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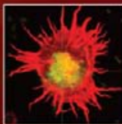
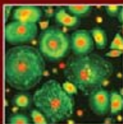
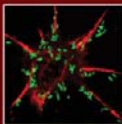
Insect Infection and Immunity

Evolution, Ecology, and Mechanisms

Edited by Jens Rolff
and Stuart E. Reynolds

Insect Infection and Immunity

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Evolution, Ecology, and Mechanisms

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Great Clarendon Street, Oxford OX2 6DP

Oxford University Press is a department of the University of Oxford.
It furthers the University's objective of excellence in research, scholarship,
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Oxford New York

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Kuala Lumpur Madrid Melbourne Mexico City Nairobi
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Published in the United States
by Oxford University Press Inc., New York

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First published 2009

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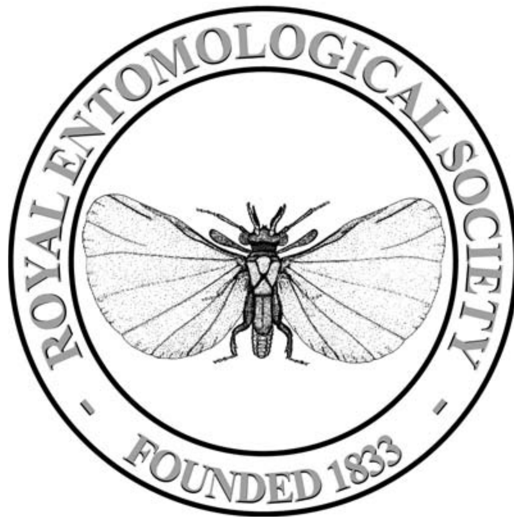
British Library Cataloguing in Publication Data
Data available

Library of Congress Cataloging in Publication Data
Data available

Typeset by Newgen Imaging Systems (P) Ltd., Chennai, India
Printed in Great Britain
on acid-free paper by
CPI Antony Rowe, Chippenham, Wiltshire

ISBN 978–0–19–955135–4 (Hbk.)
ISBN 978–0–19–955136–1 (Pbk.)

10 9 8 7 6 5 4 3 2 1



This book is published on the occasion of the Royal Entomological Society's Symposium on 'Insect Infection and Immunity' in Sheffield, July 15–17 2009. All symposium speakers contributed to this volume.

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Preface

Estimates of the total number of eukaryotes range up to about 2,000,000 species. The invertebrates constitute more than 95% of these and include a vast diversity of organisms, ranging from unicellular protozoans to the more complex echinoderms and protochordates. Insects are by far the largest group of animals within the invertebrates and the great interest in research on these animals arises from their roles as vectors for many human and animal diseases, such as malaria, and the serious harm done by insects to crops and food.

Thus an interest in the diseases, pathogens and immune responses of insects has been a longstanding research interest. Even though one might think that since most insects are short-lived they do not need a sophisticated immune response, this is not the case, and instead insects and other invertebrates have been found to possess very complex and efficient immune systems. To mention just one aspect of insect immunity; the so-called prophenoloxidase activating system and the coagulation system are activated by minute amounts (picogram) of carbohydrates from bacteria or fungi, and hence these systems are in practical use to detect bacteria and fungi as contaminants or as pathogens. It is also noteworthy that these two systems are much more sensitive in detecting products from microorganisms than is the vertebrate complement system, meaning that invertebrate animals are definitely much more efficient in detecting and responding to pathogens than vertebrates.

Professor Hans G. Boman and colleagues in 1972 were the first to clearly demonstrate that an insect, namely *Drosophila*, could respond to a challenge with dead or live bacteria, with the specific induced synthesis of antimicrobial substances. Later in 1981, the same researchers were for the first time able to sequence an antimicrobial peptide (AMP), which was named cecropin, since it was isolated from the moth *Hyalophora cecropia*. From 1981 and onwards, there was an intense interest in deciphering the pathways leading to the production of AMPs. This led to the breakthrough by Bruno Lemaitre and Jules Hoffmann and colleagues in 1996, whereby the pathway to the production of the antifungal peptide drosomycin was characterized. This opened the possibility of detailed genetic studies of the signaling pathways involved in the production of AMPs. Subsequently, the completion of the *Drosophila* genomic sequence in 2000 made it possible for an even more powerful molecular genetic analysis of the immune system in this insect.

Basically two lines of research on insect immunity have co-existed. One mainly focused on genomic, transcriptomic and proteomic analysis of the immune responses; the other line of studies being concentrated on ecological immune studies. Paul Schmid-Hempel, Joachim Kurtz, Sophie Armitage and Jens Rolff initiated several meetings between 2004–2007, where molecular biologists and ecologists met and presented their research with the main aim to bring researchers representing these lines to come together to promote discussions and collaboration. This book edited by Stuart Reynolds and Jens Rolff follows this tradition and is a mix where several chapters are devoted to molecular immune studies and others to different aspects of ecoimmunity. Recent studies have shown that insects and other invertebrates may have the capacity for immune priming and also show specificity in their immune responses. It has also been shown that there seem to be specific immune defences to different bacterial species and that the immune response can vary depending on feed, time, presence of symbionts and the natural flora of the gut. Thus it seems as if these lower animals may have

a very complex network to combat different pathogens and to avoid being killed by these pathogens. In order to discover how these networks operate, and to reveal whether some sort of adaptive immunity is at work in invertebrates, collaboration between molecular immunologists and more ecology-oriented entomologists will be necessary.

With this exceptionally broad panel of international authors and by the diversity of subjects, this book is likely to be a new and refreshing outlook on the exciting field of insects as well as invertebrate immunity.This book consequently provides an excellent opportunity to get an overview of insect immunity and its implications for the well being of insects in their natural habitats and for their natural behavior. It is well suited for scientists, post-doctoral fellows and post-graduates who wish to get a stringent overview of several aspects of insect immunity.

Uppsala March 2009
Kenneth Söderhäll
Uppsala University
Sweden

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CHAPTER 1

Introducing insect infection and immunity

Jens Rolff and Stuart E. Reynolds

> All science, no matter how arcane or irrelevant it may appear to outsiders, has broader implications, which can lead the willing scholar into some quite unfamiliar territory.
>
> Geerat J. Vermeij (2004)

1.1 Why study insect immunity?

The current great interest of researchers—not all of them entomologists—in insect immunity and infection has been driven by a variety of causes. Early attention mostly grew from economic concerns. For example, in 1835 Agostino Bassi was among the first to verify the germ theory of disease by showing that a fungus (*Beauveria bassiana*) was the cause of the white muscardine disease of the commercial silkworm (*Bombyx mori*). Later, Louis Pasteur spent the years 1865–1870 investigating pébrine (caused by a microsporidian) and flacherie (a viral disease), two further silkworm maladies that threatened to destroy the Provençal silk industry. Among the great microbiologist's discoveries was that the insects showed considerable variation in their resistance to these pathogens. In 1880, the pioneer immunologist Elie Metchnikoff was among the first to propose practical methods of microbial biological control of an insect crop pest, initiating trials of the fungus *Metarhizium anisopliae* against grain beetles (Lord, 2005).

Such applied interest continues today. Since it is estimated that insects destroy approximately 18% of the world annual crop production (Oerke and Dehne, 2004) and nearly 20% of stored food grains (Bergvinson and Garcia-Lara, 2004), the damage amounting to around US$100 billion every year (Carlini and Grossi-de-Sa, 2002), this interest is not surprising. Since populations of most insects are regulated by density dependent factors involving pathogens and parasites, to feed the world we need to understand, and if necessary manipulate, the interactions of pest insects with their natural enemies.

Insects also provide crucial ecosystem services as pollinators, as has been highlighted by recent transnational declines in the populations of both solitary and social bees (Biesmeijer *et al.*, 2006) and concern over the mysterious Colony Collapse Disorder of honey bees in North America (Oldroyd, 2007). The estimated value of US pollination services alone is between $4.1 and 6.7 billion (Nabhan and Buchman, 1997). It is possible that pollinator declines are at least in part consequences of the compromised immunity of these insects to their usual parasites and pathogens (e.g. Gregory *et al.*, 2005).

Furthermore, mosquitoes, sandflies, and many other insect vector species cause severe health and economic problems. To take only one example, of the 2.5 billion people at risk (40% of the world's population), more than 500 million become severely ill with malaria every year, and more than 1 million die from its effects (World Health Organization, 2008). To comprehend the biology of the disease, it is crucial to understand the ability of the parasite to survive the rigours of the vector's immune system.

