from the serum of infected antimals. The amount of antigen bound was estimated by a competition with peroxidase-conjugated ES antigens. This assay could detect the presence of infection in animals as early as 6 days after infection and was therefore suggested to be of value for the diagnosis of early, prepatent infections.

Antibody detection

Antibody detection assays are overwhelmingly the preferred method for immune diagnosis of fasciolosis. The reasons include the relative simplicity of the assays, and early seroconversion (usually 1–2 weeks) during primary infections as compared to 'late' patency (2–3 months). Consequently, most investigators today use ELISA and/or Western immunoblots for the immune diagnosis of fasciolosis. The preferred 'crude' antigen system is excretion–secretion (ES) products prepared by incubating live worms in culture medium for varying periods of time, at varying temperatures, in varying buffers, with and without protease inhibitors. In spite of these variations in culturing conditions, analyses of the ES products by SDS–PAGE and Western immunoblot finds them to be remarkably similar. Earlier works on ELISA for the immunodiagnosis of fasciolosis have been reviewed by Hillyer (1986, 1988, 1993).

Immune diagnosis is also of use for seroepidemiologic studies of livestock and humans. In llamas from *F. hepatica* endemic areas in South America, 16% were found positive, with seroprevalence increasing in llamas over 6 months of age (Rickard, 1995). The dot-ELISA as used in that study was an adaptation of Rivera-Marrero *et al.* (1988), which found the llamas positive by 2 weeks after infection. Sheep and cattle of the Corapata region in the Bolivian Altiplano showed high seroprevalence in ELISA, with 89% prevalence in sheep and 58% in cattle. Over 40% of the humans tested in this region were positive by ELISA (Hillyer *et al.*, 1996).

Enzyme-linked immunosorbent assay (ELISA)

Antibody levels in mice, rabbits, sheep and cattle with fasciolosis can be detected in FAST-ELISA by 1–2 weeks of infection (Hillyer and Soler de Galanes, 1991). They rise rapidly reaching a plateau by 3–6 weeks of infection. In one study with experimentally infected calves, levels remained high throughout the 13 weeks of infection (Vignali *et al.*, 1996). Egg excretion rates in infected calves peak at 14–20 weeks, drop sharply thereafter and become egg negative by one year (De León *et al.*, 1981). This decrease in eggs in the infected cattle is presumably due to the death of adult flukes, and is also followed by a decrease in ELISA absorbance values (Hillyer *et al.*, 1985). Experimentally infected goats exhibited a rise in serum antibody levels by 2–4 weeks of infection, a peak at 3 months and then a steady decline, yet

still positive, through the one year that followed. Those infected with 200 metacercariae always had antibody levels higher than those infected with half this amount (Martínez *et al*., 1996).

Isotype determination is also important. For example, IgM antibodies peak early by week 3 in infected sheep and drop sharply by week 6 post-infection; IgG antibodies peak by 4–5 weeks of infection but remain high thereafter (Chauvin *et al*., 1995). In contrast, Clery *et al.* (1996) found IgG1 to be the dominant isotype over IgM, IgG2 and IgA in both chronically infected and acutely infected, previously naive calves. O'Neill *et al.* (1998) have shown that IgG1 and IgG4 antibodies are the predominant isotypes elicited by infection in humans.

With respect to humans, Espino *et al*. (1987) found that 20 patients with confirmed fasciolosis were all positive for antibodies by ELISA using FhES as antigen. In this study, no cross-reactivity was observed using sera from patients with other parasitic infections including schistosomiasis. Hillyer and Soler de Galanes (1988) used a FAST-ELISA with FhES antigens and found that serum from humans with fasciolosis had elevated antibody levels for the entire 3 years that infection was monitored. Antibody levels in those treated with bithionol and cured of infection slowly decreased to almost normal levels 6–12 months after treatment. Those treated with praziquantel, which did not cure the infection, had unchanged antibody levels over the 20+ months followed. In this study, sera from humans with schistosomiasis mansoni had intermediate antibody levels compared to those with fasciolosis and the normal controls, suggesting the presence of antibodies reactive with FhES.

Recently, O'Neill *et al.* (1998) developed a diagnostic test for human fasciolosis based on the detection of antibodies to ES antigen or purified cathepsin L proteinase. The authors found that the assays were much improved by the use of conjugated second antibodies that detect IgG4 rather than total antibodies. Importantly, sera obtained from patients infected with schistosomiasis mansoni, cysticercosis, hydatidosis and Chagas' disease were negative in these tests. On the other hand, Osman *et al.* (1995) used an IgM ELISA to detect antibodies to ES antigens. Although all 38 patients with acute fasciolosis were positive by this test, only 77% of 14 chronic cases were positive. Therefore, an IgM ELISA may be a good indicator of early infection only.

Western immunoblot (enzyme-linked immunoelectrotransfer blot)

Using Western immunoblots Hillyer and Soler de Galanes (1988) found that the sera from humans, rabbits, cattle and sheep with fasciolosis all recognized two sharply resolved excretory/secretory antigens of 17 and 63 kDa. For humans, reactions of antibodies with these two antigens were present in sera for at least 3 years of infection. While the antibodies to the 17 kDa antigenic polypeptide disappeared in the immunoblots 6 weeks post-cure, a less clear-cut decrease in the 63 kDa antigen was observed. This study suggested that the 17 kDa FhES antigen was an excellent candidate for the immunodiagnosis of acute and chronic fasciolosis and for the early definition of chemotherapeutic cure. In a

subsequent study, which used additional sera from infected persons from Bolivia, a prominent 12 kDa band was recognized by all 20 egg-positive infected persons, and a 105 kDa band recognized by all but one; the 17 and 63 kDa markers were also important (Hillyer *et al*., 1992b).

Other important studies reveal a myriad of bands in FhES reactive with antibodies in serum obtained from *F. hepatica* infected humans, rabbits, sheep and cattle. In sheep, for example, bands with the following kilodaltons were found prominent: 12, 15, 20, 24, 27, 28.5, 30, 41, 51, 56, 69 and 156. There may be slight variations in the molecular masses reported, and fewer reactive proteins have been reported by others, but there is general agreement among investigators about the more prominent protein bands. Thus, similar banding patterns were reported using the sera from 12 fasciolosis patients in Chile (Silva *et al*., 1994), sheep in France (Chauvin *et al*., 1995), cattle in Puerto Rico (Santiago and Hillyer, 1988) and Chile (Gorman *et al*., 1994), and cattle with *Fasciola* sp. in Japan (Itagaki *et al*., 1995).

Guobadia and Fagbemi (1995) found that the 17, 21, 57 and 69 kDa protein bands were specific for *F. gigantica* infection in sheep, and that reactivity against an 87 kDa band disappeared 2 weeks after treatment, making this a predictor of cure. Further studies on this antigen are warranted. Until these bands are fully identified biochemically and recombinant antigens prepared, these reagents will be unstable and difficult to reproduce from crude antigen preparations as is done routinely today.

ELISA versus Western immunoblot?

The Western blot was originally described for the immune diagnosis of parasitic infections by Tsang *et al.* (1983). Parasitologists almost uniformly ignored this powerful tool for many years but the virologists found it extremely useful. Today, the gold standard in the United States for the definition of infection with the human immunodeficiency virus which causes AIDS is to screen by ELISA and confirm by Western blot (NIH, 1996). This approach has also been recommended for the antibody detection of infection in schistosomiasis (Tsang and Wilkins, 1991).

It is clear from the numerous articles cited herein that the ELISA in an epidemiological setting always has more 'positives' than stool examination. This discrepancy may be due to drugs or self-cure in patients or livestock and a seroreversion to negative. The Western immunoblot corrects some of these in that banding patterns will change during seroreversion. In some cases samples that are ELISA-positives show up negative by Western immunoblot. Thus, as for the diagnosis of AIDS the combination of the two techniques is preferable to arrive at a more accurate infection status.

Shaker *et al.* (1994) eliminated the *Fasciola* (species not stated) antigens cross-reactive with *S. mansoni* by immunoaffinity chromatography and then compared this antigen preparation in ELISA and Western immunoblot (reactivity to 33 and 54 kDa bands) using human infection sera. They reported a sensitivity of 100% and specificity of 93% for ELISA alone, and a sensitivity and specificity of 100% when combined with Western immunoblot.

Purified/Recombinant Antigens

Little work has been published on purified or recombinant antigens. Three *F. hepatica* purified antigens have been reported as having potential for antibody detection assays in fasciolosis. These are: (i) nFh12, a 12 kDa *Fasciola*/*Schistosoma* cross-reactive, cross-protective antigen related to fatty acid binding proteins (Hillyer, 1995); (ii) glutathione *S*-transferase(s); and (iii) cathepsin proteases.

The amino acid sequence of the *F. hepatica* fatty acid binding protein has high similarity with a protective *S. mansoni* recombinant 14 kDa fatty acid binding protein (Moser *et al.*, 1991; Rodríguez-Pérez *et al.*, 1992; Hillyer, 1995; Tendler *et al.*, 1996). Antibodies to purified nFh12 are detected by FAST-ELISA as early as 2 weeks in cattle, rabbits and mice infected with *F. hepatica*. Sera from mice infected with *S. mansoni* also cross-react with Fh12 by 6 weeks of infection (Hillyer *et al.*, 1988; Hillyer and Soler de Galanes, 1991). Antibodies to the recombinant *Fasciola* rFh15 fatty acid binding protein also appear in rabbits by the second week of infection (Muro *et al.*, 1997). Its practical use for immune diagnosis of fasciolosis needs to be investigated further.

Glutathione *S*-transferases are candidate vaccines in schistosomiasis (Capron *et al.*, 1990; Hillyer, 1991), and possibly ovine fasciolosis (Sexton *et al.*, 1990, 1994). Several cDNAs encoding *F. hepatica* glutathione *S*-transferases have been reported. Remarkably, those from Australia and Puerto Rico are virtually identical (Panaccio *et al.*, 1992; Wijffels *et al.*, 1992; Muro *et al.*, 1993). The antibody responses to *F. hepatica* GST in various experimental infections with *F. hepatica* were examined by Hillyer *et al.* (1992a). Sheep and rabbits developed antibodies to FhGST by 2 weeks of infection, but mice and cattle did not. Strain differences may also be important as Corriedale × Polworth wethers failed to produce antibodies to FhGST during infections but readily produced antibodies after immunization (Sexton *et al.*, 1990).

Cysteine proteases have been isolated from *F. hepatica* (Dalton and Hefferman, 1989; Rege *et al.*, 1989; Yamasaki *et al.*, 1989; Smith *et al.*, 1993; McGinty *et al.*, 1993; Wijffels *et al.*, 1994a, b; Heussler and Dobbelaere, 1994; Dowd *et al.*, 1994) and *F. gigantica* (Fagbemi and Hillyer, 1992) adult worms. Using a purified cysteine proteinase in ELISA, Yamasaki *et al.* (1989) correctly diagnosed 13 patients with fasciolosis, although slight cross-reactivity with sera from patients with either schistosomiasis japonica or mansoni was also observed. Two *F. hepatica* cysteine proteases were isolated from adult worm ES products and shown that they are part of the mosaic of antigens that form the Fharc2 precipitin band which was used in early studies to diagnose liver fluke infection (Cordova *et al.*, 1997). Recently, O'Neill *et al.* (1998) used purified cathepsin L1 as an antigen in ELISA to diagnose liver fluke infections in a human population in the Bolivian Altiplano. The assay had a high degree of sensitivity; >97% of patients harbouring *Fasciola hepatica* eggs were positive by this assay. Furthermore, all patients deemed negative by this ELISA were also shown to be coprologically negative for eggs. Recent data have shown that recombinant cathepsin L1, functionally expressed in yeast, shows a similar specificity and sensitivity to native antigen for the detection of fasciolosis (O'Neill, Parkinson and Dalton, unpublished).

Acknowledgements

The investigations of the author are supported by NSF-EPSCoR-III Grant No. OSR9452893 and NIH-NIGMS Grant No. 08224.

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14 Molecular Biology

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Introduction

Molecular biology is having a profound impact in most areas of the biosciences. To date, the tools of molecular biology have not been directly applied to the study of *Fasciola hepatica* to any great extent. Where they have been applied it has been with essentially two aims. These have been to utilize the ability of appropriate expression systems to produce quantities of fluke proteins for further study – generally as potential vaccines – or to make use of the unique ability of this technology to provide information relating to genetic organization and diversity. This chapter deals with the limited inroads achieved to date and uses information obtained from related organisms to obtain an insight into the mechanisms that contribute to the survival of *F. hepatica*. As the concepts underlying research in genetic diversity may not be familiar to those with a background in molecular sciences, we have included an introduction to this area which we hope will allow readers to appreciate the potential for the application of molecular biological techniques to these questions.

Genetic Diversity and its Role in the Biology of *Fasciola hepatica*

Genetic diversity within a species is the fundamental material upon which selective environmental pressures can act. It is thus essential for the process of evolution. As a digenic parasite *Fasciola* is found in both mammals and molluscs. Within the definitive mammalian host there may be great differences in the environment to which the fluke is exposed. This may range from the highly surfactant milieu of the bile duct to the physiologically less extreme environment of the gastrointestinal tract, peritoneal cavity and liver parenchyma. While physiologically less demanding these latter regions may present their own dangers for the fluke as it is here that the host's immune effector mechanisms will be most active. The free-living miracidium encounters

Fasciolosis (ed. J.P. Dalton)

a completely different environment in which it has to find and infect a snail, and within the snail the sporocysts face new pressures. This succession of contrasting environments, each with its own set of selective pressures, is itself changing as the snail and mammalian hosts will also be subject to genetic diversity and hence selection for certain traits, which may include resistance to parasitism by *Fasciola*.

There are constraints imposed on the divergence of genetic material in that it is necessary not only for the products of the genes involved to continue to function and interact with other genes of the fluke but there must also be limits beyond which if the parasite changes it will no longer be able to interact with and survive in its two hosts.

The mechanisms by which diversity can arise are the same for *Fasciola* as for other organisms but again there are certain unique points which may affect the rate at which diversity can arise. The liver fluke is a hermaphroditic species, which may confer the ability to maintain a population where the parasite is greatly overdispersed, but under these circumstances there will also be an accompanying tendency towards a genetically homogeneous population. This will be exacerbated by the production of rediae following asexual divisions in the snail (polyembryony). A single miracidium may give rise to 600 cercariae. The overdispersal of parasites, hermaphroditism, polyembryony and the need for the life cycle of the parasite to be interwoven with two hosts may all act to reduce the rate at which genetic diversity can be exhibited. However, these factors do not affect the selective pressures working towards the creation of genetically diverse populations. These selective pressures may be considered as falling into two groups.

1. *'Habitat' variability*. In *Fasciola* it could be considered to be advantageous to the spread of the parasite if there were to be minimal restrictions on the range of host species in which the parasite completes its life cycle. The definitive mammalian hosts are generally sheep or cattle but *Fasciola* does not seem to be specific to this group – almost any mammal that ingests metacercariae may be infected. The intermediate host is a freshwater snail; in Europe this is generally of the family Lymnaeidae but other snails fulfil this role elsewhere. One might expect that there would be a trend towards the selection of certain alleles associated with particular primary and secondary hosts. With the wide geographic range of *Fasciola* it might be expected that some degree of allopatric speciation (speciation due to geographic isolation) would have occurred. We might assume that fascioliosis originated in Eurasia as an infection of herbivores living in temperate climates as embryonation is inhibited below 10°C and hatching of the eggs requires a temperature of 22°C or greater. The spread of fascioliosis to the Americas and Australasia has been brought about by the introduction of infected livestock from Europe. This has necessitated *Fasciola* finding a different species of snail to serve as its secondary host in these continents.

2. *Host immune system variability*. Both the definitive and intermediate hosts possess systems to limit infection. In outbred populations there will be a variation in the effectiveness of this response which will relate to the balance

between different effector systems brought into play (see Chapter 10 of this volume). This serves as a further selective pressure on the fluke population and where the adaptive immune system is involved it might be expected to lead to the development of antigenically diverse strains. Where the hosts may be of different species in different geographical areas, as is the case with the intermediate host for *Fasciola*, then the conditions exist for the development of sympatric evolution (speciation within a geographical area) which may serve to increase diversity.

The importance of genetic diversity for *Fasciola hepatica*

An assessment of the genetic diversity of *Fasciola hepatica* has the potential to answer several questions, some of interest to the theoretical biologist while others may have important consequences for applied biologists concerned with the control of fascioliosis. Among these questions are the following:

1. Has co-evolution occurred between *Fasciola* and its hosts? If this were to be the case, then one might expect to see the development of strains which preferentially infect certain species of snail or a divergence between the flukes found in cattle and those found in sheep.
2. The life cycle of the parasite makes it extremely susceptible to founder effects when introduced into new geographical areas. Would this result in the development of unique strains in those regions (such as Australasia and the Americas) where it has been imported in historical times?
3. Genetic diversity is necessary for the selection of strains resistant to either drugs or the host's immune response. Bearing in mind the probable existence of almost clonal populations due to founder effects are we more likely to see the development of resistance occurring in Eurasian populations than in those of Australia/America?

The techniques of genetic diversity studies – parameters measured

- *Morphology*. This is the oldest technique and has been the basis of the determination of species within the fasciolids. Its limitations are illustrated by the failure of morphological techniques to allow the determination of species of *Fasciola* from Japan (Kendall, 1965). There have been occasional comments in the literature suggesting that flukes from cattle are larger than those from sheep but no definitive study has been reported.
- *Isoenzyme patterns*. Isoenzymes encoded by different loci may show, as a result of changes in amino acid sequence, differences in migration rates during electrophoresis. This provides a means of studying the variability of specific alleles encoding the enzymes whose presence may be revealed by the use of an appropriate substrate. This technique has been successfully applied to the question as to the origin of Japanese *Fasciola* species (Agatsuma *et al*., 1994).
- *Antigenic diversity*. The production of monoclonal antibodies specific for the major tegumental antigen and other proteins of the fluke (Hanna and

Trudgett, 1983; Hanna *et al*., 1988) provided the opportunity to determine whether major antigenic differences existed between populations of flukes. Although no systematic study has been carried out in this area there are no reports indicating that antigenic diversity poses a problem for potential vaccination programmes. Although diversity may be seen in the amino acid sequence of tegumental antigens (see below) the structural motif is maintained and as such it is probable that their ability to function as epitopes is not compromised.

- *Restriction enzyme mapping.* Restriction enzymes cleave DNA at sites bearing specific sequences. Thus *Eco*RI will cleave DNA at each GAATTC, *Sau*961 at each GGNCC and *Hae*III at each GGCC. The probability of these sites occurring in a sequence and hence the size of the resulting fragments is a function of the size of the site. Six-mers such as the cleavage site of *Eco*RI occur more rarely (every 4096 bases on average with an A–T usage of 50%) than four-mers (every 256 bases). The correct choice of enzyme will give a number of well-defined bands readily resolvable after agarose electrophoresis. This technique has been used to distinguish between fasciolid species but may not be of sufficient discrimination to detect variation between individuals (Blair and McManus, 1989).
- *Sequence analysis.* With the advance of molecular biology its techniques are being increasingly applied to a wide range of biological problems. Although, theoretically, sequence data provide the ultimate argument in discussions of genetic diversity caution must be exercised in the interpretation of such evidence. Generally speaking in these studies only short sequences produced by polymerase chain reaction (PCR) amplification have been sequenced and the degree of diversity that they can be expected to exhibit will vary with their function in the fluke's genome. Expressed sequences will be required to maintain structural features required for their function – this will work against the generation of diversity by genetic drift – but may also come under selective pressures which will encourage the development of variant forms. Unexpressed regions of the genome such as introns will not be exposed to these influences and therefore may not show the same rate of variability. Little is known of the genomic structure of fasciolids and although large intronic sequences have been reported for schistosomes (Markovics *et al*., 1994) it has been suggested that they may be of limited size in *Fasciola hepatica* (Panaccio and Good, 1998).

Evidence for diversity in fasciolids

In comparison to schistosomes there are few published papers which deal directly with variability within *Fasciola hepatica*. There are reports of size differences between flukes from cattle and those from sheep and one study showed that the ionic composition of flukes from cattle differs from sheep flukes (Caseby *et al*., 1995), although these findings may reflect physiological adaptations by the fluke to different environments rather than genetically

determined traits. Using restriction enzyme mapping of ribosomal DNA, Blair and McManus (1989) studied 21 flukes which differed in their geographical origin – samples being provided from Mexico, Ireland, New Zealand, Australia, Hungary and Spain. The Australian samples were from well-established strains, maintained by Professor J.C. Boray, which had been passaged through differing definitive and intermediate hosts and in some cases selected for resistance to salicylanilides. Within the species there was no variation in restriction sites but there was considerable variation in the size of the non-transcribed spacer region (NTS). NTS from individual flukes ranged in size from 5.38 kb to 8.74 kb, suggesting that considerable genetic diversity may exist in the field. Interpretation of these results is complicated, however, by the nature of the NTS. In schistosomes this is known to be composed of a series of repeats and as such is constrained in its sequence variability.