However, it has also long been recognized (the case is summarized, for example, by Wigglesworth,

1971) that insects can be tractable models of vertebrate function, and so studies of insect immune function have been undertaken with the idea of making fundamental discoveries about the mechanisms of immune defences. Cuénot studied the phagocytic function of insect blood cells as early as 1895 (cited by Munson, 1953). A paradigmatic study of humoral immunity is that of Stephens (1962), who showed that a bacteria-killing factor appeared in the haemolymph of wax moth (*Galleria mellonella*) larvae that had been injected with *Pseudomonas aeruginosa*, and that this bactericidal activity was apparently able to confer protective immunity on the living insect.

It is a fact of scientific life, however, that the payoff from basic studies can often be long in coming. In the case of insect immunity, we might identify the work of Hans Boman and his colleagues as defining a crucial experimental moment. Boman *et al.* (1972) demonstrated that the fruit fly *Drosophila melanogaster* could be induced to synthesize new antibacterial defences when injected with bacteria. These discoveries quickly began to generate an understanding of the molecular nature of the responsible antimicrobial peptides. However, recognition of the importance of studying insect immunity only took off years later, after the discovery in 1996 (Lemaitre *et al*., 1996) that Toll receptors, formerly only known for their role in embryonic development, play a crucial role in the immune signalling of flies. The subsequent discovery of an immune role of Toll-like receptors (TLRs) in vertebrates (Medzhitov *et al*., 1997) followed swiftly. This has led to a spectacular, so-called Toll rush. As a consequence, *D. melanogaster* has become established as one of the prime models for studying innate immunity, and also as a model for studying human pathogens.

Evolutionary biologists have joined this field, because it provides an excellent means in which to study host–parasite co-evolution, principles of population genetics and trade-offs shaping life histories from population to molecular levels (Schmid-Hempel, 2005). Also, insects provide tractable model species for proof-of-principle studies in evolutionary biology, as they enable researchers to use powerful approaches, such as experimental evolution or quantitative genetics. Yet insects have much wider intrinsic importance, and studying them should not be because of, and should not be driven solely by, their convenience as model systems.

Beyond these directly applied considerations, insects form the most diverse metazoan taxon. Consequently, a better understanding of how they deal with the majority of their natural enemies, parasites, and pathogens has the potential to enhance our understanding of interactions in an ecological framework and at the community level. At still another level, some insects are also beautiful, and we simply don't know enough about them. Therefore, we feel that whatever the subject and speciality of a researcher are, natural curiosity, or 'the love of insects' (Eisner, 2004) will, and often should, take centre stage.

This book, never claiming to be a complete reflection of research on insect immunity, still covers a great diversity of research interests and approaches. The first part of the book focuses mainly on mechanistic views on insect immunity, whereas the second focuses on the level of the whole organism.

1.2 How insect immunity works

Broderick *et al.* (Chapter 2) first provide an overview of the best-understood insect immune system, that of *D. melanogaster*. They introduce the reader to one of the most important outputs of the fly's immune system: the powerful array of antimicrobial peptides and proteins (AMPs) that provide a broad-spectrum systemic defence against not only prokaryotic microbes, but also eukaryotic pathogens and parasites, such as fungi and protozoa. They then review the twin transcriptional control regulatory systems, named after crucial components of the signalling pathways Toll and Imd, which govern the production of AMPs. They also take into consideration the as yet rather incompletely understood Janus kinase/signal transduction and activators of transcription (JAK/STAT) cellular control system that links Toll- and Imd-regulated responses with others (e.g. the complement-like TEP proteins and a whole host of cellular defences). In the second part of their chapter, the authors turn to the much less well-studied set of local epithelial immune responses that are presumably deployed

in most cases before the systemic response is activated. These epithelial responses include reactive oxygen species (ROS) and locally secreted AMPs. A key issue considered by the authors is how these responses are modulated to allow establishment of beneficial microbiota in the gut, and adjacent to other immune-reactive epithelia. They conclude that 'signalling between the local and systemic response, while not required in every interaction, may dictate whether microbial infection leads to tolerance, resolution, or host lethality'. We believe that immune tolerance will prove to be an important emerging theme of the next decade's research on insect immunity.

Ragan *et al.* (Chapter 3) provide an antidote to the fly-centred approach of the previous chapter by focusing on the immune roles of haemolymph proteins. These have mostly been studied by biochemical means using much larger insects such as Lepidoptera, especially the commercial silkmoth *Bombyx mori* and the tobacco hornworm *Manduca sexta*. The authors focus on microbial pattern-recognition proteins, and the complex interplay between proteinases, inactive homologues of proteinases, and proteinase inhibitors, all of which collaborate to regulate the proteolytic conversion of prophenoloxidase (proPO) to its active form, phenoloxidase (PO). It is becoming ever clearer that the proPO system is among the most rapidly deployed immune defences in insects. Its value to the host is shown by the fact that pathogens and parasites are frequently distinguished by the presence of anti-PO counter-adaptations. It is also especially interesting that vertebrate animals don't have a homologue of this system. Why not? We don't know.

In Chapter 4, Imler and Eleftherianos return to *Drosophila* for an account of how this insect responds to the threat of viral infection. Although our understanding of insect antiviral responses is less advanced, it is already evident that the mechanisms involved are different to those used against bacteria and fungi. Responses to pathogenic viruses fall into two categories: those using RNA interference (RNAi) to attack the process of viral (RNA) genome replication, and those involving upregulation of a large set of, as yet, poorly defined viral infection-related genes. The importance of the RNAi pathway is extremely clear, since mutations in the RNAi system genes render flies more susceptible to viral infections. These genes evolve at exceedingly fast rates, as would be expected if they were under strong selection. Another smoking gun is the presence of anti-RNAi genes in viruses. There are many unsolved problems: for example, the extent to which known *Drosophila* immune-signalling pathways regulate antiviral immunity, and even the extent to which such upregulation is important, are not well understood.

To what extent do innate immune responses follow a pre-patterned disposition? Das *et al.* (Chapter 5) point out that it is becoming increasingly evident that insect immunity is much more complicated than this. Focusing on the immune system of the malaria mosquito *Anopheles gambiae* to give a detailed account of the extent to which recognition of microbial surfaces can give rise to highly specific immune responses, Das *et al.* consider the varied repertoire of pattern-recognition receptor (PRR) genes, which is further diversified by splicing at the mRNA level. Detailed accounts are given for the Gram-negative binding protein (GNBP) family, the peptidoglycan-recognition protein (PGRP) family, the immunoglobulin domain family, and the fibrinogen domain immuno-lectin (FBN) family. All these genes are present in multiple copies, so that invading microbes can potentially engender differential responses according to the nature of the detecting PRR. There is only a single gene for the Dscam (Down syndrome cell adhesion molecule) protein, but the ability for differential splicing of this gene's mRNA is particularly impressive, potentially generating literally thousands of different isoforms. It is already clear that at least some of this molecular diversity can generate immune responses that are differentially directed towards particular immune challenges. The extent to which different recognition is combined with unique antimicrobial action (as is the case with vertebrate immunoglobulins) remains unknown.

The advent of comparative genomics has shed light on the evolution of insect immune defences. Comparing the *Drosophila* group with mosquitoes and honey bees, in Chapter 6, Kafatos *et al.* disentangle common themes from specific components of immunity. Honey bees, for example, have a

relatively small set of immune genes which demonstrate a high degree of conservatism. They are most intriguing when compared with non-social insects, as the differences between them are attributable to the evolution of sociality in bees and other Hymenoptera. The authors also highlight how the use of comparative genomics led to the unravelling of evolutionary novelties. Genes containing leucine-rich repeats are an example, which led to the discovery of a new complement-like mechanism in mosquitoes. This also mirrors some overlap with the mechanism of acquired immunity in lampreys and hagfish (see below), which in itself might be exciting.

To conclude the book's first part, Schneider (Chapter 7) also focuses on *Drosophila*, but from the very different standpoint of the integration of immune responses into the insect's general physiology. He points out that most studies of insect immune systems to date have regarded both immune challenges and responses as being stereotypic, whereas in fact both are highly idiosyncratic. To rectify this, Schneider focuses on the responsiveness of insect immune responses to the context in which they are elicited. Importantly, this context includes not only the nature and extent of the pathogenic or parasitic challenge, but also the state of the insect at the time of that challenge. For example, host nutritional state is known to be important in determining the outcome of infections. This is not only because immunity is expensive (and therefore competes with other physiological systems for resources), but also because immune responses themselves may alter food intake, food selection, and energy flow. Immune responses also vary in extent and quality according to the time of day (i.e. phase of circadian clock), and the insect's reproductive status and age (senescence). Even the quality and quantity of the insect's native gut microbiota is important, and it is becoming evident that we have to consider the microbial ecology of an infection in describing both the immune challenge and the consequences of the immune response. Schneider's important message is that as we have now identified so many important players in the immunological play, we need to read their parts more closely to see how their characters can develop according to the plot.