Another example of the use of a region which may be constrained in its variability is the second internal transcribed spacer (ITS1) region of ribosomal DNA. Working with this region Adlard and colleagues (1993) were able to distinguish species differences within the fasciolids but found only one nucleotide change in 600 bp of sequence from *Fasciola hepatica* specimens isolated from New Zealand, Mexico, Hungary and Australia (0.4%). Using both ITS1 and the mitochondrial gene ND1 they compared flukes from cattle and sheep and from a variety of geographical locations (Dosay *et al*., 1996). A greater amount of sequence divergence for both genes (1–7%) was found, with flukes collected in Ireland showing greater diversity than specimens from the Americas (Trudgett, personal communication). It is probably premature to conclude that this is evidence for the New World flukes exhibiting the founder effect proposed above, although recent evidence that the intermediate host for *F. hepatica* in Bolivia – *Lymnaea viatrix* – is probably of European origin may strengthen this association (JabbourZahab *et al*., 1997). Parsimony analysis of the data yielded trees with mixed groupings of flukes derived from cattle and sheep – suggesting that co-evolution with the definite hosts has not occurred to a degree that would be reflected in changes in regions of the genome not directly influenced by the selective factors.

There is, however, indisputable evidence for host effects in selecting strains of flukes. Professor Boray and colleagues have reported variability in the isoenzyme patterns of glutathione *S*-transferase (GST) from isolates of flukes from cattle, sheep and rats. Cattle flukes showed the greatest variability. The level of GST activity was lowest in those hosts which became resistant to reinfection by *Fasciola*, and was interpreted as being the result of modulation of the activity by the host immune system (Miller *et al*., 1993). A further example of diversity induced by the host immune response is seen in Fig. 14.1. The T1 antigen provides the major antigenic stimulus during the migration of the fluke in the definitive host (Hanna and Trudgett, 1983). The carboxy terminal regions of T1 isolated from different flukes show great variation in their sequence while maintaining their structural configuration and antigenicity.

These data may underestimate the degree of variability in the T1 protein, as part of the procedure by which the sequence data were obtained involved

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1.....  KEGEPENKEGEPENKERETTATGGTSRLTNHLAVLEFPLKCLGVL
2       KEGEAEKKEGEAEKKRTEATTTGGTSRLTNHMALLVFSLRSLRLIQ
3       EGKKPENKKGEPENKERETTATGGTSRLTNHLAVLEFSLRCLGLIQW
4       KPGPEPKPGPEPPGKPEENSTTGGTSRLSNHMAFLTFPLKCLGLIQW
5       PGDGDPKPEPKGDGDSNTTASSGGTSRLSNQMAFLTFPLKCLGLIQ
```

Fig. 14.1. Diversity in tegumental protein (T1) carboxy terminal sequences: 1, sequence from flukes grown in rats using metacercariae supplied by Compton; 2–5, sequences from flukes obtained from different cattle at slaughter. Conserved residues are underlined, many of the substitutions are conservative, i.e. T/S, V/L. (A. Trudgett, A.T. McNair, E. Hoey and P. Ramasamy, unpublished data.)

immunoscreening of cDNA libraries. Any variants which had changed sufficiently to destroy the majority of their epitopes would have escaped detection.

The high degree of variation seen in the TI proteins has also been confirmed in other proteins. From a cDNA library produced from Compton Paddock *F. hepatica* mRNA it is rare that totally identical cDNAs encoding the same protein can be obtained (Panaccio and Good, 1998). Over 50 GST clones were isolated and found to encode four distinct classes of GSTs, GST-1, GST-7, GST-47 and GST-51 (Panaccio *et al.*, 1992). However, each clone of the same class had minor nucleotide changes that distinguished it from other clones. This same phenomenon was also observed for cathepsin proteases. Of the ten cathepsin L1 cDNA isolated none were completely identical. It is highly likely that these differences are not the products of different genes but a reflection of the genetic diversity found within the population. Most of the molecular characterization of *F. hepatica* is based on fluke derived from Compton Paddock Laboratories which are not derived from a clonal source. It is therefore necessary to produce strains of *F. hepatica* that have been derived from a single individual before one can conclude that these observations are due to the genetic diversity found within *F. hepatica* populations.

The Practical Importance of Diversity

From the limited evidence presented above it would appear that there is genetic diversity in fluke populations in the field. The reliance by many laboratories on metacercariae derived from a single supplier, coupled with the asexual reproductive stages in the life cycle and founder effects outside Eurasia, may have served in the past to obscure this phenomenon when using traditional techniques.

Genetic variants become apparent when conditions change to favour their selection. In recent years we have seen the dominance of triclabendazole in the treatment of fasciolosis. This has to some extent overshadowed the demonstration of the development of strains of fluke resistant to other

flukicides such as the salicylanilides in field populations (Boray, 1990). More recently, and perhaps inevitably, there are now reports of resistance to triclabendazole in Australia and Europe (Overend and Bowen, 1995). In order to deal with this potentially devastating problem, those seeking control of flukes by either chemotherapy or vaccination will, in the future, be conscious of the need to develop treatment strategies which allow for genetic diversity in the target population. For this to be done in a rational manner there is a need for considerably more research to determine the extent of diversity in demes (local population of interbreeding individuals) in the regions in which control is to be attempted. This necessity promotes the study of genetic diversity from an interesting question for evolutionary biologists to a prominent position in our armoury against parasite disease.

The inability of *F. hepatica* to synthesize nucleotides *de novo* may in fact be a contributing factor to the development of genetic diversity. Using standard molecular techniques it has been known for some time that DNA polymerases of high fidelity can be forced to make errors by manipulating the concentration of each of the four nucleotides necessary for DNA synthesis. Since adult parasites have a relatively high rate of egg production, their requirements for each nucleotide must also be high. Under certain biological situations, for example in calcified bovine bile ducts, it may be extremely difficult for *F. hepatica* to scavenge from its host all the necessary raw materials to maintain its rate of egg output and hence DNA synthesis. In these situations the DNA synthesis machinery might be forced into making a high degree of errors which are then passed on to future generations.

The Application of Molecular Biological Techniques to Other Areas of the Biology of *Fasciola hepatica*

Stage-specific expression and its importance in the development of *Fasciola hepatica*

It has been known for some time that tremendous changes in fluke biology must occur during the development of mature fluke from metacercariae. The best described metabolic changes are those that occur in energy metabolism (reviewed by Tielens, 1994; see also Chapter 8 of this volume). For example, newly excysted juveniles rely solely on the Krebs cycle for energy. By day 20, aerobic acetate production is the main energy source while by day 50, the fluke rely on anaerobic dismutation. These changes in fluke biology are a function of the changing size and development stage of the fluke, the changing environment within the host and the need to evade the host's immune response.

Tkalcevic *et al.* (1997) used three specific monoclonal antibodies, FY3–1, FY3–2 and FY4–7 to demonstrate that the antigens recognized by these antibodies are expressed for only 2 days following infection. This study provides direct evidence that fluke rapidly express different proteins for short periods during development.

Molecular techniques such as differential display can be applied to rapidly identify stage-specific molecules (Liang and Pardee, 1992; Liang *et al.*, 1992). Since differential display was developed there have been many improvements

to this technique (Liang *et al*., 1993; Li *et al*., 1994). A simple differential display technique involves using a random combination of two ten-mer primers to amplify cDNA that was produced from mRNA using only one of the primers. If the resulting PCR fragments incorporate a radioactive label the resultant products can be 'displayed' on DNA sequencing gels. The power of this technique lies in the ability to perform identical displays using different starting sources of mRNA. For example, displaying side by side the PCR products derived from both mature and immature fluke allows one to identify which transcripts are common to both developmental stages and which are unique to each stage. The technique is designed to detect absolute differences in gene expression but in practice also detects some quantitative differences.

Reed (1997) used differential display to study the differences in gene expression between mature and immature fluke (14 days post-infection). For any given primer combination used to produce these displays there were, on average, 22% apparently adult-specific and 14% apparently immature-specific cDNA products able to be identified. These differences in percentages relate to the total number of bands produced from each primer combination using mRNA from each developmental stage. Assuming that an adult fluke expresses between 2000 and 5000 genes at any one time, this study suggests that approximately 400–1000 genes could be specific to the adult stage when compared with 14-day-old flukes. This number may be feasible when one considers the number of genes needed for reproduction and feeding. These results are consistent with the notion that there is a very high degree of differential gene expression during parasite development.

To confirm that the differences observed in the displays did in fact indicate differential gene expression Reed (1997) isolated several cDNA fragments specific to immature parasites and used them to screen an immature fluke cDNA library. One clone identified an abundant 400–500 bp RNA species by Northern blot that was expressed at levels at least tenfold higher in immature parasites relative to adult parasites. The remaining cDNA fragments (DD14, DD16, DISP10 and DISP2) were apparently expressed at levels below the sensitivity limits of Northern analysis, although differential expression of these transcripts was confirmed by the more sensitive reverse transcriptase PCR (RT-PCR) technique. This study demonstrates the utility of differential display to study even rare mRNA transcripts. Apart from their stage-specific expression pattern the function of DD14, DD16, DISP10 and DISP2 is not known since these sequences do not have any homology to any known genes. The systematic application of differential display and related techniques will provide an understanding of the changing patterns in gene expression during fluke development. However, currently the molecular mechanism by which fluke are able to regulate the complex changes in gene expression is not known.

Possible role of *trans*-splicing of premessenger RNA in facilitating stage-specific expression

In nematodes, many mRNAs have been shown to contain a common 5′ terminal sequence, termed a spliced leader (SL). This sequence is added to

the 5′ end of mRNA transcripts in a *trans*-splicing reaction in which the 5′ terminal exon from a SL RNA is spliced on to a mRNA transcript. The SL RNA is a trimethylguanosine capped 100 nt RNA which is bound to Sm protein and is contained within a small nuclear ribonucleoprotein particle (Thomas *et al.*, 1988; Van Doren and Hirsh, 1988; Van Doren and Hirsh, 1990). The process of *trans*-splicing is very similar to the conventional nuclear pre-mRNA splicing (*cis*-splicing) that removes introns from primary transcripts. In fact both *trans*-splicing and *cis*-splicing are catalysed by the same snRNPs (Thomas *et al.*, 1988; Hannon *et al.*, 1991), proceed through a branched intermediate (Murphy *et al.*, 1986; Sutton and Bothroyd, 1986; Bektesh and Hirsh, 1988; Thomas *et al.*, 1988) and use the same splice site border sequences (Huang and Hirsh, 1992).

Caenorhabditis elegans is the best studied system for *trans*-splicing in nematodes. At least two SL sequences have been described, SL1 (Bektesh *et al.*, 1988; Takacs *et al.*, 1988; Nilsen *et al.*, 1989) and SL2 (Huang and Hirsh, 1989). SL1 is the most commonly used splice leader sequence found on 50–80% of mRNA transcripts (Nilsen, 1993). SL1 is conserved in all nematode species studied thus far (Bektesh *et al.*, 1988; Takacs *et al.*, 1988; Nilsen *et al.*, 1989). SL2 has been found in only two mRNAs of *C. elegans* (Huang and Hirsh, 1989; Okkema and Kimble, 1991). *In vivo* experiments suggest SL1 is *trans*-spliced to primary transcripts which begin with an intron (termed outron). In contrast, SL2 is *trans*-spliced to downstream coding regions of polycistronic mRNA precursors (Spieth *et al.*, 1993).

Trans-splicing has also been shown to occur in some trematodes. In contrast to the conservation in spliced leader sequence within the nematodes, spliced leader sequences have not been found to be conserved in trematodes. Rajkovic *et al.* (1990) found that the spliced leader sequence of *S. mansoni* failed to hybridize to mRNA from *F. hepatica*. Recently, Davis *et al.* (1994) described a spliced leader gene of *F. hepatica* and provided the first evidence that *trans*-splicing occurs in *Fasciola*. The spliced leader sequence was found to be 37 nucleotides in length and as predicted by Rajkovic *et al.* (1990) is not conserved with the spliced leader found in *S. mansoni*. Trematode spliced leader sequences have not been as well conserved as within nematode species. The percentage of mRNA transcripts of *F. hepatica* that are *trans*-spliced is unknown. Davis *et al.* (1994) have identified 19 mRNA transcripts that are *trans*-spliced. Only for one of these mRNA transcripts was the protein encoded identified (Genbank Accession No. U10297).