1.3 Insect immunity and organism-level interactions

Many insects harbour bacterial symbionts, often in their guts. As Hurst and Darby review in Chapter 8, evidence is accumulating to suggest that bacterial symbionts provide protection against other natural enemies, including fungi, viruses, parasitoids, and even predators. If they provide resistance, how does the evolutionary ecology of symbiont-mediated protection differ from resistance through the host's own immune system? Although this is hardly studied, Hurst and Darby speculate that these symbionts are similar to constitutive defences: the insect always pays a metabolic cost. However, secondary symbionts can be lost easily if the selection pressure exerted by a parasitoid relaxes, for example. Aside from protection, there is another twist to the story. In most cases, these symbionts will be expressing pathogen-associated molecular patterns (PAMPs) similar to, or the same as, those of the pathogen. Moreover, the host needs to ensure that the symbionts co-operate. This establishes a very interesting perspective on the evolution of the insect's immune system: maintaining and managing symbionts could constitute a formidable selection pressure for the evolution of a policing system, such as immunity.

The interactions between hymenopteran parasitoids and their lepidopteran hosts have long held the attention of applied entomologists, insect ecologists, and evolutionary biologists. For more than 30 years, it has been known that the female wasp coats her eggs with virus particles in order to interfere with both the immune status and life history of the caterpillar host. Several polydnavirus genome sequences are now available. Moreau *et al.* (Chapter 9) review how this information has emerged as an important experimental tool to reveal the mechanisms of host immunity. The viral genome is transferred to caterpillar host cells, where it directs the expression of a variety of factors perverting host function in favour of the wasp. Effectively, the viral DNA has been captured to become an external extension of the wasp genome. Analysis of viral genes not only reveals which host genes are most important in resisting parasitic attack, but simultaneously casts light on

the co-evolutionary arms race that exists between pairs of interacting parasite and host genes.

Koella's chapter (Chapter 10) takes a theoretical perspective on the evolution of immune defences, and speculates on the conditions under which an organism can become resistant. Central to his argument are trade-offs between components of the immune system, the immune system and life history of the host. Moreover, understanding resistance evolution is made more difficult by the fact that host and parasite/pathogen genotypes interact, which might very well be genotype-specific. Using co-evolutionary models in which host and parasite are allowed to evolve, yields results different to those obtained from merely studying the host and the parasites separately. As they are tied together in a very intimate relationship, the presented theory strongly argues for empirical studies investigating both sides of the relationship.

The outcome of host–parasite interactions can also depend on the presence of other players, for example predators. Adamo's chapter (Chapter 11) shows how and why short-term immunosuppression mediated by stress occurs. The best example is that of crickets, which show a flight-or-fight syndrome. In the presence of predators or other sources of stress, these insects show lowered resistance against infection. This seems to be mediated by the demand for lipids to fuel the flight. A specific protein, apoliphorin III, is usually involved in immunosurveillance, but has now been found to be essential for lipid transport. Such physiological trade-offs are not only a cause of concern when assessing experimentally the resistance of a particular organism, but they add a further layer of complexity to understanding the evolution of resistance in its ecological context, where encounters with predators might be rather frequent. This chapter resonates, of course, with the earlier one by Schneider (Chapter 7).

The majority of insect immunity research has been carried out on *D. melanogaster*. Yet, as clarified by Kraaijeveld and Wertheim in Chapter 12, knowledge about how flies resist parasites and pathogens is mostly limited to just a few standard pathogens. Flies, almost certainly like all other insects, encounter a variety of natural enemies in the wild, ranging from nematodes, parasitic wasps and flies, bacteria, and fungi to viruses. How flies resist common natural enemies, such as nematodes and microsporidia, has hardly been investigated. Kraaijeveld and Wertheim emphasize that if we are to understand the evolution of the insect immune system, we need to quantify the strength of selection. To achieve this, information on infection rates in wild populations is paramount. Moreover, we need to understand the costs of immune functions, which puts constraints on the evolution of resistance. Although these costs are well studied at the phenotypic level, especially for parasitoid attacks, at the genomic level hardly anything is known. The authors explore the use of post-genomic tools to explore the costs of resistance. So far, ideas about costs can only be inferred indirectly from changes in the carbohydrate metabolism of infected individuals, for instance. However, the combination of experimental evolution studies combined with genomic techniques holds great promise here.

Despite the advantage of being 'immunocompetent', i.e. showing resistance against a wide range of parasites and pathogens, natural populations of hosts show great variation in parasite resistance. Junjeja and Lazzaro (Chapter 13) dissect this observation by discussing the evolution of different components from a population genetic perspective. Looking at recognition, signal transduction, and immune effectors, they show how these differ in their evolutionary trajectories within and across species, making use of data generated from whole-genome studies in more than 15 species of insect. Interestingly, the components of the immune system differ not only in how fast they evolve, but also in the way in which they change. Signalling pathways, for example, show a high degree of amino acid divergence. Yet across species, the main proteins in signalling are highly conserved; they are orthologues. By contrast, antimicrobial peptides hardly show any signatures of adaptive evolution at the amino acid level, yet different taxa of insects seem to have their own groups of antimicrobial peptides. The authors then go on to discuss how these genotypes translate into phenotypes with varying degrees of resistance. They highlight the importance of understanding trade-offs within

the immune system (see also Chapter 12 in this volume) and the way pathogens, here mostly bacteria and viruses, can interfere with the immune system. From the pathogen's perspective, the phenotype matters, because it constitutes their selective environment.

Hallmarks of vertebrate immunity are memory and specificity, and the mechanisms underlying these traits are well studied. As these components of the acquired immune system are confined to the jawed vertebrates, it has been inferred that the functional outcome, specificity and memory, must be limited to the jawed vertebrates. Pancer *et al.* (2004) recently demonstrated the existence of different diversifying mechanisms in the lampreys and hagfish, and thereby challenged this notion. In fact, transplantation experiments in the 1960s already indicated the existence of acquired immunity in lampreys. In their chapter, Sadd and Schmid-Hempel (Chapter 14) discuss examples of highly specific immune reactions in insects, as well as the nature of secondary response. Although the underlying mechanisms are elusive, the functional outcomes are clearly worth studying. They discuss a generic way of defining specificity and convincingly show that genetic diversity contributes to pathogen resistance. In social insects, this could be achieved through the mating system, and therefore at the colony level this is somewhat analogous to a simple somatic diversifying mechanism.

Wounding has frequently been assumed to be a major route for infections. As Siva-Jothy discusses in Chapter 15, it has only recently become clear that copulatory wounding occurs frequently in insects. This has implications for the evolution of wound repair, which is mediated by the immune system via clotting and melanization (Theopold *et al.*, 2004). Moreover, Siva-Jothy puts a convincing argument forward that females are in control of the timing of mating and therefore, are in a position to 'predict' their infection risks, if mating bears the risk of wounding and infection, for example, by sexually transmitted diseases. All else being equal, this leads to the hypothesis that the investment and management of immunity is tailored to meet the demands of higher risks during mating.

1.4 Emerging themes

As with any edited volume, the current one has some obvious gaps. Different readers may miss different things. Whereas these gaps come down in part to decisions by the editors as well as practical considerations and problems, there are clearly areas that have not been touched upon because the body of research is rather limited. In this section, we will briefly discuss a number of areas that we feel deserve attention by researchers, ideally garnering interest from different disciplines. These areas have rarely been studied (we also readily admit that these are subjective choices and do not pretend to give a complete coverage).

First, in parallel to speculations about the evolution of immunity in vertebrates (Hedrick, 2004; McFall-Ngai, 2007; Rolff, 2007), it is not entirely clear how the different components of the insect immune system have evolved. In Chapter 8, Hurst and Darby suggest an important role for symbionts and the need of the host to control its symbionts. Also, pathways such as the melanization pathway are involved in wound repair, and concurrent selection on this function will almost certainly have shaped the evolution of the proPO system (Cerenius *et al.*, 2008). Identifying the key selection pressures that led to the evolution of particular components of the immune system will be important, as well as understanding how selection moulded their integration into existing resistance mechanisms. To better perceive the starting origins of the insect immune system, it may be beneficial to take a closer look at homologies between the immune genes and signalling pathways of insects and those of other invertebrates, rather than just comparing them with mammalian systems. The highly diversified form of Dscam, for example, is restricted to insects and crustaceans, whereas Dscam itself is probably very old and is found in vertebrates as well (Brites *et al.*, 2008). The implications of this for understanding the insect and crustacean immune systems still need to be explored.

Second, whereas many insect parasites have been described, mechanistic understanding of insect immunity against pathogens and parasites is rather limited to a few groups (see Chapter 12 by Kraaijeveld and Wertheim). Many of the pathogens

commonly used in experiments are actually generalists, and are used to elicit and study immune responses mostly for the sake of convenience. Frequently, the parasite is injected into the host, circumventing the first line of immune defence offered by the cuticle. In order to understand the natural dynamics of infections, natural pathogens and manipulations of parasite loads via natural routes of infections are required. Moreover, insect immune genes must be presumed to have co-evolved with the virulence genes of the most frequently encountered, often specialist, parasites and pathogens. Studies of bacterial infections in *Drosophila* only very rarely use natural pathogens (but see Vodovar *et al*., 2005; Lazzaro *et al*., 2006). Macroparasites of insects are hardly studied, yet insects are hosts for parasites with complex life cycles such as tapeworms (Hurd, 1998), as well as numerous species of nematodes.

Third, it is often assumed that wounds breach the cuticle, allowing pathogens to invade. Although wound repair is quite well studied (Theopold *et al*., 2004), it is not self-evident that most microbial infections normally occur through this route. We are not aware of any data quantifying the frequencies of wounds in natural populations of insects, which at least would offer an estimate of the opportunities for infection. This has important implications, since septic systemic infections imposed on experimental insects through injection or body-wall piercing must often be necessarily 'unnatural'. Under such circumstances, the choice of the model pathogen becomes important. The virulence genes of pathogens that access the insect haemocoel directly are likely to have evolved differently from those of pathogens that enter from the gut, for example.

Yet, in the light of copulatory wounding, as discussed by Siva-Jothy (Chapter 15), wounding could well turn to be a frequent event in an insect's lifetime, and hence contribute to the selection for resistance. We need to know the identities of the pathogens concerned. Perhaps another exception here is the case of the specialist insect-pathogenic bacteria of the genera *Photorhabdus* and *Xenorhabdus*, which are symbiotic partners of entomopathogenic nematodes, and are indeed literally injected into their hosts by their worm partners (Goodrich-Blair and Clarke, 2007).

Fourth, an additional argument for studying the interactions of the insect immune system with 'natural' pathogens and parasites, as discussed by Moreau *et al*. (Chapter 9), is that those immune genes that are crucial to host defence can often be revealed by identifying virulence genes in the genomes of co-evolved parasites. While a serious start has been made on this approach with viruses, less has been done with the larger genomes of insect-pathogenic bacteria and (even less) fungi. A start on this has now been made by screening cosmid libraries of *Photorhabdus* spp. (e.g. Waterfield *et al*., 2008), and other bacterial pathogens will surely follow. The larger and often less experimentally tractable genomes of fungi pose a bigger challenge, however.

Finally, the issue of the specificity of immune responses emerges in several of the chapters of this book. To what extent are specific immune responses adaptive (Sadd and Schmid-Hempel, 2006)? How many and how much of different defensive response types should be deployed? How specific should they be? As pointed out by Sadd and Schmid-Hempel in Chapter 14, specificity can take two different forms in insects: immune responses that are specific to pathogen species/strains, and the phenomenon of immune priming, whereby the response to a second infection by the same pathogen strain is more successful. This is likely to be mediated by haemocytes (Pham *et al*., 2007). Moreover, resistance, as discussed by Koella (Chapter 10), is an outcome of the interaction between host and parasite. Whereas Dscam (see Das *et al*., Chapter 5) is considered to be a candidate for specific immune reactions in insects, specificity does not need to be restricted to the recognition level. Recognition, transduction, and effectors all show different signatures of adaptive evolution (Juneja and Lazzaro, Chapter 13). One could speculate that specificity is also determined by the interactions between these different levels of the immune system during a course of an infection (Haine *et al*., 2008).

In writing this chapter, it again became clear to us how seemingly arcane or irrelevant research can unexpectedly become important. Under the current funding climate (Braben, 2008) it would be almost impossible to secure funding to investigate

a peculiar physiological trait in an insect of no economic importance, which had nevertheless attracted the curiosity of researchers. Yet, Osama Shimomura set out in the early 1960s to study the fluorescence of the jellyfish *Aequorea victoria* (Shimomura, 1995), an investigation that ultimately led to the development of what is now an essential tool for cell biology and infection biology. In fact, most researchers that contributed to the current volume use green fluorescent protein (GFP)-labelled bacteria routinely in their research.

We hope that the inter-disciplinary nature of this volume will lead 'willing scholars' into some quite unfamiliar territory. We are confident that over the frontier lie many undiscovered human and scientific benefits, as well as much enjoyable biology. We also hope that not only insect immunologists, but scientists everywhere, will continue to insist on the importance of applying the highest scientific standards to problems of great intrinsic scientific interest, but with no apparent immediate applicability.

1.5 Acknowledgements

We would like to thank the Royal Entomological Society and Ian Sherman and Helen Eaton from Oxford University Press for embarking on this project. The book is the result of a meeting still to be held at the time of writing. All the contributors and reviewers put in a tremendous effort to make it possible to publish an academic book on time. Finally, we are grateful to Vi Nguyen who was instrumental in checking the manuscript and submitting the book.

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SECTION A

Immune mechanisms and integration

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CHAPTER 2

Recognition and response to microbial infection in *Drosophila*

Nichole A. Broderick, David P. Welchman, and Bruno Lemaitre

2.1 Introduction

Insects exhibit robust resistance to infection. Historically, this led to their use in understanding the biochemical and cellular mechanisms underlying host resistance to microbial infection. While these early studies aided in the understanding of many aspects of the innate immune response (Boman, 1995), the question of the genetic and molecular basis of these mechanisms persisted. As a tractable genetic model, *Drosophila melanogaster* has emerged as a paradigm for deciphering the innate immune response of insects (Ferrandon *et al.*, 2007; Lemaitre and Hoffmann, 2007). Overall, studies in the fruit fly have led to increased understanding of mechanisms of pathogen recognition, the molecular basis of immune signalling, and a description of the specific responses of insects to microbial infection.

In this chapter we discuss the mechanisms whereby *Drosophila* recognize foreign microbes, the signalling systems that regulate adapted responses against them, and the effector mechanisms used to control them. We will first focus our attention on the so-called systemic antimicrobial response. This response consists of the massive production of peptides or polypeptides, especially antimicrobial peptides (AMPs), by the fat body in response to the intrusion of bacteria or fungi into the haemolymph (Ferrandon *et al.*, 2007; Lemaitre and Hoffmann, 2007). To some extent this defence mechanism shares similarities with the acute-phase response of mammals that leads to the production of acute-phase proteins by the liver, an equivalent of the insect fat body. Both responses involve the upregulation of immune genes by nuclear factor κB (NF-κB) and Janus kinase/signal transduction and activators of transcription (JAK/STAT) pathways. However, there are major differences: while the acute-phase response is induced by inflammatory cytokines produced by other cells, most of the systemic response of insects is activated by pattern-recognition receptors (PRRs) that directly sense microbial elicitors. In addition, the systemic response involves the production of many antimicrobial peptides that directly target microbial invaders, while acute-phase proteins aid in the clearance of microbes by other immune defence mechanisms, such as phagocytosis and complement. This specific feature of the insect systemic response is attributed to the open circulatory system of insects, and the relatively small volume of their haemocoel that allows antimicrobial peptides to accumulate and reach active concentrations. Recently, genomic profiling studies revealed that many factors are secreted by the fat body in addition to antimicrobial peptides, suggesting that the systemic immune response impacts almost all immune reactions occurring in the haemolymph, including clotting, melanization, and phagocytosis.

In most organisms, AMPs do not act systemically, but are produced locally in specific tissues that are in contact with the external environment. This is the case for mammalian mucosa, such as the genital, respiratory, and digestive tracts. This local response also exists in insects such as *Drosophila* and mainly consists of the expression, either constitutive or inducible, of a subset of AMPs in epithelia. The second section of this chapter will focus on the

local immune response, particularly gut epithelial immunity. Though less characterized than the systemic response, more and more evidence points to the critical role of the local immune response during natural infection by *Drosophila* pathogens.

Despite this compartmentalization of the immune response, there is not complete separation between the local and systemic response. Several recent studies suggest communication between the two systems. In the final section of this chapter, we will discuss current research themes exploring the integration of local and systemic immunity, as well as their integration in host physiology.