```
FH   37-NT     AACCTTA ACGGTTCTCTGCCCTGTATATTAGTGCATG
SM   36NT      AACCGTC ACGGTTTTACTCT TGTGATTTGTTGCATG
CONSENSUS      AACC T  ACGGTT T   C  TGT   TT  TGCATG
```

The function of *trans*-splicing is currently unknown. There is strong evidence that *trans*-splicing, at least in nematodes, is not obligatory for any general aspect of mRNA metabolism (Conrad *et al.*, 1991, 1993). Nilsen (1993) suggested that *trans*-splicing may play a role in the processing of polycistronic transcription units. Davis and Hodgson (1997), using primer extension, RT-PCR

and RNase mapping, demonstrated that two closely linked genes in *S. mansoni*, the enolase gene and the gene encoding a component of ubiquol–cytochrome *c* reductase complex, are transcribed on a single RNA transcript that is processed into monocistronic mRNAs. It is interesting to note that the close linkage of these two genes is also conserved in *F. hepatica*.

The synthesis of polycistronic RNA followed by *trans*-splicing may in fact be the mechanism by which *F. hepatica* is able to achieve the rapid changes in protein expression during development. As shown in Fig. 14.2, genes that are expressed at the same developmental stage are predicted to be physically linked on the genome. Expression of the polycistronic unit will result in the simultaneous production of all proteins encoded by the unit. For example, FY3–1, FY3–2 and FY4–7, which share the same stage-specific expression pattern, are predicted to be encoded by genes that are physically linked and form part of the same polycistronic unit.

The relationship of *Fasciola hepatica* to other species as revealed by sequencing studies

Adlard *et al.* (1993), using the second internal transcribed spacer, demonstrated that the identity between *F. hepatica* and *F. gigantica* was 97.2% and that between *F. hepatica* and *Fascioloides magna* was 86.8%. They were also able to show that the *Fasciola* species from Japan was almost identical to *F. gigantica*. This work was recently confirmed by Hashimoto *et al.* (1997) who used the second internal transcribed spacer and mitochondrial cytochrome *c* oxidase subunit I sequences to show that the Japanese *Fasciola* species was a strain of *F. gigantica*.

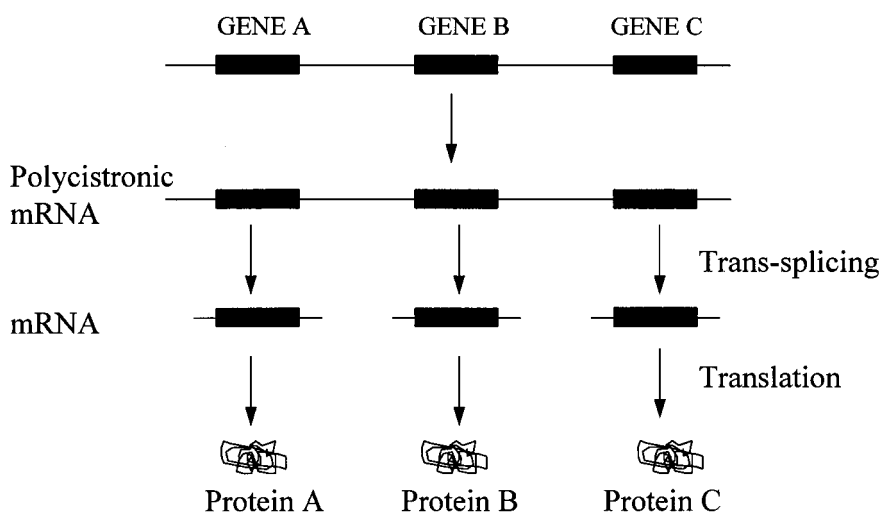

Fig. 14.2. Demonstration of how a polycistronic mRNA leads to co-expression.

A maximum parsimony tree based on 18S sequences (Panaccio and Good, 1998) revealed that the molecular phylogenetic tree was very similar to that derived on taxonomic features except for a few notable differences. A molecular tree suggests that *Fellodistomun fellis* should be in the order Echinostomida rather that Strigeatida. The suborder Paramphistomata should be in an order of its own rather than a suborder of Echinonostomida. It is questionable whether the family Schistosoma should map within the subclass Digenea.

Cloning Strategies Applied to *Fasciola hepatica*

The cloning strategies applied to the study of *F. hepatica* have been limited. The majority of workers have used antisera to identify expressed proteins from cDNA libraries. This approach has led to the isolation of GST (Panaccio *et al*., 1992), cathepsin L proteases (Yamasaki and Aoki, 1993; Wijfffels *et al*., 1994; Dowd *et al*., 1997; Roche *et al*., 1997), arc-2 (Muro *et al*., 1994), fatty acid binding protein (Rodrigues-Perez *et al*., 1992), peroxiredoxin (McGonigle *et al*., 1997) and a gut mucin-like protein (Marin *et al*., 1992).

One of the more interesting cloning strategies was employed by Heussler and Dobbelaere (1994) where they used primers homologous to the active sites of cathepsin proteases to amplify cDNA fragments encoding this enzyme. Their work was a significant advance in that they were able to demonstrate that *F. hepatica* expresses a family of cathepsin L proteases as well as cathepsin B proteases. Localization studies have demonstrated that cathepsin L proteases are expressed in the intestine and are a major component of secreted/excreted protein (Yamasaki and Aoki, 1993; Wijffels *et al*., 1994). Hollywell (personal communication) localized the site of cathepsin L expression to the Mehlis gland as well as the intestine. These data suggest that apart from playing a role in feeding, cathepsin L proteases play a role in egg production possibly through the processing of eggshell precursor proteins. Even though cathepsin B proteases had been demonstrated to play an important role in feeding in *S. mansoni* the role of this enzyme in *F. hepatica* is unclear. However, Creaney *et al.* (1996) have localized the expression of cathepsin B proteases to the gut lumen and to secretory granules within the gut epithelia in juvenile fluke.

The random cloning of transcripts containing spliced leader sequences as demonstrated by Davis *et al.* (1994) is a rapid method to identify genes that are part of polycistronic units. In nematodes, approximately 80% of all genes are predicted, based on the occurrence of spliced leader sequences, to be found in polycistronic units. The number of *F. hepatica* genes involved in *trans*-splicing is unknown. The combination of differential display and probing for spliced leader sequences will provide a rapid method for determining the potential role *trans*-splicing plays in coordinating stage-specific expression.

Expression of *Fasciola hepatica* proteins

For molecular biology to provide further functional data it is necessary that a suitable transformation is developed for the expression of *F. hepatica*

proteins. A transformation system for *F. hepatica* or any other trematode has not yet been described. The development of a reliable continuous culturing system for *F. hepatica* cells would be an important first step.

Currently studies on protein function are reliant on expression in other systems. Even though the transformation systems of choice for *F. hepatica* would be the nematode *C. elegans* due to its relatively simple transformation method, to our knowledge, expression of a *F. hepatica* protein in *C. elegans* has not been attempted. Grant (1992) successfully used parasite genes from *Trichostrongylus colubriformis* to successfully transform *C. elegans*. The availability of genetic mutants of *C. elegans* would allow complementation studies to be performed using *F. hepatica* DNA.

Escherichia coli, due to its ease of use, is the most widely used expression system. The success of obtaining functional *F. hepatica* proteins in this system has been somewhat limited. A notable exception is the expression of glutathione *S*-transferase (GST). Due to the high solubility of this protein, high yields of soluble recombinant GST have been obtained using *E. coli* expression. Not only have the rGST (recombinant GST) retained the ability to bind glutathione, enzyme studies confirmed that the rGST have the same enzymatic activities as their native counterparts (Salvatore *et al.*, 1995). The purification of active rGST has allowed the substrate specificities of the four described mu GSTs to be determined. Creaney *et al.* (1995), using rabbit sera raised to peptides specific to each GST, were able to confirm that the different GSTs have different sites of expression in adult parasites. Together these studies provide a basic understanding of the substrate specificity and site of expression of each GST.

This success is in total contrast to the failure of *E. coli* to express functional cathepsin proteinase. Even though Fhcat-L1 and Fhcat-L2 could be obtained in very high yields (30–40 mg) of purified recombinant protein per litre of culture, the protein was totally insoluble and localized to inclusion bodies. The failure to encode the prepropeptides may have significantly contributed to the failure to produce soluble protein.

Roche *et al.* (1997) were able to express active *Fasciola* cathepsin L1 in *Saccharomyces cerevisiae*. The recombinant cathepsin L1 had similar specificities for substrates with hydrophobic residues in the P2 position as the native cathepsin L1. They demonstrated that to obtain functionally active enzyme, it was necessary to express the complete prepropeptide. Dowd *et al.* (1997) used the same approach to express *F. hepatica* cathepsin L2 in *S. cerevisiae*, and obtained a functionally mature enzyme.

The expression of biologically active recombinant protein has now allowed the substrate specificities of a number of *F. hepatica* enzymes to be determined. This work is especially relevant when ascribing possible functions to members of a highly related gene family.

The More We Know the Less We Understand

Even though the amount of freely available DNA sequence data of *F. hepatica* is limited, being restricted to a total of 89 partial and complete sequences, the

data raise more questions than solutions. Of the 19 *trans*-spliced genes identified by Davis (1994) only one transcript was homologous to any known gene. This result is a reflection of both the limited size of the DNA sequence databases and the evolutionary distance *F. hepatica* is from most studied organisms. In a few cases where sequenced databases have provided a clue to the identity of a molecule, its function within *F. hepatica* often remains unclear. For example, Reed (1997) described a molecule with a high degree of identity to amoebapore, a molecule found in *Entamoeba histolytica* and involved in cell lysis (Leippe *et al.*, 1994). The role of the amoebapore-like molecule in *F. hepatica* is unknown. Likewise, Bozas *et al.* (1995) described a low molecular mass monomeric protein that had significant similarity to the Kunitz-type (BPTI) family of proteinase inhibitors. Immunolocalization studies revealed that Fh-KTM is localized to the gut, parenchymal tissue and the tegument of adult parasites. The possible role of this proteinase inhibitor in *F. hepatica* is unknown.

Conclusion

The molecular biology of *F. hepatica* is very much still in its infancy. We still do not understand the mechanism which drives the genetic diversity seen in *F. hepatica* populations and the mechanism by which flukes can coordinate the complex changes in gene expression that need to occur during development. With techniques such as differential display and *in situ* PCR the developmental stage of expression and site of expression of any gene can be quickly determined. The use of *C. elegans* as a major genetic model, together with its sequenced genome, will provide molecular biologists with a powerful tool which will greatly facilitate a better understanding of *F. hepatica*'s molecular biology.

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15 *Fasciola gigantica*: Epidemiology, Control, Immunology and Molecular Biology

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Introduction

Tropical fasciolosis caused by infection with *Fasciola gigantica* is regarded as one of the most important single helminth infections of ruminants in Asia and Africa (Boray, 1985; Fabiyi, 1987; Murrell, 1994; Anon., 1995; Harrison *et al.*, 1996; Roberts and Suhardono, 1996; Malone, 1997). Estimates of the prevalence of *F. gigantica* in ruminants range up to 80–100% in some countries (Schillhorn van Veen, 1980; Fabiyi, 1987; see also Table 15.1). Together with major nematode infections, fasciolosis is a significant constraint on the productivity of domestic ruminants throughout Asia, South-East Asia and Africa and is thus a significant impediment to global food production (Dargie, 1987; Fabiyi, 1987; Murrell, 1994). It has been estimated that world population growth will demand that food production increase threefold by the year 2050 and consumption of livestock products may rise fivefold in Asia by the year 2010: such heightened demand will result in pressure to increase the productivity of livestock with a 40–50% increase in meat production projected for developing countries (Murrell, 1994). Since tropical fasciolosis is a significant factor in limiting livestock production, the development of sustainable strategies for controlling *F. gigantica* infection is a priority. Strategic use of anthelmintics, enhancement of host resistance by genetic improvement or by the use of vaccines, biological control and better herd management all have a role to play in sustainable control of fasciolosis (Murrell, 1994; Roberts and Suhardono, 1996).

Despite the global significance of *F. gigantica* as a constraint on agricultural production, there have been relatively few studies aimed at developing vaccines for tropical fasciolosis, analysing the immunology of *F. gigantica* infection in cattle and sheep or unravelling the molecular biology of this parasite (Haroun and Hillyer, 1986; Harrison *et al.*, 1996; Spithill *et al.*, 1997). This may reflect the fact that *F. gigantica* is predominantly a parasite found in

Fasciolosis (ed. J.P. Dalton)

Table 15.1. Prevalence of *Fasciola gigantica* and estimated economic losses from fasciolosis.

Country	Prevalence (%)	Losses (million US$)	Reference
Indonesia	25–90	107	Edney and Muchlis (1962); Soesetya (1975); Beriajaya and Soetedjo (1979); Copeman (1997)[a]
Cambodia	35	51	Copeman (1997)[a]
NE Thailand	15–85		Pholpark and Srikitjakarn (1989)
Philippines	34–100	55	Tongson (1978); Copeman (1997)[a]
Vietnam	40–80		Anderson (1997)[b]
China	10		Wilson *et al.* (1982)
Burma		>40	quoted in Fabiyi (1987)
Pakistan	10–100		Kendall (1954)
India	40–60		Roy and Tandon (1992)
India (Kashmir)	25–100		Sharma *et al.* (1989)
Nepal	34–90	20	Morel and Mahato (1987)
Iran	27–91		Sahba *et al.* (1972)
Egypt	11–88		El-Azazy and Schillhorn van Veen (1983)
Sudan	up to 66		El-Azazy and Schillhorn van Veen (1983)
Tanganyika	up to 50		Hammond (1956)
Malawi	19–37		Mzembe and Chaudhry (1981)
Chad	62		Fabiyi (1987)
Mali	up to 50		Tembely *et al.* (1988)
Kenya	18–57		Bitakaramire (1973b)
Tanzania	27–47		Hyera (1984)
Nigeria	65	>40	Schillhorn van Veen *et al.* (1980); Fabiyi (1987)
Cameroons	45		Fabiyi (1987)
West Africa	up to 97		Schillhorn van Veen (1980)
Zambia	50–70		Fabiyi (1987)
Zimbabwe	50–70		Fabiyi (1987)
Uganda	up to 97 (mean 63)		Okao (1984); Fabiyi (1987)
Ethiopia	30–90		Fabiyi (1987)
TOTAL		>2000	Boray (1985)
		>3200	This chapter

[a] D.B. Copeman, Townsville, 1997, personal communication.
[b] N. Anderson, Hanoi, 1997, personal communication.

developing countries where funding for such basic studies is restricted, together with the fiscal difficulty of experimentally studying a parasite of large ruminants. The increasing recognition of human fasciolosis as a public health problem may result in a refocusing of research into fasciolosis (Maurice, 1994; Anon., 1995; Bjorland *et al.*, 1995; Esteban *et al.*, 1997a,b; Hillyer and Apt, 1997; O'Neill *et al.*, 1997).

In this chapter, we review the epidemiology of tropical fasciolosis, the economic effects of this disease on ruminant productivity as well as chemical and non-chemical methods for control. We have reconsidered the literature

on the nature of acquired resistance expressed against this parasite by ruminants and describe recent results assessing the high resistance expressed by Indonesian Thin Tail (ITT) sheep to *F. gigantica*. The nature of the immune mechanisms which may be operating in ITT sheep and initial studies on the genetic basis of this resistance are discussed. Recent vaccine studies in cattle are considered. The molecular biology of *F. gigantica* is reviewed in terms of both purified proteins, cloned cDNA sequences and the molecular taxonomy of this parasite. Other reviews have discussed some aspects of the epidemiology and economic impact of tropical fasciolosis on production (Schillhorn van Veen, 1980; Fabiyi, 1987; Dargie, 1987; Murrell, 1994; Harrison *et al.*, 1996; Malone, 1997), alternative methods of control (Roberts and Suhardono, 1996), the immunodiagnosis of fasciolosis (Hillyer, 1997) and the immunology of host resistance to *F. gigantica* (Haroun and Hillyer, 1986; Spithill *et al*., 1997).

Epidemiology

Introduction

Knowledge of the epidemiology of infection with *F. gigantica* has been clouded by the common yet erroneous assumption that it is essentially similar to that of infection with *F. hepatica*. The confusion exists because both parasites infect the liver of herbivores and have a similar form of life cycle. However, in terms of their epidemiological consequences, these similarities are far outweighed by differences between them in the ecological requirements of their snail intermediate hosts, differences in their host–parasite relationships and large economic, social and agricultural differences between tropical and temperate regions where *F. gigantica* and *F. hepatica*, respectively, are endemic. These differences justify consideration of these two parasites as epidemiologically separate entities.

A further source of confusion has arisen because most of the few publications (relative to those for *F. hepatica*) which have sought to define aspects of the epidemiology of infection with *F. gigantica* have focused on pastoral areas where infection is acquired from metacercariae in water or on vegetation at the fringes of lakes and rivers. The enormous potential for infection from irrigated rice fields after harvest has largely been ignored, yet this is likely to be the main source of infection with *F. gigantica* wherever irrigated rice is grown intensively in lowland tropical areas.

Definitive hosts of *Fasciola gigantica*

Throughout most of its range *F. gigantica* is of greatest importance as a parasite of cattle and buffalo although, regionally, infection may assume importance in goats, sheep and donkeys. Hammond and Sewell (1974) proposed that *F. gigantica* is better adapted to cattle than sheep in that it is more infective and lives longer. Wild herbivores are also susceptible; Hammond (1972) reported infection in 16 species in Africa. Laboratory

animals are not readily infected with *F. gigantica* and there are conflicting reports regarding their susceptibility. Such conflicting reports may be the consequence of differences in susceptibility of the various strains of laboratory animal used and there may also be regional differences in infectivity of strains of *F. gigantica* (see below).

There are occasional reports of human infection with *F. gigantica*, mainly case studies, and of serological tests for its detection which have recently been reviewed (Hillyer, 1997). Hammond (1974) summarized the earlier literature showing that human infections have occurred in the former USSR, Asia and Africa, with eggs of *F. gigantica* found in 2.4% of 3901 human faecal samples in Malawi. Hammond (1974) suggested that, due to inadequate diagnosis, human infections may be more common than indicated by the occasional case reports. A more recent review of human fasciolosis suggests that human disease results mainly from infection with *F. hepatica* with 2.4 million people infected and a further 180 million at risk (Anon., 1995). *F. gigantica* infections have been reported in Africa (Kyronseppa and Goldsmid, 1978), Egypt (Ali *et al.*, 1984), the USSR (Sadykov, 1988), Germany (Schwacha *et al.*, 1996) and Thailand (Kachintorn *et al.*, 1988; Tesana *et al.*, 1989). Immunodiagnosis of infection with *F. gigantica* has been reported (Khalil *et* al., 1990; Mikhail *et al.*, 1990; Youssef and Mansour, 1991; Youssef *et al*., 1991; Osman and Helmy, 1994; Shaheen *et al.*, 1994; Osman *et al.*, 1995; Maleewong *et al.*, 1996). These tests have been applied for the differential diagnosis of fasciolosis from other hepatic diseases such as schistosomiasis and amoebosis but not in population surveys, so accurate figures on prevalence of infection are not known.

Snail hosts of *Fasciola gigantica*

In his reviews of fasciolosis Kendall (1954, 1965) concluded that *F. gigantica* is transmitted worldwide by snails not readily distinguishable on morphological grounds or on grounds of their ecological requirements from the single superspecies *Lymnaea auricularia sensu lato.* Accordingly, he regarded the race of main intermediate hosts in South, West and East Africa as *L. a. natalensis* and in India, Bangladesh and Pakistan as *L. a. rufescens*. The two races merged, according to his accounts, in Oman and Lower Mesopotamia. He proposed the snail host in South-East Asia as *L. a. rubiginosa*. A similar snail, *L. ollula*, is host to *F. gigantica* in Japan (Ueno *et al*., 1975) and Hawaii (Alicata, 1938).

The major role of *L. auricularia sensu lato* as intermediate host for *F. gigantica*, proposed by Kendall (1954), has not been challenged by subsequent authors. However, a number of other species of snail have also been shown to support the development of *F. gigantica*. In a laboratory study Boray (1965) succeeded in completing the life cycle of *F. gigantica* in newly hatched *L. stagnalis*, 4-week-old *L. pergera* and *L. auricularia*, and in fully grown *L. tomentosa*, *L. truncatula* and *L. palustris.* Shahlapour *et al.* (1994) also found *L. stagnalis*, *L. pergera* and *L. palustris* (in addition to *L. auricularia*) were capable intermediate hosts for *F. gigantica*. On the other

hand, Guralp *et al.* (1964) obtained no cercariae from *L. pergera* or *L. palustris* which had been exposed to infection with miracidia of *F. gigantica*. In Nepal, Morel and Mahato (1987) concluded that both *L. a. rufescens* and *L. luteola* (*L. viridis*) act as intermediate hosts for *F. gigantica* and, in Egypt, besides *L. a. natalensis*, natural infection with *F. gigantica* occurs in *Biomphilaria alexandrina* (Farag and el-Sayad, 1995). *L. tomentosa* and *L. truncatula* were also shown in the laboratory to support development of *F. gigantica* by Dreyfuss and Rondelaud (1994, 1997) and Vareille *et al.* (1994). Kendall (1954) and Guralp *et al.* (1964), in contrast, were unsuccessful in their attempts to establish infection with *F. gigantica* in *L. truncatula* from Pakistan and Turkey, respectively. Conflicting reports of this kind are probably due to variations from place to place between strains of *F. gigantica* and snails which alter the host–parasite relationship in favour of one or the other. Furthermore, as *F. gigantica* does not occur naturally outside the range of occurrence of *L. auricularia sensu lato*, it seems reasonable to conclude that the contribution of other snails to the endemicity of this parasite is minor.

Factors affecting the population of snails

The various races of *L. auricularia sensu lato* which serve as intermediate hosts for *F. gigantica* are tropical aquatic snails which thrive in clear stagnant or slow-moving water with high oxygen content and abundant aquatic vegetation (Kendall, 1954). Such ecological situations are typically found at the fringes of rivers and lakes when water levels are stable and in irrigated rice fields throughout the humid tropics. Snails which are intermediate hosts of *F. gigantica* and *F. hepatica* are typically found in tropical and temperate regions, respectively. However, in situations such as Pakistan and Nepal where high mountains provide a gradation of temperature with altitude, an overlap has been reported, although *L. truncatula* and *F. hepatica* are mostly above about 1200 m and *L. a. rufescens* and *F. gigantica* are at lower altitudes (Kendall, 1954; Morel and Mahato, 1987). In lowland tropical areas, *L. auricularia* breeds throughout the year in favourable habitats (Chartier *et al.*, 1990). These authors also found that the population of *L. natalensis* in a river in Zaire was negatively related to rainfall. This reflects the disruption of the habitat of the snails by flooding and also their dispersion with flood water as they spend up to 70% of their time floating at the surface (Widjajanti, 1989). However, once water levels stabilize the population of snails increases and is most numerous while these conditions persist. The duration and timing of this favourable period for snails varies from habitat to habitat. It may be only a few months at the end of the wet season and beginning of the dry season in closed water bodies or persist throughout the dry season in slow-flowing rivers (Mzembe and Chaudhry, 1979; Chartier *et al.*, 1990; Tembely *et al.*, 1995). Once the water level drops too rapidly for the fringing aquatic vegetation to persist or the level of oxygen drops too low, the habitat will be rendered unsuitable for snails (Kendall, 1954).

In irrigated rice fields, in contrast, the population of snails is not directly influenced by rainfall but rather by the availability of water for irrigation and

by the stage of growth of the crop. Snails and their eggs surviving from the previous crop may colonize recently planted rice fields or they may enter with water introduced to flood the field after planting. Their numbers then increase over the next few months before declining again a few weeks prior to harvest (Widjajanti, 1989). Widjajanti proposed that this pattern may reflect the availability of algal and other food for the snails, based on the supposition that growth of such food would be stimulated by high penetration of sunlight to the substrate during early phases of growth of the crop, and reduced as the crop matured due to shading of the substrate.