2.2 The systemic immune response: AMPs

Among the various molecules produced by the fat body in response to infection, AMPs are the best characterized (reviewed in Imler and Bulet, 2005). Gene transcripts encoding AMPs are not detected in uninfected conditions, and molecular studies have revealed that their expression is induced upon infection. Over 20 AMPs, which comprise seven classes, have been identified. They are small (<10 kDa; with the exception of the 25 kDa Attacin), cationic, and exhibit a broad range of activities against bacteria and/or fungi (Figure 2.1). Insect AMPs are active at the microbial membrane, and while their precise mode of action is still unclear, the specificity of their activity in response to infection is well characterized. Diptericin, Drosocin, and Attacin are very effective against Gram-negative bacteria (Wicker *et al.*, 1990; Bulet *et al.*, 1993; Asling *et al.*, 1995). Defensin is active against Gram-positive bacteria (Dimarcq *et al.*, 1994), whereas Drosomycin and Metchnikowin exhibit antifungal activity (Fehlbaum *et al.*, 1994; Levashina *et al.*, 1995). Cecropin A1 acts against both bacteria and some fungi (Ekengren and Hultmark, 1999). Antimicrobial peptides are generally encoded by intron-less genes that are located in family clusters on the chromosome. Gene amplification by recombination and gene conversion are assumed to be the genetic forces driving the evolution of AMP genes (Sackton *et al.*, 2007). It should be noted that *Drosophila* also encodes 13 lysozymes, but they do not appear to contribute to the systemic response. Rather, they play a digestive role in the gut (Hultmark, 1996). To date, no loss-of-function study has addressed the relevance of individual peptides, but indirect evidence supports their primary role in *Drosophila* immunity (Tzou *et al.*, 2002; Liehl *et al.*, 2006).

2.2.1 Regulation of AMPs by Toll and Imd pathways

Two pathways, Toll and Imd, have been shown to regulate AMP genes (Lemaitre *et al.*, 1995, 1996). These two pathways share many common features with the mammalian Toll-like receptor (TLR) and tumour necrosis factor α (TNFα) signalling cascades, and regulate NF-κB transcription factors (Ferrandon *et al.*, 2007). The Toll pathway is triggered by the proteolytic cleavage of the Toll ligand, the cytokine Spätzle (Spz), and leads to activation of the Rel proteins Dif and Dorsal. This pathway is activated by both Gram-positive bacteria and fungi and it controls, to a large extent, the expression of AMPs with activity against fungi (e.g. Drosomycin). In contrast, the Imd pathway mainly responds to Gram-negative bacterial infection and controls antibacterial peptide genes (e.g. Diptericin) via the activation of the Rel protein Relish. Thus, the immune system of *Drosophila* demonstrates how two distinct signalling pathways can modulate the expression of genes in response to different classes of microbes, and can serve as a simple model to decipher innate immune mechanisms.

2.2.1.1 Toll pathway

The Toll pathway, as illustrated in Figure 2.2, is a conserved signalling cascade that was initially identified for its role in the establishment of dorso-ventral polarity of the embryo (Belvin and Anderson, 1996). Subsequently, it was implicated in additional developmental processes and the regulation of the systemic immune response, for which its function has been well characterized. Toll is a transmembrane receptor with an ectodomain composed of leucine-rich repeats (LRRs) and an intracellular Toll/interleukin-1 receptor (TIR) domain (Hashimoto *et al.*, 1988). Toll is activated by dimerization upon binding with a cleaved form of the secreted protein Spätzle (Weber *et al.*, 2003;

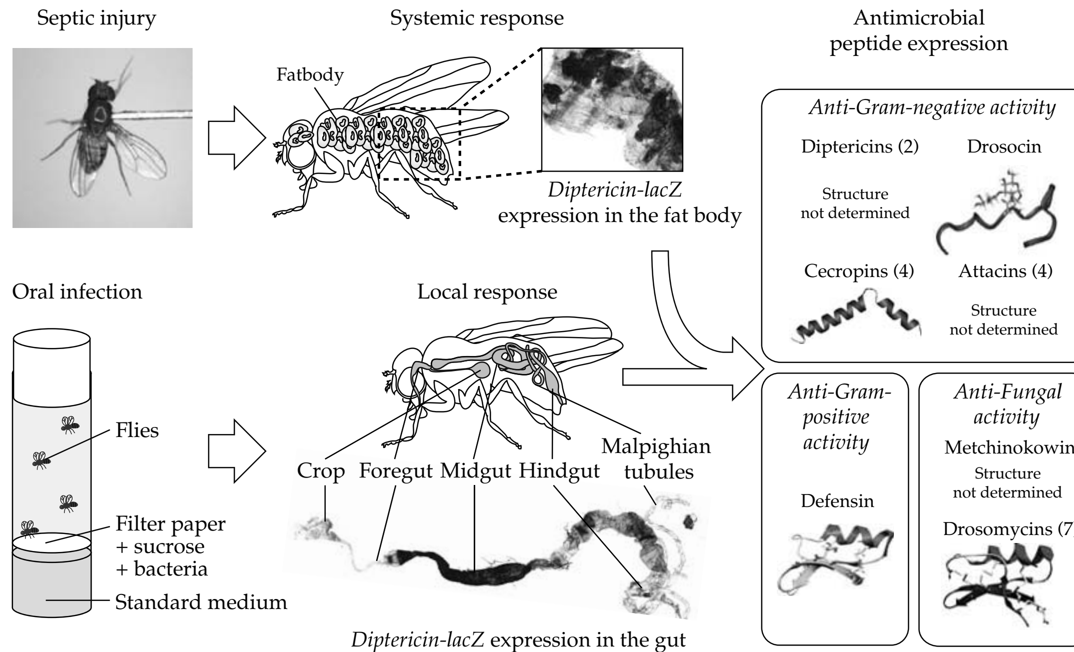


Figure 2.1 Overview of *Drosophila* systemic and local antimicrobial responses. Direct injection of bacteria into the haemolymph (septic injury) elicits a systemic immune response in the fat body, while ingestion of bacteria elicits a local immune response in the gut epithelium. Both tissues secrete a range of antimicrobial peptides with different activity spectra (classed here by their principal activity). *Diptericin-lacZ* expression in the fat body and gut is visualized by X-gal coloration.

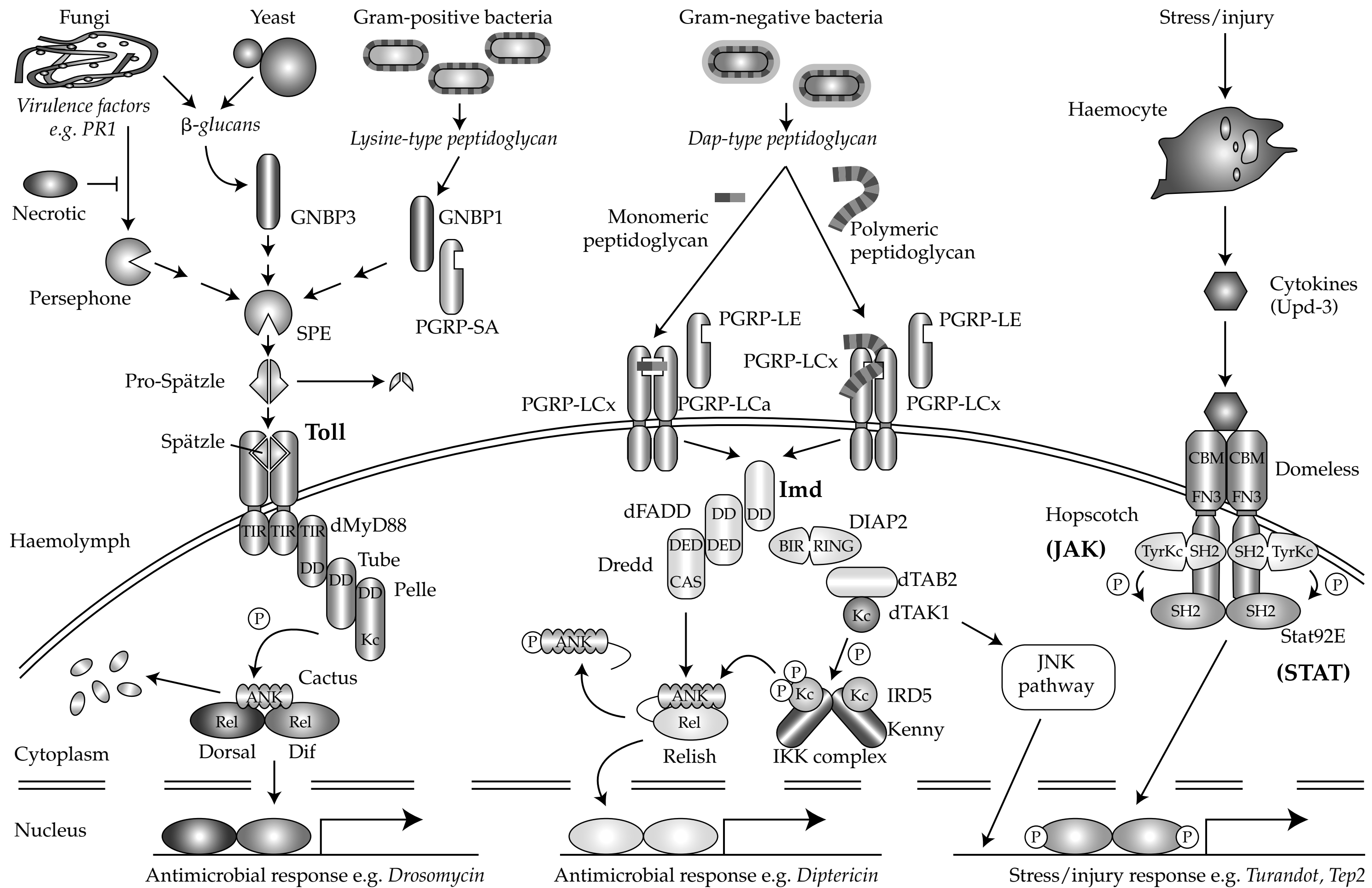


Figure 2.2 *Continued*

Hu *et al.*, 2004). Upon activation, Toll recruits a set of TIR- and/or death domain-containing adaptors that lead to activation of the kinase Pelle. Pelle, by an as yet unknown mechanism, leads to proteosomal degradation of Cactus, an inhibitor that maintains the cytoplasmic localization of two transactivators of the NF-κB family, Dif and Dorsal. The nuclear translocation of Dorsal and Dif induces the expression of many immune genes via binding to κB DNA motifs found in their promoters (Ip *et al.*, 1993; Engstrom *et al.*, 1993; Kappler *et al.*, 1993; Busse *et al.*, 2007). Flies carrying loss-of-function mutations in all components of the Toll pathway, except Cactus, are viable but extremely sensitive to infection by Gram-positive bacteria and fungi (Lemaitre *et al.*, 1996; Rutschmann *et al.*, 2002; Tauszig-Delamasure *et al.*, 2002). Importantly, these Toll-deficient flies do not exhibit proper expression of Toll-dependent proteins and peptides, such as the antifungal peptide Drosomycin.

The extracellular steps leading to the activation of Toll by its ligand Spätzle, share similarities with the coagulation proteolytic cascades of mammals or the melanization reaction of arthropods (Krem and Cera, 2002). Infection of the host by Gram-positive bacteria or fungi activates the proteolytic activity of clip-domain serine proteases (Piao *et al.*, 2005). This allows an amplification of the signal ending in the processing of the cytokine Spätzle by the terminal serine protease called Spätzle-processing enzyme (SPE) (Jang *et al.*, 2006). Three distinct cascades of serine proteases are activated by different classes of microbes functioning upstream of SPE. To date, these cascades are still poorly characterized in *Drosophila*. An *in vitro* study in another model insect, *Tenebrio molitor*, in which the entire cascade was reconstructed with purified proteases, suggests that it functions in three steps, with each active form cleaving and activating a downstream protease (Kim *et al.*, 2008; also see Chapter 3 in this volume). Direct recognition of the micro-organism by PRRs activates two of these cascades, whereas the third cascade might be activated by the proteolytic activity of virulence factors secreted by the pathogen (Gottar *et al.*, 2006). These cascades require tight regulation to avoid the potential of aberrant activation. To this end, serine protease inhibitors of the Serpin family, such as the serpin Necrotic, provide multiple layers of control to maintain proper activation of the Toll pathway (Levashina *et al.*, 1999). In this manner, regulation of the humoral response by the Toll pathway requires

Figure 2.2 Principal pathways regulating the systemic response in *Drosophila* (reviewed in Agaisse and Perrimon, 2004; Ferrandon *et al.*, 2007; Lemaitre and Hoffmann, 2007). Three distinct pathways regulate the response to microbial infection: the Toll pathway (activated mainly by fungi and Gram-positive bacteria), the Imd pathway (activated mainly by Gram-negative bacteria), and the JAK/STAT pathway activated by stress/injury sensed by haemocytes. Toll pathway (left): the Toll receptor is activated upon binding a cleaved form of Spätzle. Proteolytic cascades initiated by secreted recognition molecules (PGRP-SA and Gram-negative-bacteria-binding protein 1 (GNBP1) for Gram-positive bacteria, GNBP3 for β-glucans) or by direct cleavage of serine proteases (by fungal virulence factors) converge on Spätzle processing enzyme (SPE), which cleaves Spätzle. Spätzle binding induces Toll dimerization and subsequent recruitment of MyD88, Tube, and Pelle, leading to phosphorylation and proteasomal degradation of Cactus. Cactus degradation allows the Rel transcription factors Dif and Dorsal to translocate to the nucleus where they bind NF-κB-response elements and activate transcription of genes including *Drosomycin.* Imd pathway (centre): Imd is recruited by PGRP-LC upon direct binding to monomeric (LCx/LCa heterodimer) or polymeric (LCx heterodimer) diaminopimelic acid (DAP)-type peptidoglycan. Imd then recruits dFADD and the caspase Dredd, which might be responsible for the cleavage of phosphorylated Relish. This phosphorylation is thought to be mediated by an inhibitory κB (IκB) kinase complex (IRD5 and Kenny), itself activated by TAK1. TAK1 activation of the IκB kinase is dependent on its adaptor TAB2 and Imd and possibly dFADD and DIAP2. The precise nature of these relationships remains unclear. Phosphorylation and cleavage of its ANKyrin repeats allows the Rel domain of Relish to translocate to the nucleus where it binds different NF-κB-response elements and activates transcription of genes including *diptericin.* JAK/STAT pathway (right): JAK (Hopscotch), pre-associated with dimers of the receptor Domeless, is activated upon receptor binding of the cytokine Upd-3. Upd-3 itself is secreted into the haemolymph by haemocytes at sites of septic injury. JAK activation leads to recruitment of STATs (Stat92E), which are phosphorylated and dimerize, translocating to the nucleus to activate transcription of targets including *Turandot* genes. The stress/injury response may also be regulated by the c-Jun N-terminal kinase (JNK) pathway, which is activated by TAK1 downstream of Imd. ANK, ANKyrin repeats; BIR, baculovirus inhibitor of apoptosis repeat; CAS, caspase domain; CBM, cytokine-binding module; DD, death domain; DED, death-effector domain; FN3, fibronectin type III-like repeats; Kc, kinase domain; P, phosphate group; PGRP, peptidoglycan-recognition protein; Rel, Rel homology domain; RING, RING-finger domain; SH2, Src homology 2 domain; TIR, Toll/interleukin-1 receptor domain; TyrKc, tyrosine kinase domain.

the coordination of two building blocks, an extracellular cascade of proteases linked to PRRs and an intracellular NF-κB transactivator, that are coupled by Spätzle. Although Toll and its vertebrate counterparts, the TLRs, both play important roles in the host innate immune response, there is one major distinction between insect and vertebrate Toll functions; Toll is activated by the endogenous ligand Spatzle, while TLRs bind directly to microbial products. This has made it difficult to ascribe a common origin of immune function to the Toll receptors, despite their apparent similarities (Leulier and Lemaitre, 2008).

2.2.1.2 Imd

The Imd pathway is activated in response to Gram-negative bacteria and regulates a large set of antibacterial peptides (Figure 2.2). Mutations in genes encoding prototypic components of this pathway are fully viable, but highly susceptible to infection by Gram-negative bacteria (Lemaitre *et al.*, 1995). This increased susceptibility is associated with the inability to activate the transcription of AMP genes. The organization of the Imd pathway differs from the Toll pathway in that it is activated by a membrane-bound PRR, peptidoglycan-recognition protein (PGRP)-LC, expressed by fat body cells (Choe *et al.*, 2002; Gottar *et al.*, 2002; Rämet *et al.*, 2002). Upon Gram-negative infection, PGRP-LC recruits the Imd protein which then interacts with the adaptor dFADD via a death domain interaction (Leulier *et al.*, 2002; Naitza *et al.*, 2002). It is thought that dFADD then recruits the caspase Dredd (Leulier *et al.*, 2000). Dredd is proposed to associate with Relish (Hedengren *et al.*, 1999; Stöven *et al.*, 2000). Following Relish cleavage, the Rel transactivator domain translocates to the nucleus, whereas the inhibitory domain remains stable in the cytoplasm. Relish is phosphorylated by the inhibitory κB kinase (IKK) signalling complex (Rutschmann *et al.*, 2000; Silverman *et al.*, 2000; Lu *et al.*, 2001), which is itself thought to be activated by TAK1 and its adaptor TAB2 in an Imd- and possibly dFADD-dependent manner (Vidal *et al.*, 2001; Gesellchen *et al.*, 2005; Kleino *et al.*, 2005; Zhuang *et al.*, 2006). The Ring domain protein DIAP2 may participate via activation of dTAK1 (Gesellchen *et al.*, 2005; Kleino *et al.*, 2005; Leulier *et al.*, 2006). Although the Imd pathway is well characterized from a genetic point of view, the molecular steps that couple PGRP-LC dimerization at the cell surface to the nuclear translocation of Relish remain poorly characterized.