Despite being aquatic species the snail hosts of *F. gigantica* are able to survive periods of desiccation, leading Mahato *et al.* (1995) to conclude that such aestivation may play an important role in the epidemiology of fasciolosis in Nepal. They observed that young *L. auricularia* race *rufescens* and *L. viridis* survived in dry mud for at least 1 month. A longer period was reported by Bitakaramire (1968b) who found a proportion of *L. natalensis* survived under experimental conditions in hard dry mud for at least 24 weeks. However, these snails do not normally bury themselves in the mud as the water dries up but remain at the surface where they may be expected to be more subject to desiccation and predation than if they were buried. Nevertheless, when not exposed to the sun, they remain alive and produce eggs for some weeks after their habitat becomes dry. Widjajanti (1989) observed that desiccation of *L. rubiginosa* on the surface of the soil in shade for periods up to 1 month had an adverse effect on both survival of the snails and their egg laying, reducing mean survival time by about one-third and egg masses by about half relative to hydrated controls. However, she found no adverse effect of desiccation for 1 month, the longest period tested, on subsequent hatchability of eggs. The eggs did not hatch until rehydrated, prompting her to propose this as a possible mechanism whereby survival of the population may be enhanced in habitats subject to periodic desiccation. 'Hibernating' snails were considered by Ueno *et al.* (1975) as unlikely to play a significant role in contamination of rice fields in Japan with metacercariae even though some were shown to harbour rediae.

Larval stages in the snail

The time required for development of miracidia in eggs of *F. gigantica* varies with temperature. It was 10–11 days at 37–38°C, 21–24 days at 25°C and 33 days at 17–22°C (Guralp *et al.*, 1964). Grigoryan (1958) considered 24–26°C and pH 6.5–7 optimal and found that, under such conditions, 70–80% of eggs would develop. He found that eggs did not survive at temperatures higher than 43–44°C and that desiccation also was rapidly fatal. Eggs of *F. gigantica* do not all develop at the same rate so that, from the same batch, miracidia may hatch over a period up to 14 weeks, thus enhancing their opportunity to infect a snail (Guralp *et al.*, 1964). Guralp *et al.* (1964) also found that eggs were stimulated to hatch by exposure to sunlight or a bright light. Once released from the egg miracidia survive in water for 18–26 h (Asanji, 1988).

Development of *F. gigantica* in *L. auricularia* was described by Dinnik and Dinnik (1956, 1963, 1964). At 26°C they observed that a miracidium develops to a sporocyst in 6–8 days. The sporocyst, which contains up to six embryo balls, produces first generation rediae which in turn produce second generation rediae about 20–22 days after infection. Cercariae or another generation of rediae then develop about 26 days after infection and this pattern may continue through a number of redial generations. Development of larvae in the snail becomes slower as the temperature drops and eventually stops. Because of this, and the adverse effects of low temperature on survival of eggs and of larval stages in snails, Sazanov (1984) concluded that air temperature is the deciding factor in determining the distribution of *F. gigantica* and the presence of a suitable intermediate host an obligatory but secondary factor. At temperatures below 16°C, only a succession of daughter-redial generations are produced, but they switch to production of cercariae when the mean temperature is raised to 20°C (Dinnik and Dinnik, 1964). The definitive host may also influence rate of subsequent development of *F. gigantica* in snails. Al-Kubaisee and Altaif (1989) observed that isolates of *F. gigantica* from sheep had a lower rate of infection in *L. auricularia*, slower larval development and fewer cercariae than isolates from buffalo, which had larger eggs, miracidia and metacercariae.

Cercariae are shed in up to 15 waves (usually three or fewer) 1–8 days apart over a period of about 7–50 days (Grigoryan, 1958; Da Costa *et al*., 1994; Dreyfuss and Rondelaud, 1994). At 25–27°C, Sharma *et al.* (1989) reported shedding commenced as early as 20 days after infection of snails, but maximum shedding in this optimal temperature range occurs about 46–50 days after infection (Dinnik and Dinnik, 1963; Asanji, 1988). This interval gets longer as the temperature drops and periods of up to 197 days were recorded by Dinnik and Dinnik (1963) in the highlands (about 2000 m altitude) of Kenya. About 80% of cercariae are shed at night (Guralp *et al*., 1964; Da Costa *et al.*, 1994). At each wave of shedding 50–70 cercariae are released (Da Costa *et al*., 1994). The total number of cercariae produced per snail is thus usually a few hundred but this varies from fewer than 100 to some thousands. Bitakaramire (1968a) recovered a mean of 653 metacercariae of *F. gigantica* per snail from laboratory infection of *L. natalensis* but Grigoryan (1958) reported that up to 2700 cercariae per snail may be produced and Guralp *et al.* (1964) counted 7179 cercariae released from a snail over a period of 75 days.

After release from the snail cercariae encyst as metacercariae. About two-thirds attach to objects within 6.4 cm of the surface of the water (Ueno and Yoshihara, 1974). The remainder do not attach but become 'floating cysts' (Dreyfuss and Rondelaud, 1994). Da Costa *et al.* (1994) found 35% of cercariae released in the first two waves became floating cysts, the percentage declining in subsequent waves. Size of the snail at the time of miracidial infection also influenced the proportion of floating cysts (Vareille *et al.*, 1994). These authors found 38% of metacercariae from large snails became floating cysts in comparison with 18.2% from small snails. The proportion of floating cysts is higher for *F. gigantica* than for *F. hepatica* (Dreyfuss and Rondelaud, 1997), suggesting that such metacercariae may be

more important as a source of infection with *F. gigantica* than they are for *F. hepatica*, when stock drink from habitats where cercariae are being released. Furthermore, the floating cysts may move with the flow of water to be a source of infection at sites where the habitat is unsuitable for snails and therefore presumed safe from infection. This possibility was demonstrated by Suhardono, J.A. Roberts and D.B. Copeman (Bogor, 1996, unpublished data) who successfully infected sheep with *F. gigantica* with the sediment of water flowing from a rice field containing *L. rubiginosa* infected with *F. gigantica*.

The duration of survival of metacercariae is inversely related to temperature of storage and directly related to the degree of hydration. Unpublished results of Suhardono, J.A. Roberts and D.B. Copeman (Bogor, 1996, unpublished data) in Indonesia demonstrated that metacercariae survive longer in water than out of water. At 20°C these authors found survival in water was high for 5 weeks but then dropped quickly, with a low proportion of metacercariae still viable after 10 weeks. Furthermore, at 26, 30 and 35°C, the periods during which a high proportion of metacercariae remained viable dropped progressively to 3, 2 and 1 weeks, respectively, with a few remaining viable for 10, 5 and 2 weeks, respectively. These times are more conservative than those reported by Boray and Enigh (1964) who demonstrated that metacercariae in water survived 21 days at 35°C but 114 days at 30°C, and also Grigoryan (1959) who found a few metacercariae survived 6 months but none survived 10 months at 16–21°C. The host of origin may also affect duration of survival of metacercariae in water as al-Kubaisee and Altaif (1989) found that 69.4% of metacercariae of sheep origin survived 80 days at 4°C in comparison with 89.8% for metacercariae of buffalo origin.

When metacercariae are stored out of water, the duration of their viability is directly related to relative humidity and inversely to temperature and exposure to sunlight. In Japan, Kimura and Shimizu (1978) showed some metacercariae remained viable over winter on rice stems in a barn (2–28°C and relative humidity 37–88%) for 120 days but not 150 days. However, the period of survival drops sharply as ambient temperature rises. A few metacercariae survived in lucerne hay for 15 days at ambient temperature of 21–32°C and relative humidity of 30–50% but all were dead by day 35 (Grigoryan, 1959). A similar result was obtained for survival of metacercariae in rice straw in Indonesia by Suhardono, J.A. Roberts and D.B. Copeman (Bogor, 1996, unpublished data) and in silage (Grigoryan, 1959; Gupta and Kamra, 1987). Desiccation of metacercariae in a dish was even more rapidly fatal, all being found dead by day 15 at 24–29°C and 60–75 % relative humidity (Grigoryan, 1959). However, when exposed to direct sunlight under such conditions, all were dead within 8 h (Suhardono, J.A. Roberts and D.B. Copeman, Bogor, 1996, unpublished data).

In lowland equatorial regions, therefore, aquatic habitats should be safe to graze about 2 months after death of snails but this period will be extended in cooler habitats for up to 6 months. Similarly, metacercariae which become dry on aquatic vegetation as a result of receding water levels or on hay are likely to be no longer infectious after about 5 weeks in lowland tropical areas but may survive up to about 4 months in cooler climates.

Factors influencing exposure of the definitive host

Reports of the duration of the period during which animals are exposed to infection with *F. gigantica* vary between habitats. In almost all cases, however, the rate of infection is not constant throughout the year but concentrated over a relatively few months. The pattern in any particular area is a reflection of the timing and duration of ecological circumstances favourable to the population of snails and survival of metacercariae, as well as the management of livestock which permits dung from infected stock to enter the habitat of the snails and allows stock to drink water or eat fringing vegetation from such sites when cercariae have been shed.

The resurgence of the population of snails which accompanies stabilization of water levels in rivers and lakes towards the end of the wet season is accompanied, in pastoral areas, by the forced return of stock to such sites to drink as temporary surface water dries up. Snails then become infected with miracidia which hatch from the eggs of *F. gigantica* deposited with faeces in or washed into the water. Cercarial shedding in such habitats thus commences about 2 months later, usually early in the dry season in lowland tropical areas. Furthermore, water plants at the fringes of rivers or lakes are likely to become increasingly attractive as food for stock as the dry season progresses and surrounding vegetation dries off, thus increasing the likelihood of infection at this time. This pattern of infection of snails and stock was described by Schillhorn van Veen (1980) in his review of infection in pastoral areas of West Africa. He reported that the population of snails and their prevalence of infection with *F. gigantica* were highest at the beginning of the dry season, leading to outbreaks of fasciolosis in sheep at the end of the dry season. Complementary findings were presented by Ogunrinade (1985) in Nigeria in an abattoir study which revealed that prevalence of infection in cattle was highest in April to June (the beginning of the rainy season) indicating they became infected during the previous dry season. In Pakistan, too, Swarup and Pachauri (1987) found the highest prevalence of infection of snails after the end of the wet season in October, with larval development in snails during the following spring months followed by infection of stock in summer from March onwards. As a consequence, the highest prevalence of infection with adult flukes in buffalo occurred from June to October during the wet season. A similar pattern was described in Bangladesh by Chowdhury *et al.* (1994b) who found the highest prevalence of infection in snails occurred after the end of the monsoon season, from July to September.

The duration of the period during which animals are exposed to infection will vary between habitats, depending on their continuing suitability for snails, persistence of viable metacercariae and grazing management of livestock.

Infection of snails in irrigated rice fields with *F. gigantica* is promoted by the common practice in many tropical countries of using animal faeces as fertilizer. In West Java, Suhardono, J.A. Roberts and D.B. Copeman (Bogor, 1996, unpublished data) found that rice fields with the highest prevalence of infection with *F. gigantica* in *L. rubiginosa* were those adjacent to a village,

especially those which received drainage from a nearby cattle pen. No snails infected with *F. gigantica* were found in fields further than 200 m from a village. Furthermore, there was minimal transfer of infected snails or miracidia between adjacent fields with the flow of irrigation water. Animal dung as fertilizer is mainly used in irrigated rice fields during preparation for planting and on the young growing crop. Consequently, it may be anticipated that the new generation of snails produced by the colonizing population in the field will become exposed to infection with *F. gigantica* within the first 2 months after the crop has been planted and will commence shedding cercariae some weeks prior to harvest. Infection from this source is only likely to occur in animals grazing the stubble after harvest, drinking water from the field or being fed fresh rice stalks. Furthermore, in lowland tropical areas, if the field remains dry for about 6 weeks after harvest, or rice stalks are stored dry for 5 weeks, these sources of fodder would then be safe to eat. Where the climate is cooler, however, metacercariae may remain viable on such fodder for periods up to 6 months.

Infection in the definitive host

Newly encysted metacercariae require at least 24 h to become infective (Boray, 1969). Larval flukes develop in the hepatic parenchyma and enter the bile ducts about 89 days after infection in cattle (Guralp *et al*., 1964). Reports of the duration of the prepatent period vary, usually from about 12 to 16 weeks (Grigoryan, 1958; Guralp *et al*., 1964; Sewell, 1966; Prasitirat *et al*., 1996). Reasons for this variation may include the sensitivity of the method used to detect eggs in faeces, the number of flukes in the infecting dose, breed of host and strain of *F. gigantica*. The output of eggs rises for the first 4 to 12 weeks after eggs appear in the faeces (Sewell, 1966; Prasitirat *et al*., 1996) then falls to low levels, reducing the value of faecal egg counts as an indicator of level of infection (Sewell, 1966). With the same infecting dose faecal egg counts are up to 80% lower in buffalo than in cattle (Prasitirat *et al*., 1996; E. Wiedosari and D.B. Copeman, Bogor, 1997, unpublished data). Counts also vary between breeds of cattle, with figures in Bali cattle about 45% less than those in Ongoles with the same exposure to infection (E. Wiedosari and D.B. Copeman, Bogor, 1997, unpublished data). Hammond and Sewell (1975) found the number of *F. gigantica* in cattle begins to fall about 28 weeks after infection. Most adult parasites survive less than a year but some survive at least 3 to 4 years (Alicata and Swanson, 1941; Hammond and Sewell, 1975).

Biological diversity of *Fasciola gigantica*

There is evidence for the existence of isolates of *F. gigantica* which differ in biological properties. In a comparison of the behaviour of *F. gigantica* isolates from buffalo and sheep from Iraq, al-Kubaisee and Altaif (1989) showed that the two isolates differed in their infection rate in snails, rate of larval development and size of eggs, miracidia and metacercaria. Infection

experiments in different laboratories have revealed differences in the ability of *F. gigantica* isolates to infect small animals. Mango *et al.* (1972) showed recoveries of an East African isolate of *F. gigantica* in rats was 0–1% and 4–9% in rabbits yet observed no egg production in faeces of infected rats, rabbits, guinea pigs, hamsters and mice. This contrasts with the results of others who observed egg production in rabbits infected with South African and Hawaiian isolates of *F. gigantica* (quoted in Mango *et al.*, 1972). Rats have been reported to be refractory to infection by East African, Indian and Zambian isolates with recoveries of 0–5% (Mango *et al.*, 1972; Gupta and Chandra, 1987; Itagaki *et al.*, 1994) yet Yoshihara *et al.* (1985) observed a 17% recovery with an isolate of unreported origin. Sahba *et al.* (1972) found rabbits and gerbils were susceptible to infection, while Asanji (1988) successfully infected one of three rats but neither of two rabbits. Furthermore, Gupta and Chandra (1987) reported that the rabbit and guinea pig were susceptible to infection and the mouse refractory whereas Khajuria and Bali (1987) successfully infected only four of five guinea pigs, one of five rabbits and none of four mice. Such observations suggest that there is variation in the biological properties of *F. gigantica* which may reflect the genetic variation revealed by molecular genotyping (see below). It will be of interest to conduct a more detailed comparison of the relationship between parasite genotype and virulence in different hosts to gain further knowledge of the basis for the behaviour of *F. gigantica*.

Economic Importance

Introduction

Accurate assessment of the economic loss from infection with *F. gigantica* is hampered by several factors: incomplete information of the extent to which meat, milk and fibre production, as well as mortality, reproduction, draught output, feed conversion efficiency and appetite are adversely affected by infection; the variation in importance of each of these productive indices from place to place; variation between animal breeds in their resilience and resistance to infection; and the extent to which productive loss is influenced by level of infection, level of nutrition, age, sex and concurrent infection of exposed animals with other parasites and infectious agents. Moreover, extrapolation of information derived from the more numerous studies with *F. hepatica* is likely to be unreliable due to the significant differences in host–parasite relations between *F. gigantica* and *F. hepatica*.

Weight gain

Major difficulties with estimating the extent to which weight gain is reduced by infection with *F. gigantica* and with comparing results between studies are created by the differences between studies in level of infection, the age and sex of animals, the interaction between level of nutrition and pathogenic effect, and the difference between breeds and within breeds in resistance and

resilience to infection. Possibly the most often quoted estimate of the effect of infection with *F. gigantica* on weight gain is that of Sewell (1966). He reported a linear relationship in yearling zebu cattle between burden of adult *F. gigantica* and weight gain, each fluke reducing the potential annual gain by about 200 g. Although the nutritional level was not specifically mentioned, some indication may be inferred from data presented from which it may be calculated that mean daily growth of fluke-free controls was 400 g per day. While this may seem low for those used to weight gains of stock grazing temperate pastures, it is a fair result for tropical pasture which, at its best, is only capable of sustaining growth rates in yearling cattle of about 700 g per day.

The demonstration by Sewell (1966) of a linear relationship between fluke numbers and their effect on weight gain of young cattle appears at first glance to be at odds with reports from others whose results suggest the existence of a threshold effect, the size of the threshold depending on the quality of nutrients (especially protein) fed. For example, Graber (1971) was able to negate the weight loss, cachexia and death which occurred in cattle infected with *F. gigantica* on a deficient diet by provision of an adequate diet. Similarly, Nour *et al.* (1979) found that sheep fed a high-protein diet were only slightly affected by infection with *F. gigantica*, whereas they observed more severe depression of weight gain and level of haemoglobin in animals fed a basal diet with or without a mineral supplement. The apparent threshold effect may be illusory, however, as the experiments of Graber (1971) and Nour *et al.* (1979) were not designed to investigate the relationship between level of infection and response. The most plausible conclusion from these studies is that the extent to which weight gain is affected is proportional both to the level of nutrition and to the size of the infection.

Variation between breeds and among individuals within a breed in resistance and resilience to infection with *F. gigantica* are other factors which confound comparison between reports on clinical effects of infection. The only comparative study found is the unpublished work of E. Wiedosari and D.B. Copeman (Bogor, 1997, unpublished data) who monitored for 36 weeks the effects of infection with 15 metacercariae of *F. gigantica* given twice weekly for 32 weeks to seven water buffalo, seven Bali cattle and seven Ongole cattle. Four animals of each breed were maintained as uninfected controls. All were about 6 months old and free of detectable fluke eggs in their faeces, and all were dosed with triclabendazole and ivermectin at the commencement of the study. Food was fresh young elephant grass *ad libitum* plus a concentrate of cereal, protein and minerals which gave a mean weight gain in the uninfected control calves of 405 g per day. Infection significantly depressed weight gain in Bali and Ongole calves ($P < 0.05$) by means of 160 g per day and 98 g per day, respectively, relative to their controls but infected and control buffalo calves had similar weight gains. At slaughter 36 weeks after commencement of the trial, infected Ongole calves, with mean ± standard deviation of 214 ± 78 flukes of all ages, had significantly more flukes ($P < 0.007$) than either buffalo (83 ± 57) or Bali calves (100 ± 32). From this it might reasonably be concluded that buffalo

and Bali calves are more resistant to infection with *F. gigantica* than Ongole calves. However, the relationship between weight depression and adult fluke numbers (those > 2 cm long) presented a different pattern of resilience to infection, with mean potential annual loss per fluke in Bali, Ongole and buffalo calves of 987, 234 and 114 g, respectively. The magnitude of these differences between breeds thus highlights the importance of breed as a determinant of the effects of fasciolosis and the necessity to take this into account when estimating the likely magnitude of the effect on weight gain of infection with *F. gigantica*.

Age, too, is a significant determinant of the extent to which body weight of cattle is affected by infection with *F. gigantica*. The few studies that have been reported indicate that adults are more resilient to the effects of infection with *F. gigantica* than yearlings. In Zimbabwe, treatment of infected cows each 8 to 12 weeks with nitroxynil or rafoxanide gave no measurable benefit in terms of body weight (except in one herd where the level of nutrition was low), weaning weight of calves or reconception rate (Needham, 1977). Srikitjakarn *et al.* (1988) also found no beneficial effect on body weight of infected buffalo treated with niclofolan at the end of the dry season in Thailand and, in South-West Java, where annual incidence of infection with *F. gigantica* approaches 100%, Suhardono *et al.* (1991) found that treatment of adult Ongole cattle each 8 weeks with triclabendazole for 12 months conferred no weight advantage. Furthermore, in the same region, Suhardono, J.A. Roberts and D.B. Copeman (Bogor, 1996, unpublished data) found that a single treatment with triclabendazole in July in two successive years had no measurable effect on weight of adult Ongoles. However, the mean weight gain of treated yearlings (250 g per day) was significantly higher ($P < 0.05$) than that of untreated yearlings (195 g per day).

No report has been found linking sex as a determinant of the effect of infection with *F. gigantica* on body weight. This possibility should be explored, however, as Asanji and Williams (1984) found a higher prevalence of infection in female than in male cattle, suggesting that a difference in susceptibility between the sexes may exist.

Draught performance

No studies have been reported which quantify the adverse effects of infection with *F. gigantica* on draught performance. However, in an unpublished study in Indonesia in 1991, J.A. Roberts, B. Bakrie, D.B. Copeman and E. Teleni (Bogor, 1991, unpublished data) measured the work output of five pairs of water buffalo infected with *F. gigantica* in comparison with that of three other pairs that were not infected. Only one infected pair showed clinical signs of anaemia and low body weight; they also had the lowest work capacity. A second infected pair which were anaemic but had intermediate body condition had the second lowest work capacity. It was estimated that anaemia from fasciolosis in this study reduced work output by 7–15%. A further reduction of 20% in potential work capacity (and body weight) in infected adults was proposed based on the direct relationship between work

capacity and body weight and the likely reduction in growth of 75 kg due to infection with *F. gigantica* over a growing period of 2.5 years. This expectation was formulated on reduced weight of 200 g per fluke per year (Sewell, 1966) and the average of 125 adult flukes recovered from the infected buffaloes (J.A. Roberts, B. Bakrie, D.B. Copeman and E. Teleni, Bogor, 1991, unpublished data).

Estimates of cost of the reduced draught capacity which results from infection with *F. gigantica* may be measured in terms of the opportunity cost to a farmer as a consequence of the increased time taken by infected animals to perform the work that must be done; this amounts to about 27–35% more time with buffaloes according to the conclusions of J.A. Roberts and D.B. Copeman (Bogor, 1991, unpublished data). As the average draught animal in Indonesia is used in land preparation for growing rice only about 23 days per year, the opportunity cost for a farmer with infected buffalo in this situation is thus the value of his labour for about 7 days per year.

Further evidence that infection with *F. gigantica* adversely affects draught capacity was collected by Suhardono, J.A. Roberts and D.B. Copeman (Bogor, 1996, unpublished data) in Indonesia from a survey of farmers during the second year of a trial to measure the effects in Ongole cattle of a single treatment with triclabendazole administered in July, about 6 weeks after harvest of the second seasonal rice crop in the area. The survey revealed that treated animals were used twice as many days as untreated animals for preparing land for planting rice. This result suggests that farmers recognized that the treated cattle performed better than those that were untreated. Furthermore, those with untreated animals avoided the opportunity cost associated with increased time to prepare their land by hiring animals that had received treatment. Thus, where this hiring option is available, the economic cost associated with reduced work capacity in animals infected with *F. gigantica* may be the cost of hiring replacement animals for land preparation rather than the opportunity cost of a farmer's labour.

Fertility

While the limited evidence available tends to support the conclusion that infection with *F. gigantica* is likely to have the same level of adverse effect on fertility of cattle as that reported for *F. hepatica*, the extent and pathogenesis of the reduced fertility have not been documented. In a study of fertility in rural cows in Uttar Pradesh, Kumar and Sharma (1991) found that anoestrus cows and repeat-breeder animals had significantly lower haemoglobin concentration values than normally cycling cows. Because chronic fasciolosis induces anaemia they concluded that infection with *F. gigantica* might be one of the contributing factors responsible for the infertility observed. Suhardono, J.A. Roberts and D.B. Copeman (Bogor, 1996, unpublished data) also observed a link between infection with *F. gigantica*, anaemia and fertility. There were significantly longer intercalving intervals and lower packed cell volume in Ongole cows in Indonesia infected with *F. gigantica*, than in those treated with triclabendazole each year in July for 2

years. In their study, treated cows had a mean intercalving interval of 18.5 months whereas in untreated cows the interval was 31.5 months. It is thus reasonable to conclude that infection with *F. gigantica* is likely to adversely affect reproduction. Furthermore, the extent may be proportional to the degree of anaemia induced, an outcome that varies according to the level of nutrition, level of infection and breed.

It is now recognized that sexual maturity in heifers is determined by weight rather than age *per se* (reviewed by Entwistle, 1978). The retardation of growth of young females induced by infection with *F. gigantica* could thus be expected to prolong time to reach maturity, but no reports of studies which measured this have been found. However, an estimate may be made. Fluke-free heifers growing at 400 g per day could be expected to reach a mature weight of about 280 kg from a birth weight of 35 kg in about 612 days. Using Sewell's (1966) figure of 200 g reduction of potential weight gain per fluke per year, a heifer with a moderate burden of 100 flukes could be expected to grow about 55 g per day more slowly and thus take an extra 100 days to reach sexual maturity. Furthermore, considerably longer delays could be anticipated in animals with heavy fluke burdens on a low plane of nutrition.

Lactation

It has generally been assumed that infection with *F. gigantica* will reduce milk yield and quality in lactating animals based on extrapolation of information from infection with *F. hepatica* where yield may be depressed by up to 13.3% (Black and Froyd, 1972; Randell and Bradley, 1980). However, Dargie (1987), in his review of the topic, was critical of the design of many of the studies and cited studies by others (Whitehead, 1976; Castagnetti *et al.*, 1982; Hope Cawdery, 1984) which showed no or minimal effects of infection on milk yield in cattle. There have been few studies with *F. gigantica* to determine the magnitude of the effect. Kumar and Pachauri (1989) reported that treatment of buffaloes infected with *F. gigantica* with albendazole increased milk yield by a mean of about 5.5 litres per animal per week and also improved the quality of the milk. Needham (1977), on the other hand, found no measurable difference in weaning weight of calves (an indicator of milk output of their dams) from cows infected with *F. gigantica* and those treated every 8 to 12 weeks with an adulticide. Such contradictory reports are to be expected due to differences between studies in factors likely to affect milk output such as level of nutrition, size of infection and differences in resilience to infection between individuals and breeds. However, at present, too few studies have been undertaken to enable meaningful prediction of the extent to which any of these determinants affects lactation in animals infected with *F. gigantica*.

Estimates of the cost of tropical fasciolosis

There are reports from most regions where infection with *F. gigantica* is endemic which state that fasciolosis is one of the most important, if not the

most important, disease(s) of livestock, especially of cattle and buffalo (Kendall, 1954; Edney and Muchlis, 1962; Sewell, 1966; Tongson, 1978; Okao, 1984; Swarup and Pachauri, 1987; Touratier, 1988; Chowdhury *et al*., 1994a; Harrison *et al*., 1996; reviewed in Fabiyi, 1987). However, most reports are anecdotal or based on calculations from prevalence proportions derived from abattoir or faecal egg examination studies. With few exceptions, estimates of loss have been narrowly based, usually on the value of infected livers condemned at slaughter as unfit for human consumption (Hyera, 1984; Okao, 1984; Morel and Mahato, 1987) or on the value of meat lost through lower carcass weights of infected animals (Edney and Muchlis, 1962; Sewell, 1966; Preston and Castelino, 1977; Ollerenshaw and Graham, 1986; Srihakim and Pholpark, 1991; Harrison *et al*., 1996). Most authors recognized the inadequacy of their estimates but lacked the information to make more complete assessments. Vassilev and Jooste (1991), on the other hand, in the most comprehensive study of its type with *F. gigantica* so far, estimated the benefit risk–cost ratios per cow unit in Zimbabwe based on use of one or two anthelmintic doses. They took into account the anticipated reduction in mortality and liver condemnation, improved weight gain and reproductive performance, and the anticipated improvement in feed conversion efficiency and milk yield. Their assessment also included the influence of quality of the diet and the presence of concurrent infection with gastrointestinal nematodes. Where information for *F. gigantica* was lacking, estimates of loss were taken from studies on *F. hepatica*. Annual loss from infection with *F. gigantica* per cow unit was estimated to vary from Z$30 to Z$84, Z$44 to Z$119 and Z$79 to Z$179 for animals on high, medium and low planes of nutrition, respectively, and from Z$30 for animals infected only with *F. gigantica* to Z$72 for those with *F. gigantica* and nematodes. These figures are roughly comparable to estimates of annual loss in cattle infected with *F. hepatica* in Florida of US$112 by Simpson *et al*. (1985) and in Germany of DM300 to 400 (Oostendorp and Over, 1985, cited by Vassilev and Jooste, 1991).

It should be kept in mind, however, that the estimated cost of infection with *F. gigantica* of Vassilev and Jooste (1991) has been calculated for farming and economic conditions which prevail in Zimbabwe and does not necessarily apply in circumstances where these conditions differ. Their calculations were also based largely on extrapolation of effects attributed to infection with *F. hepatica* even though there is little evidence to support this. In most areas where *F. hepatica* is endemic animals are kept for profit, measurable in terms of output of meat, fibre, milk or reproductive efficiency. Increased output of these productive indices as the result of control is readily compared with the cost of control to justify its use. In contrast, in many countries where *F. gigantica* is endemic, animals are kept primarily for reasons other than direct profit such as to give social status to their owner, to produce dung for fuel or fertilizer, to provide draught power, or to sell at times when money is required for special or unforeseen events. Consequently, it should not be assumed that the costs and benefits of treatment can be calculated in the same way for *F. gigantica* as they are for *F. hepatica*. Moreover, the perceived importance of derived benefits and the basis for their calculation are likely to differ from place to place.

Global economic impact of tropical fasciolosis

The global economic losses due to fasciolosis are difficult to estimate due to the uncertainties in the total number of animals infected and the relative intensity of infection. It is possible that the true prevalence of fasciolosis in tropical countries is underestimated since serological tests, which have been developed to date for *F. hepatica* in developed countries to diagnose infection (e.g. Yamasaki *et al.*, 1989; Dumenigo *et al.*, 1996; Hillyer, 1997), have not yet been widely applied in the field (Fagbemi *et al.*, 1997; Guobadia and Fagbemi, 1997; Viyanant *et al.*, 1997). The reported prevalence of *F. gigantica* in several countries and regions is shown in Table 15.1 together with published estimates of economic loss where available. It is clear that fasciolosis is widespread, endemic in many countries and causes significant losses to agricultural producers and small farmholders.

In Indonesia, liver fluke infection of cattle and buffalo is one of the most important parasitic diseases (Partoutomo *et al.*, 1985) and the impact of fasciolosis in Indonesia has been estimated in several studies. Surveys of the incidence of liver fluke in Indonesia have reported prevalence rates of 25–90% in cattle and buffalo (Edney and Muchlis, 1962; Soesetya, 1975; Beriajaya and Soetedjo, 1979). Estimates of the annual economic losses due to fasciolosis in Indonesia have been made by several authors (Table 15.1). The figure of A$96 million (US$65 million) (Winrock Report, 1986) only accounted for the cost of lost meat production. The total annual cost of lost meat production, lost draught power and reduced fertility in infected cattle/buffalo has subsequently been assessed at A$158 million (US$107 million) (D.B. Copeman, Townsville, 1997, personal communication). This represents an annual loss per animal of A$63 (US$42).

The losses in other countries in Asia can be estimated since the farming practices in countries such as Cambodia, Vietnam and the Philippines are similar to Indonesia and prevalence rates for fasciolosis are comparable. In 1997 the size of the cattle/buffalo herd in Asia alone was estimated at 589 million (FAO, 1997) with the Indonesian herd (15 million) representing 2.5% of the estimated numbers in Asia. Using a conservative scenario with prevalence estimates of 10% and loss per infected animal of US$42 (D.B. Copeman, Townsville, 1997, personal communication), economic losses in cattle and buffalo alone exceed US$2.4 billion in Asia. Similar calculations for the African cattle herd of 201 million animals (FAO, 1997), where prevalence rates are similar to Asia, predict losses at US$0.84 billion bringing total world losses to at least US$3.2 billion. The worldwide annual loss due to fasciolosis is a substantial figure by any valuation and possibly greater than the earlier estimates of US$2 billion by Boray (1985). These figures highlight the nature of the problem caused by chronic fasciolosis.

Control

Introduction

Most textbook recommendations for control of *F. gigantica* are vague generalizations based on methods applied in temperate countries to control *F.*

hepatica. Words such as strategic anthelmintic treatment, grazing management, application of molluscicide, and fencing off or draining swampy areas are used. However, their relevance to control of *F. gigantica* is limited by the paucity of epidemiological information about *F. gigantica* on which application of such strategies is based, the restricted economic and agricultural options available to most farmers in areas where infection with *F. gigantica* is endemic and the lack of information about the benefits of implementing measures for control. Another shortcoming with such recommendations is their failure to distinguish between methods appropriate for animals raised in pastoral areas or in areas where irrigated rice is cultivated intensively. This distinction is necessary as they represent the two broad farming systems where most infection with *F. gigantica* occurs. The determinants of infection with *F. gigantica*, management of animals, economic constraints and social practices in these two broad ecosystems are sufficiently different that methods of control appropriate for one do not necessarily apply in the other.

Most recommendations for control of *F. gigantica* and *F. hepatica* in the literature are based on chemotherapy; however, whereas anthelmintic is commonly the mainstay in programmes to control *F. hepatica*, it is not widely used against *F. gigantica*. Ideally, control measures for *F. gigantica* should be low cost, readily available and applicable with little disruption to existing agricultural practices. A number of strategies such as use of molluscicides, feeding management and other means of biological control offer alternatives to, or may be used in conjunction with, anthelmintics to achieve control but they require knowledge of the farming system and when and where animals are becoming infected, for their implementation. The absence of this information in most areas where *F. gigantica* is endemic may be one reason so little control is practised. The widespread adoption of measures to control infection with *F. gigantica* will, however, ultimately depend on demonstration to farmers that what they regard as benefits from control justify the costs of implementing them. This information also is lacking.

Chemotherapy

Most reports are of studies designed to determine the efficacy of a particular drug against *F. gigantica* rather than strategies for its use in the field. They show that drugs effective against *F. hepatica* have similar activity against *F. gigantica* (see Table 15.2). Because of their relatively low therapeutic index in comparison with more modern alternatives, continuing use of carbon tetrachloride, tetrachlorodifluroethane, hetol and hexachloroethane as treatment for fasciolosis can no longer be justified. All of the tabulated compounds have high efficacy against adult flukes, whereas only triclabendazole and clorsulon also have high efficacy against mature and immature *F. gigantica*. It should also be noted that the dose rate of triclabendazole required for buffalo is double that recommended for cattle (Estuningsih *et al.*, 1990).

A few authors have made recommendations for frequency and timing of drug treatment based on knowledge of when animals and snails become infected. A general strategy for control using anthelmintic was proposed by

Table 15.2. Efficacy of drugs against *Fasciola gigantica*.

Drug	Host	Dose	Efficacy	Reference
Albendazole	Buffalo	2.4 g per animal	Zero epg 28 dpt	Swarup and Pachauri (1986)
	Buffalo	15 mg kg^{-1} po	97% reduction in epg	Misra *et al.* (1989)
	Cattle	15 mg kg^{-1} po	96% reduction in epg	
	Goat	7.5 mg kg^{-1} po	96% reduction in epg	
Bilevon M	Sheep	4 or 6 mg kg^{-1} po	No effect 2 or 5 wpi	Hildebrandt (1968b)
	Sheep	6 mg kg^{-1} po	82%, 100% at 8, 10 wpi respectively	
Bithionol SO_4	Sheep	80 mg kg^{-1} po	Effective	Chompoochan *et al.* (1996)
Clorsulon	Cattle	2 mg kg^{-1} po	100% against adults; 92% against immatures	Robin *et al.* (1986)
Closantel	Goat	10 mg kg^{-1} po	63% at 6 wpi	Yadav *et al.* (1995)
		15 mg kg^{-1} po	75% at 6 wpi	
		20 mg kg^{-1} po	100% at 6 wpi	
	Goat	20 mg kg^{-1} po	85% at 4 wpi 96% at 6 wpi	Gupta and Yadav (1994)
Hexachlorophene	Sheep, goat	30–50 mg kg^{-1} po	100% >40 dpi (toxic at 40 mg kg^{-1})	Guralp *et al.* (1964)
	Cattle, buffalo	25 mg kg^{-1} po	100% >40 dpi	
Hilomid	Sheep	60 mg kg^{-1} po	39%, 92%, 100%,99%, 100% at 6, 8, 10, 12 or 16 wpi respectively	Hildebrandt (1968a)
	Sheep	30 mg kg^{-1} po	79%, 99%, 100% at 10, 12, 16 wpi respectively	Hildebrandt (1968a)
Niclofolan	Buffalo	0.8 mg kg^{-1} sc	Effective	Srikitjakarn *et al.* (1988)
	Sheep	2 mg kg^{-1} im	Effective	Ali *et al.* (1985)
		10.5 mg kg^{-1} po	Ineffective	
Nitroxynil	Sheep, cattle, buffalo	10 mg kg^{-1} sc	100% at >6 wpi	Roy and Reddy (1969)
Oxyclozanide	Sheep	15 mg kg^{-1} po	11%, 20.8, 98.7% at 8, 11, 16 wpi respectively	Hildebrandt and Ilmolelian (1968)
		60 mg kg^{-1} po	87.5%, 100%, 100% at 8, 11, 16 wpi respectively	
	Cattle, buffalo	10 mg kg^{-1} po	100%	Ratnaparkhi *et al.* (1993)
	Goat	15 mg kg^{-1} po	100%	
Rafoxanide	Sheep	5 mg kg^{-1} po	> 95% at 75 dpi	Horak *et al.* (1972)
		7.5 mg kg^{-1} po	>90% at 50 dpi	
		15 mg kg^{-1} po	64% at 25 dpi	
Triclabendazole	Buffalo	1.5 mg kg^{-1} day^{-1} ir	Effective	Sanyal and Gupta (1996a)
		12 mg kg^{-1} po	Ineffective at 2, 6 and 10 wpi	Estuningsih *et al.* (1990)
		24 mg kg^{-1} po	100% at 2 and 10 wpi	Sanyal and Gupta (1996b)
		24 mg kg^{-1} po	100%	Mahato *et al.* (1994)
		12 mg kg^{-1} ir	8–9% at 2 and 10 wpi	Sanyal (1996)
	Cattle	12 mg kg^{-1} ir	100% at 2 and 10 wpi	Suhardono *et al.* (1991)

dpi = days post-infection; dpt = days post-treatment; epg = eggs per gram of faeces; im = by intramuscular injection; ir = by intraruminal injection; po = orally; sc = by subcutaneous injection; wpi = weeks post-infection; wpt = weeks post-treatment.

Boray (1991). He recommended treatment at the end of a period of ecologically reduced activity of the parasite and snail intermediate host, followed by treatment 1 to 2 months after the expected peak of infection in hosts, and an additional treatment in heavily infected areas or where infection may be acquired throughout the year. While this strategy is theoretically sound, it is unlikely to be widely adopted due to the high cost of anthelmintic; moreover, in most areas, lack of information about when animals become infected and benefits likely to be derived from treatment will also mediate against its use.

Where the source of infection is herbage and water at the fringes of rivers and streams in pastoral areas, most authors in the northern hemisphere have recommended anthelmintic treatment about September and again in February to treat infection acquired from about July to December (Schillhorn van Veen, 1980; Morel and Mahato, 1987; Srikitjakarn *et al*., 1988; Rai *et al*., 1996). Guralp *et al.* (1964), on the other hand, recommended treatment each 40 days from mid-September to late February in Turkey and Srihakim and Pholpark (1991) in Thailand recommended that all cattle and buffalo older than 8 months be treated in September and again in April in areas with high prevalence, especially stock in poor condition. To achieve control in a similar habitat in the southern hemisphere (Malawi) Mzembe and Chaudhry (1981) recommended treatment in January, April and September. As with the recommendation of Boray (1991) such strategies may be effective but are not widely used, possibly because of the cost but also due to the lack of information on benefits which would justify the outlay for anthelmintic.

Recommendations made for pastoral areas do not apply to agricultural areas where the main source of infection is residual water and vegetation in irrigated rice fields and fresh rice stalks after harvest. Where anthelmintic is used for control in rice-growing areas, it would seem logical to treat animals with anthelmintic about 3 months prior to planting the rice crop so that stored and fresh faeces, used as fertilizer in the young crop, would be free from eggs of *F. gigantica* and thus the snails would not become infected. Animals grazing the stubble after harvest would then not be exposed to reinfection. On the other hand, the advice of Traore (1989) to treat animals before grazing rice stubble does not seem logical as it will not affect the rate of acquisition of new infection by grazing stock. A better effect might be anticipated with treatment 6 weeks after harvest if the field remains dry (this is sufficient time for metacercariae on stubble to die) using triclabendazole or clorsulon as they are effective against immature and mature flukes. This strategy was followed in West Java where infection is largely confined to the period from January to June when rice from the two annual crops is harvested. Suhardono, J.A. Roberts and D.B. Copeman (Bogor, 1996, unpublished data) achieved good control with a single treatment with triclabendazole in July, applied 6 weeks after the last of the seasonal rice harvest. Had only an adulticide been available for use, it would have been necessary to treat in July and September to achieve a similar level of control. The success of this strategy for a single treatment with triclabendazole in July also relied on there being a period of no or little natural transmission during the following few months, enabling the generation of snails in the first

seasonal rice crop, planted from September onwards, to remain virtually free from infection and thus break the annual cycle of transmission. A high level of control was achieved, with more than 80% of animals still with no detectable fluke eggs in their faeces 12 months after treatment (Suhardono, J.A. Roberts and D.B. Copeman, Bogor, 1996, unpublished data).

In areas where irrigation allows continuous or asynchronous cropping of rice throughout the year, there would be the opportunity for continuous exposure of animals to infection with *F. gigantica*. In such situations Griffiths (1967) and Tongson (1978) recommended anthelmintic treatment each 3 months as the means of control. However, it should be possible to extend this period to 4 months if all farmers who share grazing on their newly harvested rice fields treat their animals with an anthelmintic which is effective against immature and adult flukes. Such a programme would ensure that faeces of animals would remain virtually free from fluke eggs (due to the 14 to 16 weeks prepatent period of *F. gigantica*) with the consequence that snails would remain fluke free and the rice crops would not be infective for grazing stock. The high cost of anthelmintic and cooperative effort required make it unlikely that such a programme would be sustainable over many years. However, even if maintained for one year, it should substantially reduce the level of infection in the group of participating animals to low levels which could then be maintained with grazing management or using other biological control methods.