2.2.2 Synergistic interaction between Toll and Imd pathways

Toll and Imd form two distinct pathways that can be activated independently (Lemaitre *et al.*, 1996; Georgel *et al.*, 2001; De Gregorio *et al.*, 2002). However, both pathways usually function in synergy. Injection of microbes directly into the haemocoel generally leads to an activation of both pathways, although at different levels corresponding to the type of micro-organism injected (Lemaitre *et al.*, 1997). The most important mechanism of integration of the two pathways occurs at the gene-promoter level via the presence of different Toll and/or Imd κB-responsive elements (Busse *et al.*, 2007; Tanji *et al.*, 2007). In addition, many components of these pathways are regulated at the transcriptional level, which provides an additional layer of regulation that both amplifies the immune response and fosters cross-talk between the two pathways (De Gregorio *et al.*, 2002). Thus, a mutation that constitutively activates the Toll pathway results in faster activation of the Imd pathway upon subsequent infection of Gram-negative bacteria (Lemaitre *et al.*, 1996).

Recent large-scale analyses, at the transcriptome and proteome levels, have revealed that in addition to AMPs, the production of many peptides and proteins is activated following septic injury (Figure 2.3) (De Gregorio *et al.*, 2001; Irving *et al.*, 2001). Further genetic evidence demonstrated the Toll and Imd pathways to be the major regulators of this response (De Gregorio *et al.*, 2002). Some of these target genes are involved in the regulation of the systemic immune response itself (e.g., signalling components). Others participate in distinct defence mechanisms as components of the melanization cascade or clotting system, or opsonins. A third group of proteins includes putative immune effectors. Among this group are 17 members of the *Drosophila* immune molecule (DIM) family

Recognition

PGRP-LB
PGRP-LC
PGRP-LF
PGRP-SA
PGRP-SB1
PGRP-SC2
PGRP-SD
GNBP-like
GNBP-like 3
Idgf1
Idgf3

Signalling

Imd	*Toll*		*JNK*
IMD	Necrotic	Pelle	d-Jun
Relish	spirit	Cactus	Puckered
Pirk	SPE	Dorsal	
	Spätzle	Dif	
	Toll		

Small peptides

Known antimicrobials	*Peptides of unknown function*
4 Attacins	
4 Cecropins	DIM1
Defensin	DIM2
2 Diptericins	DIM4
Drosocin	DIM23
2 Drosomycins	13 other DIMs*
Metchnikowin	21 other peptides

Other responses

Stress
Turandot
Frost

Iron metabolism
Zip3
Transferrin

ROS metabolism
Peroxidase (IRC)

Enzymes
3 Carboxylesterases
β-Galactosidase
Lip1

Humoral responses

Melanisation
Pale, MP1, Punch, MP2, Dhpr, Serpin27A, Ddc, Serpin28D, yellowf, laccase-like, Cp19

Coagulation
Fondue

Opsonisation
Tep2
Tep4
α2M-receptor-like

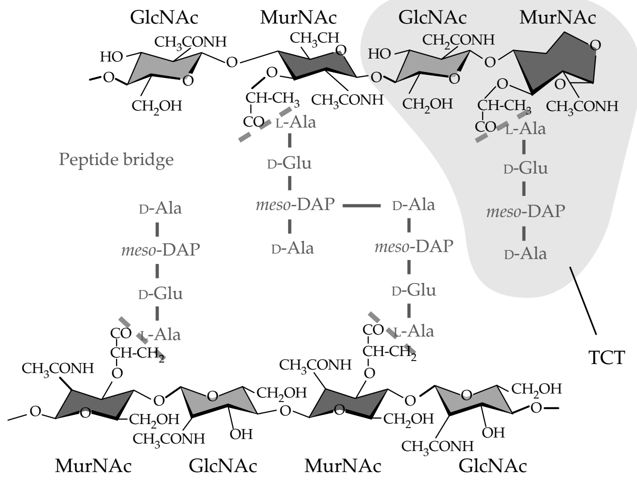
Unknown function
3 Serpins
14 Serine proteases

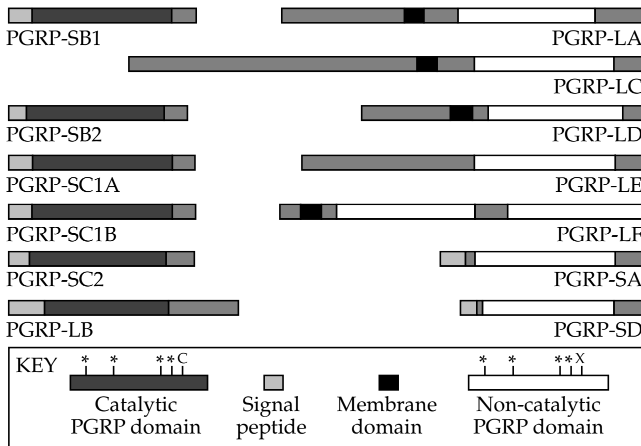
Figure 2.3 *Drosophila* immune-regulated genes. Microarray analysis has identified the repertoire of genes regulated upon septic injury with a mixture of Gram-positive and Gram-negative bacteria or natural fungal infection (De Gregorio *et al.*, 2001; Irving *et al.*, 2001). Only upregulated genes are shown, organised by their putative or determined functions. **Drosophila* immune molecules (DIMs) were identified as peptides induced by the immune response, rather than upregulated transcripts (Uttenweiler-Joseph *et al.*, 1998).

and eight Turandot proteins, which are small peptides secreted by the fat body with a possible role in the host stress response (Uttenweiler-Joseph *et al.*, 1998; Ekengren *et al.*, 2001). Furthermore, one catalase gene, two transferrin genes, and one iron-transporter gene are also induced following septic injury, suggesting a role for reactive oxygen species (ROS) and iron sequestration to limit microbial development (De Gregorio *et al.*, 2001; Irving *et al.*, 2001). Iron is essential for most invading micro-organisms during the course of an infection, and both animals and plants have evolved elaborate immune strategies to limit iron availability to micro-organisms. In addition, many uncharacterized genes are upregulated upon infection. A rapid survey of target genes of the Toll and Imd pathways suggests that the Toll pathway has a predominant role in providing the secreted immune components that function in the haemolymph, such as clotting and melanization factors and has a specific antimicrobial role against invading fungi. In contrast, the Imd pathway appears to orchestrate the antibacterial responses via the regulation of most antibacterial peptides. Accordingly, the Imd pathway is activated with more rapid kinetics, peaking at 6 h, while Toll regulates many late responsive genes (24–48 h). Despite our knowledge of the target genes of each pathway, their specific contribution to host defence is not completely understood and remains a major challenge in the field.

2.2.3 Two pathways, specific microbial elicitors

The initial observation that different classes of micro-organisms induced specific patterns of AMP expression, implies that *Drosophila* is able to sense and discriminate between microbes (Lemaitre *et al.*, 1997). The basis of this specificity is through host recognition of microbial molecules by proteins called PRRs. These receptors interact directly with conserved motifs of micro-organisms referred to as pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1997). In *Drosophila*, recognition of bacteria is achieved largely through the sensing of specific forms of peptidoglycan by PGRPs (Figure 2.4) (reviewed in Steiner, 2004; Royet and Dziarski, 2007). Peptidoglycan is an essential component of the cell wall of both Gram-negative and Gram-positive bacteria. It consists of long glycan chains of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues that are cross-linked to each other by short peptide bridges. Peptidoglycan is a highly complex and fast-evolving molecule with marked differences from one bacterium to another. However,





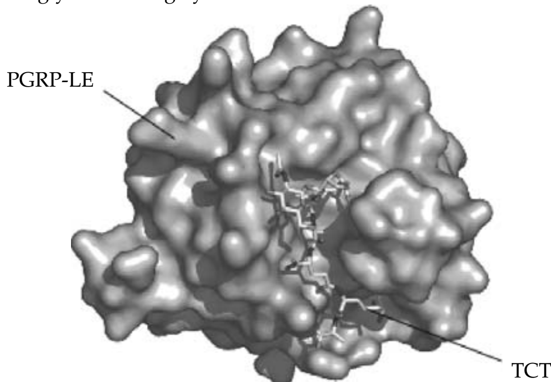


Figure 2.4 Peptidoglycans and PGRPs. Recognition of bacteria is mediated by the detection of peptidoglycan, a critical bacterial cell wall component. Peptidoglycan is a peptide cross-linked polymer of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). Depicted is the structure of *Escherichia coli* peptidoglycan, which, as in all Gram-negative bacteria, contains a *meso*-DAP residue in the peptide bridge, where most Gram-positive bacteria have an L-lysine. The terminal peptidoglycan monomer (highlighted) of all *E. coli* peptidoglycan glycan chains contains a unique variant of MurNAc with an internal 1,6-anhydro bond. This monomer is the minimal signature for detection of peptidoglycan and is referred to as tracheal cytotoxin (TCT). PGRPs bind peptidoglycan by a conserved 160 amino acid PGRP domain (related to bacteriophage T7 lysozyme). The *Drosophila* genome contains 13 PGRP genes, divided into Short and Long classes by their transcript length and the presence or absence, respectively, of a signal peptide. Catalytic or amidase PGRPs conserve zinc-binding residues (*), including a diagnostic C-terminal cysteine, required for the amidase activity which cleaves the peptidoglycan peptide bridge from the sugar backbone (dashed line on the top peptidoglycan molecules). PGRPs lacking these residues are referred to as recognition PGRPs. Illustrated is the three-dimensional structure of PGRP-LE binding to TCT (Kaneko *et al*., 2006).