It is apparent that the strategic use of anthelmintic has the potential to provide an effective means of control of infection with *F. gigantica* in both pastoral and agricultural settings. Furthermore, the minimal usage of anthelmintics against *F. gigantica* to date has ensured that their efficacy has remained high with no reports of development of resistant strains. Anthelmintics are, however, unlikely to achieve a high degree of acceptance in control of *F. gigantica* until more information is available of the costs and relevant benefits associated with their use. Tailoring of rational regional strategies for control is also dependent on there being sufficient knowledge of the pertaining agricultural cycle, social structures and determinants of infection with *F. gigantica*. This information is rarely available.

Alternatives to anthelmintics

The literature on alternatives or adjuncts to anthelmintics for control of *F. gigantica* has been reviewed by Roberts and Suhardono (1996). The available information is scanty despite the desirability for development of such options. Published recommendations include: use of grazing management (Schillhorn van Veen, 1980); molluscicides (Dinnik and Dinnik, 1963; Preston and Castelino, 1977; Mzembe and Chaudhry, 1981; Harrison *et al.*, 1996); predator/competitor snails (Nguma *et al.*, 1982); predation with fish (Gupta *et al.*, 1986) or ducks (Touratier, 1988; Rai *et al.*, 1996); and breeding resistant livestock (Roberts *et al.*, 1997b). However, none has been widely adopted for control.

Molluscicides have mainly been used or recommended for use in dams to control the snails which are intermediate hosts of *F. gigantica*, perhaps

because the more extensive habitats such as rivers and irrigated rice fields make the cost prohibitive and also because of the adverse effects of some molluscicides on non-target animals and plants in the habitat. Dinnik and Dinnik (1963) recommended use of molluscicide in highland areas of Kenya at intervals less than the minimum period for development of egg to cercaria (69 to more than 100 days, depending on the temperature). A strategic approach was also recommended by Mzembe and Chaudhry (1981), with application of molluscicide just before cercarial shedding commenced in June, and repeated in September. Preston and Castelino (1977), on the other hand, used aerial and hand spraying with molluscicide to eliminate snails from a dam and achieve control of fasciolosis over a period of 2 years. The unpublished finding by Suhardono, J.A. Roberts and D.B. Copeman (Bogor, 1996, unpublished data) that, in irrigated rice fields, snails which have the highest prevalence of infection with *F. gigantica* are those in fields adjacent to a cattle pen or village, opens the possibility for selective use of molluscicide in such areas. Two applications, 5 and 10 weeks after the rice crop is planted, should have the effect of preventing cercarial shedding in lowland tropical areas, whereas only one treatment 7 weeks after planting may be effective in cooler regions where larval development in the snail is slower. Another possibility that has not been trialled, which may be more applicable in pastoral areas than rice fields, utilizes the molluscicidal properties of eucalyptus leaves (Harrison *et al.*, 1996). These authors postulated that the leaves falling from such trees growing around the periphery of habitats of snails might provide a sustainable means of snail control.

In areas where the main source of infection is irrigated rice fields after harvest, feeding management to deny stock access to viable metacercariae and biological control of infection with *F. gigantica* in snails are particularly relevant strategies for control. The effectiveness of storage of rice stalks as hay at room temperature in Indonesia (about 28°C) for 1 month to kill metacercariae was confirmed by Suhardono, J.A. Roberts and D.B. Copeman (Bogor, 1996, unpublished data) who also showed that metacercariae were killed by exposure to sunshine for 8 h. The bottom third of rice stalks (the portion previously immersed where metacercariae encyst) can thus be made safe to eat by exposure to sunlight; infection can be avoided by feeding animals only the top two-thirds of the stalk which Suhardono, J.A. Roberts and D.B. Copeman (Bogor, 1996, unpublished data) showed was safe to use as fresh fodder. Another recommendation from this work was that high-risk rice fields adjacent to villages or cattle pens should not be grazed until they have been dry for at least 6 weeks after harvest, by which time most metacercariae should be dead.

The possibility of successful biological control of fasciolosis by echinostome flukes was also demonstrated by Suhardono, J.A. Roberts and D.B. Copeman (Bogor, 1996, unpublished data). The ability of larval echinostomes to aggressively displace other larval flukes from their snail hosts and parasitic castration of snails by larval echinostomes is well documented (Lie *et al.*, 1966, 1973; Kwo *et al.*, 1970; Lie, 1973; Estuningsih, 1991). However, previous workers were unable to devise a practical

method of applying this concept for control of *F. gigantica* in the field. This was achieved by Suhardono, J.A. Roberts and D.B. Copeman (Bogor, 1996, unpublished data) by adding faeces from five to ten ducks naturally infected with *Echinostoma revolutum* to bovine faeces used as fertilizer in rice fields, or by locating the duck pen over the effluent drain from a cattle pen before it entered an adjacent field. To maximize the competition between miracidia of *F. gigantica* and *E. revolutum* for snails it is important to ensure the duck and bovine faeces enter the rice field at the same time and place. This strategy was found to almost eliminate metacercariae from rice fields close to cattle pens or villages that would otherwise constitute the greatest potential source of infection for stock. However, there has been resistance in West Java to application of this novel means of control because village ducks may be concurrently infected with schistosomes, the cercariae of which cause dermatitis when they penetrate the skin of rice-field workers.

Free-ranging ducks or geese which eat snails have also been proposed as a possible means for biological control of *F. gigantica* (Touratier, 1988; Rai *et al.*, 1996) but the degree to which control is likely to be achieved has not been measured. Effective control would require that ducks were present in sufficient numbers to eat most snails in a habitat before they shed cercariae. This may be achievable along limited stretches of the shore of lakes and streams where stock drink but large numbers of birds are unlikely to be acceptable in irrigated rice fields prior to harvest. Furthermore, the common practice of allowing large flocks of ducks to glean recently harvested rice fields (and eat the snails) is not likely to reduce the availability of metacercariae for animals which graze the stubble as they encyst prior to harvest.

Immunology of *Fasciola gigantica* Infection

Cattle/goats

Acquired resistance to a secondary *F. gigantica* infection following a primary infection or vaccination has been demonstrated in cattle, goats and sheep (reviewed in Haroun and Hillyer, 1986). In cattle, using irradiated metacercariae as the immunizing vaccine, Bitakaramire (1973a) reported a 98% reduction in worm burdens in vaccinated calves. Younis *et al.* (1986), using a range of immunizing regimes, showed that vaccination of zebu calves with irradiated metacercariae reduced worm burdens by 45–68%. In goats, vaccination with a primary exposure to irradiated metacercariae reduces fluke burdens by 43% (El Sanhouri *et al.*, 1987) and 82–85% (Yadav and Gupta, 1989). Protection levels of 56% and 53% were observed in goats vaccinated by one or two mature rafoxanide-abbreviated infections, respectively (Haroun *et al.*, 1989); a non-significant 45% reduction in worm numbers was reported in cattle similarly sensitized (Haroun *et al.*, 1988). Such observations suggest that ruminants are capable of mounting immune responses which can kill *F. gigantica*.

Sheep

It is well established that sheep do not acquire resistance to *F. hepatica* as determined from the observed yields of mature parasites after primary and secondary infections with *F. hepatica* (Table 15.3; reviewed in Boray, 1969; Rickard and Howell, 1982; Haroun and Hillyer, 1986; Boyce *et al.*, 1987). In European sheep, yields of *F. hepatica* ranged from 16 to 38% after primary infection, and from 13 to 31% after secondary infection, indicating that resistance to *F. hepatica* does not develop in these sheep breeds (Boyce *et al.*, 1987). In contrast, acquired resistance to *F. gigantica* has been observed in sheep. A'Gadir *et al.* (1987) reported a significant reduction in parasite numbers in Sudanese desert sheep vaccinated with irradiated metacercariae of *F. gigantica* where the recovery of adult parasites was reduced from 17% in control animals to 3.4% in the vaccinates. Wiedosari and Copeman (1990) showed that Indonesian Thin Tail (ITT) sheep were highly resistant to *F. gigantica* based on a comparison of the relative yield of parasites from a primary infection, compared with the yields published in other studies with this parasite in other breeds of sheep. The nature of the high resistance expressed by ITT sheep to *F. gigantica* has recently been assessed.

Indonesian Thin Tail sheep acquire high resistance to *F. gigantica*

Wiedosari and Copeman (1990) originally reported that recoveries of flukes in ITT sheep at 8–16 weeks after a primary infection with *F. gigantica* ranged from 0 to 11% with a mean recovery of only 2.4%. Recently, Roberts *et al.* (1997a) confirmed these observations and showed that, in naive ITT sheep challenged with *F. gigantica*, the recovery of adult parasites at 21 weeks post-infection was 1.1% of the infective dose in a primary infection and was significantly lower (0.32%, $P < 0.05$) in a secondary infection in previously exposed ITT sheep. Analysis of body weight and blood packed cell volume data from infected sheep suggested that the resistance of ITT sheep was expressed within 8 weeks of infection (Roberts *et al.*, 1997a). In a second experiment in naive ITT sheep similar low recoveries (1.4%) of parasites were observed (Roberts *et al.*, 1997b). These studies show that ITT sheep are highly resistant to a primary infection with *F. gigantica* and acquire a further significant level of resistance following a secondary challenge.

A subsequent study of the dynamics of the early phase of *F. gigantica* infection in ITT and Merino sheep was performed in an attempt to determine the time period after challenge at which killing of parasites occurs (Roberts *et al.*, 1997c). At 3–4 weeks post-infection, the plasma levels of GLDH (a marker for damage to liver parenchyma cells) were elevated in both naive ITT and Merino sheep whereas GLDH levels were low in the exposed ITT sheep undergoing a secondary challenge. These observations suggested that significant numbers of parasites were invading the liver in naive ITT sheep but that significantly fewer parasites were reaching the liver in the exposed ITT sheep. When parasites were recovered at 3–4 weeks post-challenge from livers of ITT or Merino sheep the number of parasites was similar to that recovered at 6 weeks after challenge: this suggests that killing of most of the

Table 15.3. Recoveries of flukes in different sheep breeds infected with *Fasciola gigantica* or *Fasciola hepatica*.

	F. gigantica[a]		*F. hepatica*[a]	
Breed	Primary	Secondary	Primary	Secondary
ITT	0.8–5%[b]	0.32–1.1%[c]	31%[d]	39%[d]
Indonesian Fat Tail[e]	6.9–9.7%	nt	nt	nt
Sudanese Desert	5%[f], 17%[g]	3.4%[g]	nt	nt
Awassi[h]	16–21%	nt	nt	nt
W. African Dwarf[i]	6–21%	nt	nt	nt
Dorper[j]	24%	nt	nt	nt
Merino	15–25%[k]	12%[k]	23–53%[j,l]	23–53%[l]
Black-headed Persian[m]	31%	nt	nt	nt
S. African Mutton Merino[n]	48%	nt	nt	nt
Merino/Corriedale[o]	62%	nt	nt	nt
St Croix	6.3, 6.7%[p]	nt	16%[q]	13.7%[q]
Finn/Rambouillet[q]	nt	nt	24%	31%
Florida Native[q]	nt	nt	27%	16%
Barbados Blackbelly[q]	nt	nt	38%	30%
Clun[r]	nt	nt	57%	nt

nt; not tested.
[a] mean % recoveries of flukes.
[b] Wiedosari and Copeman (1990); Roberts *et al.* (1997a,b,c); T.W. Spithill, S.E. Estuningsih, S. Widjajanti and S. Partoutomo, Bogor, 1997, unpublished data; flukes recovered at >6 weeks post-infection.
[c] Roberts *et al.* (1997a,c).
[d] Roberts *et al.* (1997a).
[e] Roberts *et al.* (1997c); T.W. Spithill, S.E. Estuningsih, S. Widjajanti and S. Partoutomo, Bogor, 1997, unpublished data.
[f] Ali *et al.* (1985).
[g] A'Gadir *et al.* (1987).
[h] Abbas *et al.* (1986).
[i] Ogunrinade (1984).
[j] Horak *et al.* (1972).
[k] Roberts *et al.* (1996, 1997c).
[l] Boray (1969); Sexton *et al.* (1990); Wijffels *et al.* (1994b); Creaney *et al.* (1996).
[m] Hildebrandt (1968b).
[n] Swan *et al.* (1984).
[o] Hammond (1973).
[p] Roberts *et al.*, (1997a,b).
[q] Boyce *et al.*, (1987).
[r] Sinclair (1968).

parasites had occurred and was completed within 3–4 weeks of infection. Only 3% and 0.5% of parasites were present at week 6 post-challenge in livers of ITT sheep undergoing a primary or secondary challenge, respectively (Table 15.3) (Roberts *et al.*, 1997c). These results suggest that naive ITT sheep have an innate (or rapidly acquired) capacity to resist a primary *F. gigantica* infection, within 3–4 weeks of challenge, and that ITT sheep acquire a further level of resistance after exposure.

In order to determine whether the resistance expressed by ITT sheep was immunologically based, the effect of the immunosuppressive drug

dexamethasone on the resistance of ITT sheep previously exposed to *F. gigantica* was assessed (Roberts *et al.*, 1997c). Four groups of ITT sheep were treated with dexamethasone beginning 2 days before infection or at 3, 6 and 12 days post-infection, respectively, and continuing until the sheep were killed at 72 days post-infection. This protocol was designed to test whether significant killing of parasites occurs within a few days of infection, as seen in rats previously exposed to *F. hepatica* (Hayes and Mitrovic, 1977). Dexamethasone treatment substantially reduced the resistance of ITT sheep with the parasite burdens being significantly elevated in the treated sheep (6.4–13.8% recovery) relative to the burdens recovered from untreated control sheep (1.1% recovery) (Roberts *et al.*, 1997c). The recovery of parasites from sheep immunosuppressed from day 12 post-infection (7.4%) was similar to that recovered from susceptible Indonesian Fat Tail sheep (9.7%); this suggests that a large number of parasites had survived in the ITT sheep for at least 12 days after infection and were rescued by the immunosuppression. The surviving parasites at day 12 were eventually killed by day 72 post-infection, as evidenced by the reduction in worm numbers recovered from control ITT sheep. This result suggests that significant parasite attrition is occurring after 2 weeks of infection. Together with the parasite recovery data which suggest that most killing takes place by 4 weeks post-infection, it would appear that there is a window for killing of significant numbers of immature *F. gigantica* between 2 and 4 weeks post-infection in exposed ITT sheep (Roberts *et al.*, 1997c). The resistance expressed by ITT sheep to *F. gigantica* appears to differ from that expressed by rats to *F. hepatica* since, in the rat model, resistance is expressed within 24 h of infection in rats previously exposed to *F. hepatica* (Hayes and Mitrovic, 1977).

Roberts *et al*. (1997a) also described results which suggested that some adult parasites were eliminated in ITT sheep later in infection, between 15 and 21 weeks post-challenge. Thus, resistance in ITT sheep may involve two phases of parasite attrition which may reflect the action of two separate immune effector responses.

The acquisition of resistance to *F. gigantica* infection has been extended to European sheep. Merino sheep exposed to a secondary infection with *F. gigantica* showed a significant level of resistance to reinfection with the recovery of flukes in a primary infection (25%) being reduced to 12% in the exposed sheep (Table 15.3) (Roberts *et al.*, 1996). In contrast, the resistance of ITT sheep to *F. gigantica* does *not* extend to *F. hepatica* since ITT sheep previously exposed to a primary *F. hepatica* infection showed no resistance to a secondary *F. hepatica* challenge (Roberts *et al.*, 1997a). ITT and Merino sheep are thus both competent to mount a protective acquired immune response against *F. gigantica*, but not *F. hepatica*, although ITT sheep express a much higher level of resistance to *F. gigantica* than the Merino breed.

The recovery of *F. gigantica* and *F. hepatica* in various breeds of sheep is summarized in Table 15.3. These recovery data are reported by different laboratories which have usually recovered parasites from a single sheep breed following infection with different strains of *F. gigantica* metacercariae.

Since the viability of the metacercariae used to produce the infection is not known, it is difficult to quantitatively compare yields of parasites in different laboratories. The data in Table 15.3 should be seen as a comparative indicator of the relative susceptibility of breeds to *F. gigantica* only, except in these reports which have directly compared sheep breeds (Boyce *et al.*, 1987; Roberts *et al*, 1996, 1997a,b,c).

The results clearly indicate that the behaviour of the two *Fasciola* species in various sheep breeds is different, raising the important notion that these two species differ in some critical, probably biochemical, way. Differences in infectivity between species of *Fasciola* have also been reported in rats where the recovery of *F. hepatica* (20–30%) and Japanese *Fasciola* spp. (36–47%) is greater than the recovery of *F. gigantica* (0–5%) (Itagaki *et al.*, 1994). Mango *et al.* (1972) and Gupta and Chandra (1987) also observed low recoveries of *F. gigantica* from rats. These results suggest that the infectivity of *F. gigantica* for a number of animal hosts is diminished relative to *F. hepatica* but the biochemical basis for this difference in virulence is not yet established (see below).

Mechanisms of resistance to *F. gigantica* in ITT sheep

The nature of the mechanisms of resistance operating in ITT sheep early post-infection remains to be determined. The major component of the resistance in ITT sheep is either *innate* (i.e. expressed in naive animals on first contact with the parasite) or *rapidly acquired* and expressed within a few weeks of infection. Innate resistance could result from *non-immune* characteristics expressed by the host (i.e. an unusual physiology, biochemistry or anatomy) (Wakelin, 1992) or from expression of an *innate immunity*. Innate immunity involves proteins encoded by the germ line (such as the macrophage mannose receptor and lipopolysaccharide (LPS) receptor, the lectin-like receptor on natural killer (NK) cells and complement) which usually recognize carbohydrate structures such as LPS or microbial cell wall saccharides (Fearon and Locksley, 1996). *Acquired immunity* involves B and T cells which express an antigen receptor which has arisen by somatic gene rearrangement. The innate immune system plays a key role in controlling the nature of the subsequent acquired immune response which is mediated by B and T cells. It is now clear that the innate system controls not only the initiation but also the type of immune response (Th1 or Th2) that is activated by regulating the expression of co-stimulatory activity on antigen presenting cells and instructing the acquired immune system to release effector cytokines (Bendelac and Fearon, 1997; Medzhitov and Janeway, 1997).

INNATE RESISTANCE. Innate resistance could result from several mechanisms as discussed by Wakelin (1992) but two possibilities are evident:

1. *Immune response*. ITT sheep control infection by constitutive expression of an innate immune response that debilitates the parasite or leads to a rapidly induced effector response. Naive ITT sheep must express the innate immune response against the juvenile/immature fluke since parasite attrition

occurs within 3–4 weeks of infection (Roberts *et al*., 1997c). This innate response could involve the activation of macrophages, NK cells or complement that recognize particular carbohydrate signals released by juvenile *F. gigantica* and subsequently directly debilitate the parasite via inflammatory responses or complement activity. Inflammatory responses have been shown to play a role in the resistance of rats to infection with *F. hepatica* (Hayes and Mitrovic, 1977; Baeza *et al*., 1994a,b) and activated macrophages have been shown to kill juvenile *F. hepatica in vitro* (Piedrafita, 1995; D. Piedrafita, T.W. Spithill, R.M. Sandeman, P.R. Wood, S.E. Estuningsih, S. Partoutomo, J.C. Parsons, Melbourne, 1998, unpublished data). However, no binding of complement C3 was found on the surface of *F. hepatica* larvae recovered from rats (Davies and Goose, 1981) and bovine complement does not promote damage to juvenile *F. hepatica* (Duffus and Franks, 1980); the role of complement in the resistance to *F. gigantica* expressed by ITT sheep remains to be determined.

The innate cytokine response occurring at the outset of infection is a critical component of the subsequent acquired immune response which can control the production of cytokines in the microenvironment of the responding lymph node (Reiner, 1994; Kaufmann, 1995; Abbas *et al*., 1996; Fearon and Locksley, 1996). For example, genetically determined differences in innate IL-4 production or IL-12 responsiveness by T cells would dramatically alter the cascade which leads to the acquired Th1/Th2 response (Pond *et al.*, 1992; Abbas *et al*., 1996). A study of cytokine expression in tissues of ITT and susceptible sheep during *F. gigantica* infection will help unravel the role of the innate cytokine response in the early immune responses in ITT sheep.

2. *Non-immune response*. ITT sheep express some physiological (i.e. non-immunological) barrier, for example at the level of vascularity or gut physiology, which inhibits migration of the juvenile flukes. Ford *et al*. (1987) described shunting of microspheres from the portal system to the systemic circulation in *F. hepatica*-infected rats, suggesting that physiological changes occur in animals infected with *Fasciola*. However, as discussed below, evidence from our studies suggests that the major component of the resistance in ITT sheep is immunologically based.

ACQUIRED RESISTANCE. Acquired resistance could result from an immune response, the induction and expression of which occurs within a few days or weeks following infection. It is clear that there are genetically determined differences between animals in the rate of induction of protective immune responses to parasite infection (Wakelin, 1992; Gray and Gill, 1993). For example, rapidly induced immune responses have been demonstrated to play a role in determining the resistance of different mouse strains to infection with *Taenia taeniaeformis* (Mitchell *et al*., 1980). Resistance to *Trichinella spiralis* is correlated with the early activation of IFN-γ secreting T cells and poor activation of IL-4/IL-5 secreting T cells (Pond *et al*., 1992).

The nature of the immune responses which may play a role in immune elimination of *F. gigantica* in ITT sheep is under study. *In vitro* studies have shown that *F. gigantica* larvae are susceptible to antibody-dependent cellular

cytotoxicity (ADCC) involving LPS-stimulated peritoneal lavage cells (predominantly macrophages) from ITT sheep and antibodies in sera collected at 7–8 weeks post-infection with *F. gigantica* (T.W. Spithill, D. Piedrafita, S, Partoutomo, D. Clery, S.E. Estuningsih, Bogor, 1998, unpublished data). Killing is not mediated by nitric oxide since ITT lavage cells do not produce detectable levels of nitrite *in vitro* in response to LPS stimulation and the killing is not inhibited by the addition of the nitric oxide synthase inhibitor L-NMMA. Moreover, larvae of *F. hepatica* incubated under identical conditions are resistant to ADCC killing, suggesting that larvae of these two species of *Fasciola* differ in some fundamental trait. These observations are consistent with the higher resistance of *F. hepatica* to killing by ITT sheep *in vivo* (Roberts *et al.*, 1997a).

Recent studies in our laboratory have analysed the isotype of the antibody responses in naive ITT and Merino sheep infected with *F. gigantica* (D. Hansen, D. Clery, S. Widjajanti, S.E. Estuningsih, S. Partoutomo, T.W. Spithill, Bogor, 1998, unpublished data). Specific IgM and IgG_1 responses were observed in ITT and Merino sheep. IgE antibodies showed a biphasic response in both ITT and Merino sheep, characterized by a first peak of antibodies 14 days post-infection, followed by a second peak of a greater magnitude between days 30 and 65. Merino sheep showed a high IgG_2 antibody response which starts to develop soon after infection and reaches a peak by day 30. In marked contrast, this IgG_2 response was virtually absent in ITT sheep. We have confirmed this observation in an analysis of ITT and St Croix sera from the trial reported by Roberts *et al.* (1997b). These results suggest that resistance to *F. gigantica* in ITT sheep may result from a dramatic downregulation of the IgG_2 response to fluke antigens.

The low expression of parasite-specific IgG_2 antibodies in infected ITT sheep has implications for the nature of effector mechanisms which can be expressed against *F. gigantica* in this breed. In sheep, macrophages have Fc receptors for IgG_1 but not IgG_2 (Watson *et al.*,1994), raising the important possibility that high IgG_2 levels could *block* IgG_1-mediated macrophage-dependent effector functions. In contrast, sheep neutrophils have Fc receptors for IgG_2 and IgM but not for IgG_1 (Watson *et al.*, 1994): our data imply that neutrophils may not play a role in any IgG_2-mediated ADCC against juvenile *F. gigantica* in ITT sheep. These observations suggest that IgG_2 could potentially act as a blocking antibody for ADCC mediated by macrophages and suggest the hypothesis that ITT sheep, by downregulating IgG_2 expression, have an enhanced capability to express macrophage-mediated ADCC reactions against *F. gigantica*.

Thus, resistance to *F. gigantica* in ITT sheep may result from the relative *absence* of a specific IgG_2 response. This is reminiscent of observations from studies analysing antibody isotype responses in humans exposed to *S. mansoni* where positive correlations were observed between intensity of reinfection and the presence of IgG_2 antibodies to carbohydrate epitopes present on antigens from both eggs and schistosomula (Butterworth *et al.*, 1987; Demeure *et al.*, 1993; Butterworth, 1994). Blocking IgG_4 and IgM antibodies have also been demonstrated in human schistosomiasis and IgG_{2_c}

blocking antibodies have been demonstrated in a rat model of *S. mansoni* (Capron *et al.*, 1987; Butterworth, 1994).

In cattle, the production of the IgG_2 isotype appears to be regulated by type 1 helper (Th1)-like cells that produce γ-interferon (Estes *et al.*, 1994). If the same is true in sheep, the downregulation of IgG_2 responses in resistant ITT sheep suggests that Th1-like responses may be suppressed in this breed during *F. gigantica* infection, raising the possibility that resistance may result from Th2-like immune responses. This contrasts with the observations in cattle (discussed below) which have suggested a role for a Th1-like response in resistance to *F. hepatica* in cattle (see also Chapter 10 of this volume). These observations suggest the hypothesis that immune resistance mechanisms against *Fasciola* differ between sheep and cattle with Th1-like responses responsible for killing of *Fasciola* in cattle and Th2-like responses important in sheep. A corollary of this hypothesis is that parasites which develop defence mechanisms against one arm of the immune effector response expressed in one animal species could evolve a specific host–parasite relationship with that host.

Four lines of evidence from our studies to date support the hypothesis that an acquired immune response is involved in determining the resistance of ITT sheep to *F. gigantica*:

1. The resistance of both naive and previously exposed ITT sheep is significantly reversed by dexamethasone immunosuppression (Roberts *et al.*, 1997c; T.W. Spithill, J.A. Roberts, S.E. Estuningsih, S. Widjajanti, S. Partoutomo, Bogor, 1996, unpublished data). These results suggest that the high resistance expressed by ITT sheep appears to involve an immune mechanism which is suppressible by corticosteroids. Corticosteroids have been used as immunosuppressants in a range of animal species, and man, and have been shown to cause a decrease in the number of circulating immune cells, suppression of inflammatory responses, decrease in T cell proliferation and a decrease in serum IgM, IgA and IgG (reviewed in Cupps and Fauci, 1982). Dexamethasone has been shown to reverse the resistance exhibited by *F. hepatica*-sensitized rats to a secondary challenge (Hayes and Mitrovic, 1977) and to suppress the resistance expressed by naive rats (Baeza *et al.*, 1994a). Dexamethasone has been previously used to suppress the resistance of sheep to *Trichostrongylus colubriformis* (Emery and McClure, 1995).

2. We have observed antibody responses to juvenile fluke antigens within 4 days of infection of naive ITT sheep and IgM, IgG_1 and IgE responses develop in ITT sheep within 2 weeks of infection (D. Clery, D. Hansen, S. Widjajanti, S.E. Estuningsih, S. Partoutomo, T.W. Spithill, Bogor, 1998, unpublished data). In addition, we have observed a significant elevation in eosinophilia in ITT sheep, relative to Merino sheep, at days 8–25 post-infection. Thus, a rapid induction of humoral and cellular responses occurs in ITT sheep coinciding with the time post-infection when parasites are killed, suggesting that these responses may play a role in the expression of resistance.

3. The observed significant differences in the level of expression of IgG_2 between ITT sheep and susceptible Merino or St Croix sheep infected with *F.*

gigantica suggest that variation in the expression of the IgG_2 isotype correlates with resistance to infection. Moreover, there is a significant *positive* correlation between IgG_2 levels and worm burdens (D. Hansen, D. Clery, S. Widjajanti, S.E. Estuningsih, S. Partoutomo, T.W. Spithill, Bogor, 1998, unpublished data). Such immunological correlations suggest a role for the acquired immune response in resistance in ITT sheep.

4. Studies *in vitro* have shown that juvenile *F. gigantica*, but not *F. hepatica*, are susceptible to ADCC by peritoneal lavage cells (PLCs) from naive ITT sheep (T.W. Spithill, D. Piedrafita, S, Partoutomo, D. Clery, S.E. Estuningsih, Bogor, 1998, unpublished data). These observations show that larvae of *F. gigantica* are susceptible to immune effector responses to which the parasite may be exposed *in vivo*. This ability of ITT cells to kill *F. gigantica*, but not *F. hepatica*, *in vitro* correlates with the ability of ITT sheep to control infection with *F. gigantica* but not *F. hepatica*, supporting a possible role for an ADCC response in controlling *F. gigantica in vivo.*

Further studies are in progress to define the immune effector responses involved in determining resistance to *F. gigantica* in ITT sheep.

Biochemical basis for differences between *F. gigantica* and *F. hepatica* in susceptibility to sheep immune responses

There are several possible explanations which may explain the inability of sheep to acquire resistance against *F. hepatica*:

1. *The acquired immune response is not induced by* F. hepatica *since the key protective antigens in* F. gigantica *are not expressed in* F. hepatica. A comparative study in *F. gigantica/F. hepatica* of antigen expression and recognition during infection in sheep is warranted in order to determine whether certain key antigens expressed by *F. gigantica* are either not expressed by *F. hepatica* or do not induce immune responses in sheep. It is feasible that during the evolution of *F. gigantica* and *F. hepatica* the expression of certain antigens has been lost in *F. hepatica* due to variation in selection pressures operating at the level of the host. A molecular approach would be to use differential display or subtractive hybridization to compare gene expression patterns in the two species to search for sequences expressed differently: we have used differential display to identify sequences which vary in abundance between immature and adult stages of *F. hepatica* (Reed *et al.*, 1998).

2. *The acquired immune response is induced following* F. hepatica *infection but is ineffective due to expression of defence mechanisms in this species which are missing or downregulated in* F. gigantica. Recent studies have shown that juvenile *F. hepatica* are susceptible to killing by nitric oxide and high levels of reactive oxygen intermediates (ROI) (Piedrafita, 1995; D. Piedrafita, T.W. Spithill, J.P. Dalton, P.J. Brindley, R.M. Sandeman, P.R. Wood, J.C. Parsons, Melbourne, 1998, unpublished data). Resistance to immune killing expressed by *F. hepatica* could result from elevated expression of oxidant scavenger enzymes which protect the parasite from free radical damage. For example, inhibition of antioxidant enzyme activity sensitizes *S. mansoni* to killing by ROI (Mkoji *et al.*, 1988a,b) and similar observations

have been made with larvae of *F. hepatica* (Piedrafita, 1995; D. Piedrafita, T.W. Spithill, J.P. Dalton, P.J. Brindley, R.M. Sandeman, P.R. Wood, J.C. Parsons, Melbourne, 1998, unpublished data). Interestingly, glutathione *S*-transferase (GST) levels are elevated in *F. hepatica* adult parasites isolated from susceptible hosts (sheep, mice) and lowered in parasites from resistant hosts (cattle, rats) (Miller *et al.*, 1993). Piedrafita (1995) observed levels of superoxide dismutase and glutathione peroxidase in juvenile *F. hepatica* which were up to tenfold higher than those reported for schistosomula (Mkoji *et al.*, 1988a; Nare *et al.*, 1990); such high levels of defence enzymes may explain the relative resistance of juvenile *F. hepatica* to killing by chemically generated ROI (Piedrafita, 1995; D. Piedrafita, T.W. Spithill, J.P. Dalton, P.J. Brindley, R.M. Sandeman, P.R. Wood, J.C. Parsons, Melbourne, 1998, unpublished data). These results show that defence enzyme expression is variable in *Fasciola* and may imply that the level of GST is one of the factors determining the ability of the parasite to survive in different hosts. A comparative study of defence enzyme expression in *F. hepatica* and *F. gigantica* would help clarify the role of these enzymes in determining the relative virulence of these species.

Cell-mediated killing of *F. hepatica* juvenile fluke *in vitro* by rat macrophages is antibody dependent and involves attachment of effector cells to the surface tegument of the larvae to deliver the lethal effector molecules (Piedrafita, 1995; D. Piedrafita, T.W. Spithill, M.R. Sandeman, P.R. Wood, S.E. Estuningsih, S. Partoutomo and J.C. Parsons., Melbourne, 1998, unpublished data). We have similarly observed that *in vitro* killing of *F. gigantica* larvae by sheep macrophages is dependent on antibody: moreover, under conditions where we observe significant killing of *F. gigantica* larvae, larvae of *F. hepatica* are unaffected (T.W. Spithill, D. Piedrafita, S, Partoutomo, D. Clery, S.E. Estuningsih, Bogor, 1998, unpublished data). These results show that the two *Fasciola* species differ in their ability to resist immune killing *in vitro*, implying that some fundamental biochemical difference(s) between the parasites is determining susceptibility to ADCC. The corollary of this hypothesis is that the biochemical differences will result in *F. gigantica* differing from *F. hepatica* in biological behaviour which may be identifiable *in vitro*. In this regard, it is of interest that we have found that, during *in vitro* culture, newly excysted juvenile flukes of *F. hepatica* are more robust than those of *F. gigantica* and can survive for a longer period in culture (T.W. Spithill, D. Piedrafita, S. Partoutomo, D. Clery, S.E. Estuningsih, Bogor, 1998, unpublished data).

Fasciola contains a surface tegument rich in carbohydrate and glycoprotein which is actively synthesized by juvenile flukes and which can be surface labelled with ^{125}I (Dalton and Joyce, 1987). Antibody and cells bound to the tegument of *F. hepatica* are rapidly shed *in vitro* with a half-life of about 1–8 h (Duffus and Franks, 1980; Hanna, 1980). Resistance of *F. hepatica* to ADCC killing could thus result from differences between *Fasciola* species in surface glycocalyx turnover which allows *F. hepatica* to shed bound antibody and immune cells and escape damage. Accordingly, it would be of interest to compare the glycocalyx turnover of juvenile larvae of *F. hepatica* and *F. gigantica*.

3. *The acquired immune response is induced following* F. hepatica *infection but is actively suppressed during infection due to the action of some factor(s) released by the parasite.* Immunosuppressive effects on T cell proliferation have been observed in cattle and sheep infected with *F. hepatica* (Zimmerman *et al.*, 1983; Oldham and Williams, 1985; Chauvin *et al.*, 1995). In sheep, T cell proliferation was suppressed in a biphasic manner at weeks 4 and 10–11 post-infection (Zimmerman *et al.*, 1983). Injection of rats with products excreted/secreted (ES) from *F. hepatica* has been shown to suppress DTH (Cervi *et al.*, 1996). ES products inhibit Con-A-induced sheep T cell proliferation, are mitogenic to sheep PBL *in vitro* and inhibit the oxidative burst by sheep neutrophils (Jeffries *et al.*, 1996, 1997). These observations show that *F. hepatica* actively suppresses immune responses during the first 12 weeks of infection and suggest that ES products may be involved in the suppression. The ability of *F. gigantica* to similarly suppress immune responses has yet to be determined; clearly, variation in immunosuppressive activity between *Fasciola* spp. could explain differences in infectivity between species.

Studies on the immune responses to *F. hepatica* infection suggest that, in cattle chronically infected with *F. hepatica*, a Th2-like response is dominant (Clery *et al.*, 1996). Brown *et al.* (1994a) have shown that, in infected cattle, antigen-specific T cell clones express a Th0- or Th2-like phenotype and Th1-like cells were not detected; Th1 cells were isolated from other genetically identical cattle infected with *Babesia bovis*, suggesting a downregulation of Th1 responses in *Fasciola*-infected cattle (Brown *et al.*, 1994b). These results suggest that induction of parasite-specific Th1-like cells is inversely correlated with chronic *F. hepatica* infection and suggest that Th2-like responses are promoted in chronic fasciolosis in cattle. Mulcahy *et al.* (1998) have recently shown that, in cattle vaccinated with cathepsin L2 and haemoglobin from *F. hepatica*, a negative correlation was observed between the IgG_2 titres elicited and fluke burden in these cattle. These observations suggest that IgG_2 antibodies may play a role in protective immune responses to *F. hepatica* in cattle and are consistent with the hypothesis that protective immune responses may be of the Th1-type. Similar studies in cattle infected with *F. gigantica* have not been performed. Since *F. hepatica* actively suppresses immune responses, it is feasible that, during *F. hepatica* infection in cattle, the parasite induces Th2-like responses at the expense of Th1-like responses and thereby escapes immune attrition. A comparative study of immunosuppressive activity of *F. gigantica* and *F. hepatica* in cattle would be informative.

Genetics of Resistance to *Fasciola gigantica* in ITT Sheep

A putative major gene for resistance to *Fasciola gigantica*

Relevant information on genetic variation in resistance to *F. gigantica* infection in large ruminants is relatively poor. Wiedosari and Copeman (1990) first showed that indigenous ITT sheep expressed high resistance on challenge with metacercariae from *F. gigantica*. Roberts *et al.* (1997b) recently compared

the resistance of ITT sheep, St Croix sheep and cross-bred ITT/St Croix sheep in order to obtain evidence for the heritability of the resistance expressed by ITT sheep. In this experiment, the ITT sheep had a parasite take 20% of that shown by the St Croix breed, equivalent to a 3 standard deviation difference based on within-breed phenotypic variation (Fig. 15.1). The St Croix breed is reported to express a low degree of resistance to *F. hepatica* (Boyce *et al.*, 1987; Mozzala, 1990; Gamble and Zajac, 1992).

Another unique feature of the genetic aspects of *F. gigantica* resistance in ITT sheep is the possibility that a major single gene, or a gene with large effect, may be determining resistance (Roberts *et al.*, 1997b). Although the data are of a preliminary nature only, the performance of F_2 and F_3 animals from the ITT × St Croix cross showed over-dispersion in resistance when compared with the within-breed variance; also shown was strong dominance based on the average performance of the F_2/F_3 compared with the theoretical mean performance of the ITT and St Croix data. There is a suggestion of

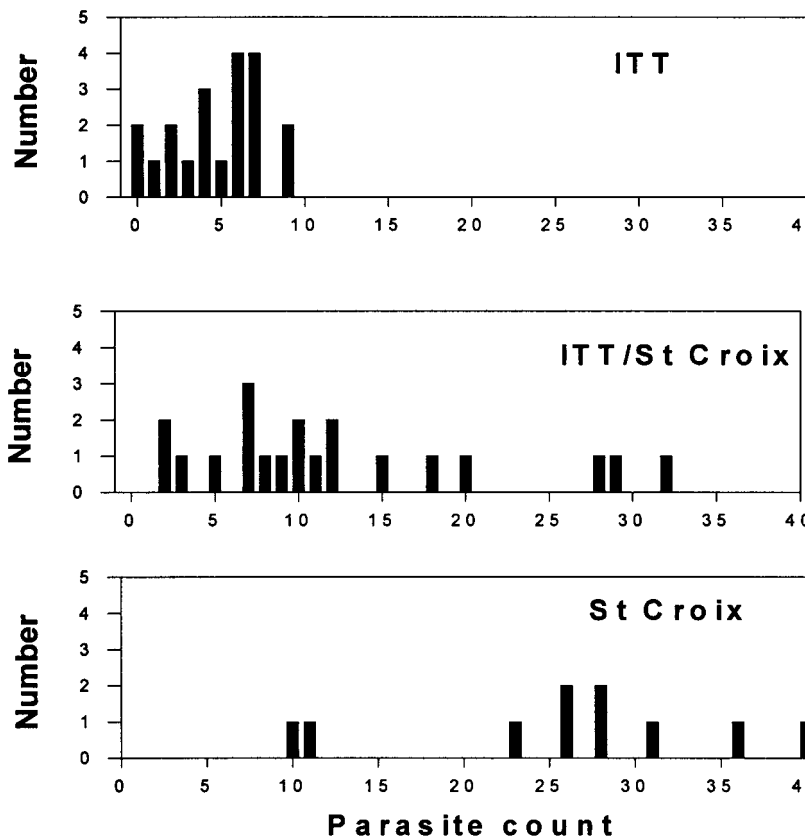

Fig. 15.1. Distribution of parasite counts recovered from ITT, ITT/St Croix and St Croix sheep challenged with 350 metacercariae. (From Roberts *et al.*, 1977b.)

resistance segregating as a major gene based on the distribution of resistant and susceptible phenotypes according to presumed Hardy–Weinberg expectations (Roberts *et al*., 1997b). This is shown in Fig 15.1, where 16 F_2/F_3 animals had moderate to high resistance (< 20 parasites) and four F_2/F_3 were as susceptible as the St Croix parental line. It should be noted that the comparative St Croix animals used in this experiment were only seven-eighths purebred, and were derived from a repeated backcross involving the Sumatra Thin Tail as foundation breed which may account for the partial resistance of the St Croix in this experiment.

The recovery of parasites in ITT, St Croix, Merino and other sheep breeds is shown in Table 15.3. The results show that the Merino appears to be the breed most susceptible to *F. gigantica* and the ITT the most resistant. Figure 15.2 shows comparative recovery data obtained in several experiments in which naive ITT or Merino sheep, and ITT sheep exposed to *F. gigantica*, were challenged with 200–500 metacercariae (Roberts *et al.*, 1996, 1997a,b,c; T.W. Spithill, S.E. Estuningsih, S. Widjajanti and S. Partoutomo, Bogor, 1997, unpublished data). It is clear that the ITT and Merino breeds differ in susceptibility to infection with *F. gigantica*. However, as with most breed studies, the origin of the animals tested is unknown and the sheep were not raised contemporaneously. Since the sample of sires which have generated the test samples is unknown or relatively small, this confounds definitive breed comparisons; we should therefore be cautious in interpreting breed comparisons until studies are conducted with animals of known history.

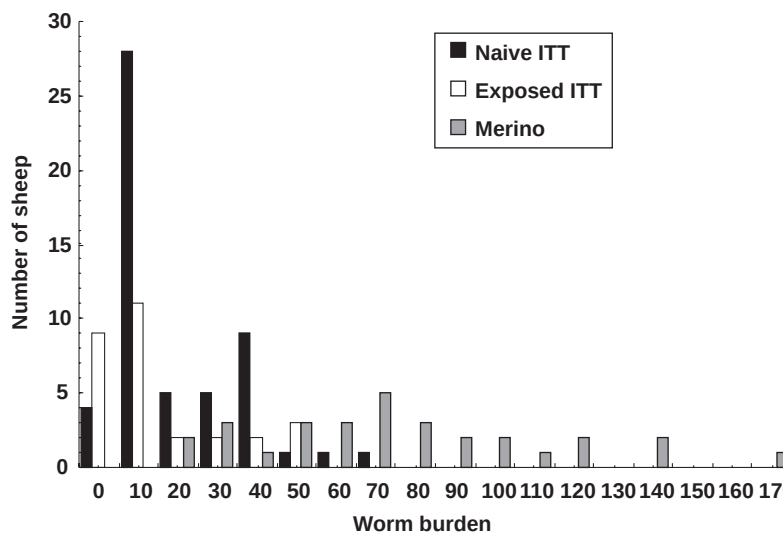

Fig. 15.2. Relationship between the number of naive ITT (black bar), ITT exposed to *F. gigantica* (white bar) or naive Merino (grey bar) sheep carrying the indicated parasite burden. Animals are grouped according to parasite counts (*n* = 0, 1–10, 11–20, etc.). Sheep were challenged with 200–500 metacercariae. Naive Merino sheep are more susceptible to infection with *F. gigantica*.

Nevertheless, the results suggest that it may be possible to use the dominant genetic resistance of ITT sheep to introduce resistance into other sheep breeds by cross-breeding and selection for resistance (Roberts and Suhardono, 1996). The improvement of the genetic potential of livestock to increase productivity is a major focus of the strategic plan of the Indonesian Ministry of Agriculture (AARD, 1994).

Major Genes for Resistance to Trematode Infection and Cross-resistance Studies

The possibility of a *major* gene influencing resistance to a complex internal parasite such as *F. gigantica* is intriguing. Major genes have been implicated in human resistance to malaria (Mims, 1982), other protozoa and *Schistosoma* (reviewed in McLeod *et al.*, 1995; Abel and Dessein, 1997). A single gene (*Rsm-1*) has been shown to be involved in determining induction of protective immunity to *S. mansoni* in mice (Correa-Oliveira *et al.*, 1986). However, to date the chromosomal location of this gene has not been determined. Further evidence for a major gene influencing resistance to internal parasites comes from the human literature where, intriguingly, a major gene has been identified that determines the acquisition of resistance to infection by *S. mansoni* (Abel *et al.*, 1991). Recently, the genetic locus for this gene (SM1) was mapped by a genome-wide genotype screen using 11 Brazilian families (Marquet *et al.*, 1996). Significant linkage was obtained on chromosome 5q31–q33 with the colony stimulating factor 1 receptor (CSF1R, M-CSF R) gene. CSF1 regulates the production of mononuclear phagocytes, osteoclasts and the function of cells in the female reproductive tract, and stimulates the survival, proliferation and differentiation of macrophages from bone marrow precursor cells. CSF1 production is stimulated by parasitic infection and interacts with the CSF1 receptor present on mononuclear phagocytes, priming macrophages for an inflammatory response. The CSF1 receptor is a tyrosine kinase (c-fms) which modulates the activities of a series of intracellular proteins that function in relaying biochemical signals to cells thereby modulating macrophage function. Thus, the observation that SM1 is closely linked to the CSF1R gene may suggest that resistance to *S. mansoni* infection in humans involves genetic polymorphisms in the CSF1 receptor which, through modifying macrophage production and/or activity, leads to enhanced killing of schistosome larvae. The presence of a major gene determining susceptibility to *S. mansoni* on chromosome 5q31–q33 has been independently confirmed in a separate study in Senegal (Muller-Myhsok *et al.*, 1997).

These observations are of particular significance to the problem of fasciolosis as there are species of schistosomes (*S. bovis, S. japonicum, S. mattheei*) which infect livestock (Taylor, 1987). Acquired resistance between *Fasciola* and *Schistosoma* spp. has been reviewed (Haroun and Hillyer, 1986). Acquired resistance to *F. hepatica* has been described in ruminants exposed to *S. bovis* and *S. mansoni* (Monrad *et al.*, 1981; Sirag *et al.*, 1981; Haroun and Hillyer, 1986). Yagi *et al.* (1986) and Haroun and Hillyer (1986) described heterologous cross-resistance to *F. gigantica* and *S. bovis* in cattle exposed to

S. bovis or *F. gigantica*, respectively. Cross-resistance to *F. hepatica* has been reported in mice exposed to *S. mansoni* (Haroun and Hillyer, 1986). Interestingly, cross-resistance to *S. mansoni* infection in animals vaccinated with antigens from *F. hepatica* has also been described (Hillyer *et al.*, 1977, 1988a,b; Hillyer, 1979) and, conversely, an antigen from *S. mansoni* (the Sm14 fatty acid binding protein, FABP) has been shown to induce complete protection in mice against *F. hepatica* challenge (Tendler *et al.*, 1996). Similarly, *Fasciola* homologues of known protective schistosome antigens (GST and/or FABP) have been shown to protect both sheep (Sexton *et al.*, 1990) and cattle (Hillyer *et al.*, 1987; Morrison *et al.*, 1996: Estuningsih *et al.*, 1997) against *Fasciola* challenge. These results suggest that immune elimination of *Schistosoma* and *Fasciola* may, at least in part, involve responses to homologous antigens (such as FABP) which are shared between these trematodes, implying that these two parasites may be susceptible to the same or similar immune effector responses. It is thus feasible that the major resistance gene for *S. mansoni* (SM1) described in humans may have some relevance to the *F. gigantica* resistance expressed in ITT sheep.

The observations that resistance to parasites such as *S. mansoni* and *F. gigantica* may be determined by major genes is intriguing since resistance to nematode parasites appears to be genetically complex (Gray and Gill, 1993). It may be that trematodes are uniquely susceptible to particular immune effector responses and, under the appropriate conditions which prevail in certain genetically endowed hosts, these responses are expressed in a qualitative or quantitative manner that is highly effective. It will be intriguing to determine the precise identity of the SM1 gene and the nature of the effector pathway which this gene controls or to which it contributes.

The availability of ITT sheep as a model to study the immune responses expressed in animals resistant to *Fasciola* is thus unique and allows us to test hypotheses by comparing immune responses in different sheep breeds (ITT, Merino, St Croix) which vary in resistance to *F. gigantica*.

Immunodiagnosis of *Fasciola gigantica* Infection in Ruminants

The diagnosis of *F. gigantica* infection in ruminants has involved the analysis of antibody responses to fluke antigens as well as the detection of circulating antigens using defined sera and monoclonal antibodies. Antibody responses and circulating antigens have been detected in sera from infected goats (Mbuh and Fagbemi, 1996). In cattle, antibodies to fluke antigens have been detected using indirect immunofluorescence on juvenile parasites and cryostat sections of flukes (Hanna and Jura, 1977; Schillhorn van Veen and Buys, 1979); total antibody and IgG_1 responses to somatic adult antigen have been reported (Ogunrinade, 1983; Fagbemi and Obarisiagbon, 1990; Fagbemi *et al.*, 1995). In sheep exposed to *F. gigantica*, antibody responses specific to adult parasite excretory–secretory (ES) antigens of size 17, 21, 57 and 69 kDa were detected within 6 weeks of infection (Guobadia and Fagbemi, 1995). Total antibodies to a 28 kDa cysteine protease of *F. gigantica* were detected in infected cattle, sheep and goats (Fagbemi and Guobadia, 1995).

Antibodies to defined antigens of *F. gigantica* have been prepared and used in immunodiagnosis (Fagbemi, 1995; Maleewong *et al.*, 1997; Fagbemi *et al.*, 1997; Guobadia and Fagbemi, 1997; Viyanant *et al.*, 1997). A circulating antigen of size 88 kDa and ES antigens of 25 and 40 kDa were detected in cattle sera within 3 weeks of infection (Fagbemi *et al.*, 1995, 1997). A monoclonal antibody to a 66 kDa surface tegumental antigen of adult flukes has been developed as a diagnostic assay in cattle: the assay detected antigen in sera from 1 to at least 32 weeks post-infection with a sensitivity of at least 86% (Viyanant *et al.*, 1997). Total parasite antigen was detected in sera from infected sheep within 2 weeks of infection using polyvalent antiserum from infected rabbits (Guobadia and Fagbemi, 1996). Monospecific antisera to ES antigens of 17 kDa and 69 kDa have been used to detect circulating antigen in sheep within 1 and 4 weeks post-infection, respectively (Guobadia and Fagbemi, 1997). The 17 kDa antigen showed high sensitivity for detecting *F. gigantica* although some cross-reaction with antigens from other flukes was observed. The development of an immunodiagnostic test for quantitating current *F. gigantica* worm burdens, by detection of antigen in sera or faeces, will greatly facilitate epidemiological studies and assist in the implementation of chemotherapy.

Development of Defined Vaccines Against *Fasciola gigantica*

Vaccination of ruminants against *F. gigantica* has been obtained using irradiated metacercariae, as discussed above and previously (Haroun and Hillyer, 1986) and rats have been vaccinated with extracts of metacercariae of *F. gigantica* (Yoshihara *et al.*, 1985). To date, there is a single report of the use of defined antigens as vaccines against *F. gigantica* (Estuningsih *et al.*, 1997). Four purified antigens from *F. gigantica* (fatty acid binding protein (FABP), glutathione *S*-transferase (GST), cathepsin L (CatL) and paramyosin) have been tested as vaccines in Brahman-cross cattle in Indonesia.

The FABP antigen is the homologue of the Fh12 antigen described by Hillyer (reviewed in Spithill *et al.*, 1997). Fh12 is the major component of the FhSmIII(M) complex which has been shown to induce a 55% reduction in mean worm burdens in cattle and 69–78% protection in mice (Hillyer, 1985; Hillyer *et al.*, 1987). The Fh12 cDNA sequence predicts a protein with high similarity to a family of FABPs (Rodriguez-Perez *et al.*, 1992) suggesting that Fh12 may play a role in the intracellular transport of long-chain fatty acids and their acyl-CoA esters. The FABP fraction from *F. gigantica*, formulated with Freund's adjuvant, was tested as a vaccine in cattle and a low but significant 31% reduction in worm burdens was observed (Table 15.4) (Estuningsih *et al.*, 1997): however, a recombinant FABP cloned from *F. gigantica* cDNA expressed in *E. coli* (Smooker *et al.*, 1997) did not elicit protection. Since there appears to be a family of FABPs in *Fasciola* (Bozas and Spithill, 1996; Smooker *et al.*, 1997), it is possible that the immune responses induced by the native FABP mixture differ from that induced by a single recombinant FABP, due to subtle conformational differences, or that the particular FABP molecule which is the target of the immune response differs from the sequence which was

Table 15.4. Efficacy of vaccines against *F. gigantica*.

Antigen	Proposed function	Form	Host	Mean vaccine protection (%)
FABP	Transport of fatty-acids	Native	Cattle	31 ($P < 0.026$)
		Recombined	Cattle	
GST	Detoxification	Native	Cattle	18 (ns)
Cathepsin L	Extracellular protease, Egg production?	Native	Cattle	0
Paramyosin	Muscle function?	Native	Cattle	0

[a] Data from Estuningsih *et al.* (1997).
Abbreviations: FABP, fatty acid binding protein; GST, glutathione *S*-transferase; ns, not significant.

cloned. Alternatively, it is feasible that the use of the N-terminal polyhistidine tag which was used to allow purification of the rFABP protein may interfere with the induction of the protective immune response. A corollary of this hypothesis is that the protective epitope(s) on FABP may be encoded in the N-terminal amino acid sequence. The mechanism of protection induced by the FABP vaccine in cattle remains to be determined.

The GSTs of *F. hepatica* have been shown to confer resistance to *F. hepatica* in both sheep (Sexton *et al.*, 1990) and cattle (Morrison *et al.*, 1996). When the efficacy of GST from *F. gigantica* was assessed in Brahman-cross cattle no significant reduction in worm burdens or faecal egg counts was observed despite the use of the same adjuvants previously shown to induce protection against *F. hepatica* with GST (Estuningsih *et al.*, 1997). These results suggest that vaccine formulations protective for *F. hepatica* may not be effective against *F. gigantica* and the possible basis for this difference in efficacy has been discussed (Estuningsih *et al.*, 1997).

Cathepsin L of *Fasciola*, formulated in Freund's adjuvant, has been shown to protect cattle against *F. hepatica* (Dalton *et al.*, 1996) and to induce a high (>70%) reduction in the output of eggs by the parasites in vaccinated sheep (Wijffels *et al.*, 1994b) and cattle (Dalton *et al.*, 1996). In Brahman-cross cattle, using DEAE Dextran/SM as adjuvant, no protection or reduction in faecal egg count was observed despite the induction of high total antibody titres (Estuningsih *et al.*, 1997). These results suggest a critical role for Freund's adjuvant in vaccine efficacy using cathepsin L which may relate to the induction of a certain arm of the immune response.

Paramyosin, an antigen shown to elicit high levels of protection against *Schistosoma* (Kalinna and McManus, 1997), has also been evaluated as a vaccine against *F. gigantica*. When formulated in DEAE Dextran/SM, paramyosin failed to induce protection in cattle under conditions where high antibody titres were observed (Estuningsih *et al.*, 1997). Such results suggest that high total antibody titres *per se* are not sufficient for expression of immunity in cattle against *F. gigantica*. It may be that particular isotypes of antibody are required for immune elimination of *F. gigantica* since Mulcahy *et al.* (1998) have shown that reduction of *F. hepatica* worm burdens correlates with both the level of IgG_2 antibodies as well as antibody affinity.

Molecular Biology of *Fasciola gigantica*

Knowledge of the molecular biology of the tropical liver fluke has lagged far behind that of the temperate counterpart *F. hepatica* and, indeed, studies of the molecular aspects of *F. gigantica* are in their infancy. Much of what has been found is the result of applying the knowledge previously gained from *F. hepatica* to *F. gigantica*. The formative state of the molecular biology of *F. gigantica* can best be appreciated by perusal of the protein and nucleic acid databases: in each, there is only one reference to a specific *F. gigantica* cDNA – that of a fatty acid binding protein isolated in our laboratory (Smooker *et al.*, 1997). While the application of knowledge gained from one species to a closely related species is obviously a valid approach, work detailed elsewhere in this chapter demonstrates that the two *Fasciola* species behave very differently in terms of resistance/susceptibility to immune attack. The mechanisms behind these differences are the subject of ongoing research and hence the definition of the differences between the parasites at the molecular genetic level become important. One area in which molecular biology has been used is in the molecular taxonomy of *Fasciola* and, in particular, the assignment of a Japanese *Fasciola* sp. as a probable *F. gigantica* strain. As will be detailed below, traditional targets for molecular taxonomy (the rRNA gene cluster) as well as some mitochondrial gene targets have been employed for this purpose. Molecular biology of the parasite generally refers to the characterization of the genetic material but we will include a brief summary of the defined proteins which have been reported for *F. gigantica*.

Protein Characterization

Cathepsin proteases

Some of the most characterized of all *Fasciola* proteins are the proteolytic enzymes present in both excretory–secretory (ES) material and in somatic extracts (reviewed in Spithill and Dalton, 1998). The cysteine proteases, in particular the cathepsin L proteases, have been well studied in *F. hepatica* and the occurrence of these enzymes in *F. gigantica* has been described. Fagbemi and Hillyer (1991, 1992) examined the proteases of adult *F. gigantica* worms and identified at least 15 bands with proteolytic activity on gelatine substrate gels. The proteases were inhibited by leupeptin and PMSF, but not by EGTA, indicating that they largely comprise cysteine or serine proteases. Further purification of the somatic extracts yielded a protease activity of molecular mass 26–28 kDa which was inhibited by the cysteine-protease-specific inhibitors E64 and iodoacetamide whereas serine and aspartic acid protease inhibitors were less effective. This protease activity has an acidic pH optimum of 4.5, within the range usually observed for *F. hepatica* cathepsin proteases (pH 3.5–4 and 7) (Chapman and Mitchell, 1982; Smith *et al.*, 1993; Dowd *et al.*, 1994; Wijffels *et al*., 1994a). In our laboratory we have demonstrated the occurrence of cysteine proteases in the ES of adult *F. gigantica* which migrate

as a triplet of bands of molecular mass 27–28 kDa and shown that these proteins cross-react with antisera raised to *F. hepatica* secreted proteases (Estuningsih *et al.*, 1997; P. Smooker, S.E. Estuningish and T.W. Spithill, Melbourne, 1996, unpublished data). As detailed below, cDNA clones encoding secreted cathepsin proteases have also been isolated.

Fatty acid binding proteins

The second major characterized family of *F. gigantica* proteins are the fatty acid binding proteins (FABPs). These are a large family of proteins, originally characterized from mammalian tissues, but now known to be present in a variety of invertebrates including *F. hepatica* (Rodriguez-Perez *et al.*, 1992). The main feature of these proteins from the point of view of trematode biology is the *S. mansoni/F. hepatica* protein Sm14/Fh15. Immunization of mice with either of these proteins results in the development of an immune response which is protective against the heterologous trematode (Hillyer *et al.*, 1988b; Tendler *et al.*, 1996). This ability to protect against heterologous challenge has made the FABPs one of the most promising vaccine candidates against trematodes. FABPs from *F. gigantica* were isolated from a soluble fraction of *F. gigantica*; Western blotting against sera raised to a *F. hepatica* FABP showed the presence of two dominant immunoreactive species which differ slightly in apparent molecular mass (Smooker *et al.*, 1997). The presence of more than one FABP isoform was not surprising: in *F. hepatica* three sequences with similarity to the FABP family have been identified (Rodriguez-Perez *et al.*, 1992; Chicz, 1994, unpublished; Bozas and Spithill, 1996). It thus appears that *F. gigantica* also contains multiple FABPs.

GST

The GSTs of *F. hepatica* have been long studied, both in terms of their biochemistry and vaccine potential (Brophy and Pritchard, 1994; Spithill *et al.*, 1997; Spithill and Dalton, 1998). GSTs from *F. gigantica* were evaluated as vaccine molecules in Brahman-cross cattle (Estuningsih *et al.*, 1997) and biochemically analysed (P. Smooker, S. Vaiano and T.W. Spithill, Melbourne, 1995, unpublished data). *F. gigantica* GSTs were shown to be of similar molecular mass and immunologically cross-reactive with those from *F. hepatica* (Estuningsih *et al.*, 1997). The N-terminal sequence of *F. gigantica* GST (FgGST) was compared to that observed for *F. hepatica* GST Fh26 (Wijffels *et al.*, 1992). In the 17 amino acids for which sequence was obtained only two differences are apparent at positions 9 and 15:

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                              9              15
FgGST      P A K L G Y W K  K  R G L Q Q  Q
                           I        A
Fh26 GST   P A K L G Y W K  I  R G L Q Q  P
                           L
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The immunological cross-reactivity, together with the similarity in size and N-terminal sequence, shows that the GSTs from the two *Fasciola* species have

common properties. The substrate specificity of FgGST was also determined and compared to that found for FhGST (Salvatore *et al.*, 1995). As expected, FgGST exhibited activity against CDNB (1-chloro-2,4-dinitrobenzene), *trans*-4-phenyl-3-butene-2-one, ethacrynic acid and *trans*-2-nonenal, with enzymatic-specific activities similar to those detected by Salvatore *et al.* (1995) for FhGST. Additionally, we tested the activity against 1,2-dichloro-4-nitrobenzene and found significant levels of activity, which were not previously detected for GST isolated from *F. hepatica* by Brophy *et al.* (1990). It remains to be determined if this activity is restricted to *F. gigantica* and missing from *F. hepatica*, suggesting a key difference in substrate utilization between *Fasciola* species, or whether the strain of *F. hepatica* from which FhGST was isolated by Brophy *et al.* (1990) is deficient in the GST responsible for this activity.

Characterization of cDNAs from *Fasciola gigantica*

We have isolated RNA from *F. gigantica* (from flukes collected from cattle at an abattoir in Jakarta, Indonesia) and used this RNA both as a template for RT-PCR and for the construction of an expression cDNA library. The former approach enabled the isolation of a number of *F. gigantica* partial cDNA clones. One of these was the *F. gigantica* FABP cDNA (Smooker *et al.*, 1997) isolated by using PCR primers derived from the corresponding *F. hepatica* sequence (Rodriguez-Perez *et al.*, 1992). The *F. gigantica* cDNA clone was shown to be 94% identical to the *F. hepatica* homologue, containing a similar signature sequence near the N-terminus and, interestingly, the same stretch of amino acids between residues 108 and 113 which correspond exactly to those found at the same region in the rat bile-acid binding protein (Gong *et al.*, 1994). Given that adult flukes reside in the bile ducts, it may be that *Fasciola* FABPs can bind bile acids. The *F. gigantica* FABP cDNA clone was expressed in the pET vector system and soluble protein purified from *E. coli* lysates (Smooker *et al.*, 1997). The recombinant protein was shown to have B cell epitopes in common with the *F. hepatica* FABP (Smooker *et al.*, 1997).

Other cDNA segments isolated by RT-PCR from *F. gigantica* RNA include a clone comprising the homologue of *F. hepatica* cathepsin B (mature coding region; Wilson *et al.*, 1998) and a short segment of the paramyosin cDNA. These cDNAs have yet to be fully characterized; however, the presence of these sequences derived from RNA shows that these homologues of *F. hepatica* sequences are transcribed in adult *F. gigantica*.

A library was constructed from adult fluke RNA by reverse transcription and screened with both nucleic acid probes (derived from *F. hepatica*) and antisera generated to both *F. hepatica* and *F. gigantica* proteins. Several full length clones encoding cathepsin L were isolated, using both *F. hepatica* cathepsin cDNA sequences and antisera raised to the corresponding proteins (Wijffels *et al.*, 1994a). Two clones were completely sequenced and shown to comprise two homologues of *F. hepatica* cathepsin L1 (Wijffels *et al.*, 1994a). The amino acid sequences predicted from the two *F. gigantica* cDNA clones are 94% identical to each other and 93–94% identical with the *F. hepatica* cDNA. Both clones contain sequences predicted to function as a signal

peptide, suggesting that the encoded proteins are secreted from cells and may encode some of the secreted ES cathepsin proteases previously discussed. The library was also screened with antisera raised to *F. gigantica* paramyosin and several clones encoding the carboxy terminal half of the related protein myosin were isolated (P. Smooker, S.E. Estuningsih and T.W. Spithill, Melbourne, 1995, unpublished data).

Molecular Taxonomy

Molecular biology techniques have enabled the identification of, and in particular the discrimination between, parasite species which are so closely related as to be difficult to distinguish by morphological methods (McManus and Bowles, 1996). These techniques have been applied to *Fasciola*. A case illustrating this was originally reported by Blair and McManus (1989) who demonstrated that a *Fasciola* isolate from Japan was identical (by RFLP mapping) to a *F. gigantica* isolate from Malaysia but had significant differences when compared to an Australian *F. hepatica* isolate. This work was extended by Adlard *et al.* (1993), studying Australian *F. hepatica*, Malaysian *F. gigantica* and Japanese *Fasciola* spp., by comparison of the nucleotide sequence of the second internal transcribed spacer (ITS2) region of the ribosomal RNA gene cluster. A strain from a second genus, *Fascioloides magna*, was included to examine intergenus variation. The results showed that *F. hepatica* and *F. gigantica* differed by 2.8% in DNA sequence, and that each differed substantially more with *Fascioloides magna* (13.2% and 16% variation respectively). The sequence from the *Fasciola* spp. from Japan, however, had seven nucleotide differences from *F. hepatica* over the region analysed, but only one difference from *F. gigantica*. Hence, this Japanese species is most probably a strain of *F. gigantica*. It had not been possible to assign the species of this strain by morphological examination. A second finding from this report was the very low sequence variation seen between isolates of the same species. In fact, the two strains of *F. gigantica* analysed (Indonesian and Malaysian isolate) were identical in sequence. This indicates that the ITS2 region of the rRNA gene cluster is in fact very highly conserved within the species.

Other genes have also been utilized for taxonomy studies. Hashimoto *et al.* (1997) again compared *F. hepatica* from Australia, *F. gigantica* from Malaysia and a Japanese *Fasciola* spp. by firstly repeating the sequence analysis of the ITS2 region. They found no differences between the Japanese and Malaysian *F. gigantica* isolates, and six differences between these and the *F. hepatica* Australian isolate. This confirmed the results of Adlard *et al.* (1993). The cytochrome *c* oxidase subunit I (COI) mitochondrial sequence was also compared in the different isolates. As may be expected from a sequence in the rapidly evolving mitochondrial genome (see Moriyama and Powell, 1997), the variation was more extensive than for the ITS2 sequence. The Australian *F. hepatica* sequence differed from the other two sequences at 25–28 sites whereas the Malaysian *F. gigantica* and the Japanese isolate differed at only four to five sites. Intraspecies variation between two

Malaysian isolates was seen at one site. These results, taken with additional mitochondrial RFLP analysis also reported in this paper, strongly imply that the Japanese species is a strain of *F. gigantica*. In another report, the relationship between the Japanese *Fasciola* species and *F. gigantica* and *F. hepatica* was examined using PCR-SSCP, with the COI and NDI genes as targets (Itagaki *et al.*, 1995). Seven samples of each were tested and intraspecies variation was found in each case. However, when comparing species, the Japanese *Fasciola* species yielded patterns with more similarity to that of *F. hepatica* than *F. gigantica*, which is at odds with the data reported above. However, the application of PCR-SSCP to an investigation of genetic divergence may not be ideal as not all single polymorphisms within genes are detected (see for example Ravnik-Glavac *et al.*, 1994) and the presence of multiple polymorphisms may affect electrophoretic mobility in unpredictable ways.

It would appear that a sequence analysis of highly conserved sequences (e.g. ITS2) will be suitable for taxonomic studies on closely related species, whereas the sequences of the more rapidly diverging mitochondrial sequences (COI, NDI, etc.) will be used preferentially to determine intraspecific variation. We have initiated such a study examining the ITS1, COI and NDI gene sequences of Indonesian isolates of *F. gigantica* (J. Andrade, J. Irving, S.E. Estuningsih and T.W. Spithill, Melbourne, 1998, unpublished data). Remarkable conservation of the ITS1 sequences was found with no variation in the sequences from the 62 isolates examined. Conversely, considerable variation was found between the mitochondrial gene sequences, in particular COI sequences where sequence divergence was observed. The results have been examined relating the sequence divergence in the COI and NDI genes to a number of parameters including geographical location and host specificity of the isolates studied. In terms of the latter, it is particularly interesting that one COI polymorphism observed (in 15 of 22 isolates tested) was only found in *F. gigantica* isolated from cattle or buffalo and not in 29 isolates from sheep. This is preliminary evidence for the presence in Indonesia of strains of *F. gigantica* which differ in host specificity. More studies will be undertaken to determine if this polymorphism can act as a marker for parasites which preferentially infect cattle or buffalo.

In view of the high resistance exhibited by ITT sheep to *F. gigantica*, we have recently analysed the genetic divergence in isolates recovered from ITT sheep compared with isolates recovered from susceptible Merino sheep to determine whether there is selection for parasite genotypes which differ in virulence in sheep. The results to date do not demonstrate the presence of a unique *F. gigantica* genotype (as defined by the COI and NDI sequences examined) which is selected for survival during passage in ITT sheep (J. Andrade, J. Irving, S.E. Estuningsih and T.W. Spithill, Melbourne, 1998, unpublished data). Thus, the high resistance expressed by ITT sheep against *F. gigantica* would appear to be a property intrinsic to this sheep breed and does not result from the selection during infection for a certain parasite genotype which is unusually susceptible to the ITT immune response.

Future Directions

As will be apparent to the reader little is known of the molecular biology of *F. gigantica* and what has been found is largely based on precedents from *F. hepatica*. However, now that biological differences between the two parasites are becoming clearer, molecular biology can be employed to define these differences at the molecular level. Hence the differences between *F. gigantica* and *F. hepatica* in, for example, the expression of defence enzymes or proteins which modulate host immune responses can be assayed under a number of different conditions *in vitro*. Additionally, techniques such as differential display could be employed to look at differences in the overall pattern of gene expression between the two species, as has been done for different stages of *F. hepatica* (Reed *et al.*, 1998). Further work at the molecular taxonomic level is also required to clarify further the relationships between genotype and host specificity and the geographic distribution of strains of *F. gigantica*. Such studies will reveal new knowledge on the relationship between *F. gigantica* and *F. hepatica* which will assist in unravelling the intriguing differences these parasites show in their host–parasite relationships.

Acknowledgements

This work was supported by the Australian Centre for International Agricultural Research (Canberra), Monash University, Agriculture Victoria and James Cook University.

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