peptidoglycan from Gram-negative bacteria differs from most Gram-positive peptidoglycan by the replacement of lysine with *meso*-diaminopimelic acid (meso-DAP) at the third position in the peptide chain. There is, however, a subclass of Gram-positive bacteria including *Bacillus* species, which possess DAP-type peptidoglycan. Additionally, the localization of peptidoglycan in the cell wall is different between Gram-negative and Gram-positive bacteria. Gram-negative peptidoglycan consists of a single layer and is hidden in the periplasmic space underneath the outer membrane and lipopolysaccharide layer, whereas peptidoglycan from Gram-positive bacteria is multilayered and exposed at the bacterial surface. Studies using highly purified bacterial compounds have shown that the specificity of Imd to Gram-negative bacteria is a result of receptor activation by DAP-type peptidoglycan, whereas the Toll pathway is activated by Lys-type peptidoglycan (Leulier *et al.*, 2003). In contrast to vertebrates, lipopolysaccharide endotoxin, the major component of the Gram-negative cell envelope, has no effect on Toll and Imd pathway activity, and previous results implying an interaction are explained by the presence of peptidoglycan contaminants in commercial lipopolysaccharide preparations (Leulier *et al.*, 2003; Kaneko *et al.*, 2004). More in-depth analysis of the Imd-dependent PGRP response to DAP-type peptidoglycan has demonstrated that both polymeric and monomeric forms are capable of activating the Imd pathway. Specifically, the GlcNAc-MurNAc (anhydro)-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala monomer, also known as tracheal cytotoxin (TCT), was identified as the minimal peptidoglycan motif capable of efficient induction of the Imd pathway (Kaneko *et al.*, 2004; Stenbak *et al.*, 2004). TCT provides an ideal 'signature' for Gram-negative bacteria, as this muropeptide is positioned at the end of the peptidoglycan strand and is released from peptidoglycan (as reviewed by Cloud-Hansen *et al.*, 2006). In contrast, the minimum structure needed to activate the Toll pathway is a muropeptide dimer of Lys-type peptidoglycan (Filipe *et al.*, 2005; Kim *et al.*, 2008).

2.2.3.1 PGRPs

PGRPs are highly conserved from insects to mammals and share a 160 amino acid domain (the PGRP domain) with similarities to bacteriophage T7 lysozyme, a zinc-dependent N-acetylmuramoyl-L-alanine amidase (Yoshida *et al.*, 1996; Kang *et al.*, 1998; Royet and Dziarski, 2007). Sequence analysis of the 13 *Drosophila* PGRPs points to the existence of two subgroups with either recognition or enzymic properties (Figure 2.4). Members of the first group lack zinc-binding residues required for amidase activity, but still retain the ability to bind and recognize peptidoglycan. Most of them function as PRRs. Genetic and molecular studies have shown that PGRP-LC (where L stands for long form) is the receptor of the Imd pathway involved in the sensing of DAP-type peptidoglycan (Choe *et al.*, 2002; Gottar *et al.*, 2002; Rämet *et al.*, 2002). Importantly, the PGRP-LC locus encodes three isoforms that all differ by their external PGRP domain (Figure 2.4). Cell culture assays have shown that polymeric DAP-type peptidoglycan is sensed by a homodimer of PGRP-LCx, while TCT is recognized by a heterodimer of PGRP-LCx with PGRP-LCa, the latter functioning as an adaptor (Kaneko *et al.*, 2004). PGRP-LE encodes a PGRP with affinity to DAP-type peptidoglycan and is expressed both extra- and intracellularly (Takehana *et al.*, 2004). A fragment of PGRP-LE corresponding to the PGRP domain alone functions extracellularly to enhance PGRP-LC-mediated peptidoglycan recognition on the cell surface (Kaneko *et al.*, 2006). A full-length form of PGRP-LE is also present in the cytoplasm and acts as an intracellular receptor for monomeric peptidoglycan that can activate AMP expression without the requirement for PGRP-LC (Kaneko *et al.*, 2006). Although not part of the systemic antimicrobial response, it should be noted that this full-length form of PGRP-LE also appears to be essential in haemocytes to prevent intracellular growth of *Listeria monocytogenes* through its ability to induce autophagy. The discovery that PGRP-LE functions as an intracellular sensor of DAP-type peptidoglycan, indicates the presence of specific immune defences of *Drosophila* to intracellular bacteria (Yano *et al.*, 2008).

The Toll pathway is activated by Gram-positive bacteria via the secreted PRR PGRP-SA (where S stands for short form) in interaction with Gram-negative-bacteria-binding protein (GNBP)1 (Michel *et al.*, 2001; Gobert *et al.*, 2003). The exact role of GNBP1 is still a matter of debate. It has been proposed that GNBP1 cleaves peptidoglycan to

promote its recognition by PGRP-SA (Filipe *et al.*, 2005; Wang *et al.*, 2006), while studies in other insects suggest that this protein links PGRP-SA to a downstream serine protease (Park *et al.*, 2007).

The tertiary structures of four PGRPs have now been solved (Kim *et al.*, 2003; Chang *et al.*, 2004, 2005, 2006; Reiser *et al.*, 2004; Lim *et al.*, 2006). A prominent feature is the presence of an extended surface groove in the PGRP domain, which includes a zinc-finger cage in the catalytic PGRP-LB. The structures of PGRP-LE and PGRP-LCa/x in complex with TCT show an interaction between the peptide stem of peptidoglycan and the PGRP groove (Lim *et al.*, 2006; Chang *et al.*, 2006). In contrast to other PGRPs, the PGRP domain of PGRP-LCa does not possess a typical peptidoglycan docking groove, in agreement with its role as a co-receptor sensing monomeric DAP-type peptidoglycan (Chang *et al.*, 2005; Mellroth *et al.*, 2005).

2.2.3.2 Gram-negative-bacteria-binding proteins (GNBPs)

GNBPs form an important family of insect PRRs that contain both a glucan-binding site and a mutated glucanase domain (Lee *et al.*, 1996). There are three GNBPs in *Drosophila* and one of them, GNBP1, participates in the sensing of Lys-type peptidoglycan (Figure 2.2). In contrast, genetic studies indicate that GNBP3 is a PRR acting upstream of the Toll pathway in the sensing of glucans derived from fungi (Gottar *et al.*, 2006). The serine protease cascade that links GNBP3 to SPE has not yet been deciphered.

2.2.4 Sensing of virulence factors and endogenous stress signals

The activation of the Toll and Imd pathways by PGRP and GNBP recognition of microbial elicitors supports the concept of PRRs originally proposed by C. Janeway (Janeway, 1989). Current research in the field is aimed at understanding how bacteria or fungi are detected during the natural course of infection and how different microbial elicitors reach their specific PRR. Another important question is the existence of other modes of recognition that do not involve PRRs. An alternative mode of sensing is based on direct sensing of virulence factors, in a manner analogous to the guard system of plants. This mechanism appears to be central to the sensing of entomopathogenic fungi such as *Beauveria bassiana* and *Metharizium anisopliae* (Gottar *et al.*, 2006). Spores from these fungi have the capacity to germinate on the fly and produce hyphae that can penetrate the cuticle of insects. This direct mode of entry is mediated through the abundant production of proteases, lipases, and chitinases by the fungus. It has been proposed that the presence of *Beauveria bassiana* is detected, independent of GNBP3, through direct activation of the Toll pathway by a fungal protease PR1. PR1 would cleave the host serine protease, Persephone, which leads to Toll-pathway activation (Figure 2.2) (Gottar *et al.*, 2006). Thus, entomopathogenic fungi would be recognized by the presence of proteases produced in order to enter the insect.

It is important to note that sterile injury, in the absence of micro-organisms, also weakly activates the Imd and Toll pathways. It is not yet clear whether this activation is due to the presence of microbial products or to the detection of host molecules released at the wound site. However, the existence of endogenous ligands is supported by the observation that larvae with melanotic tumours induce significant AMP gene expression (Ligoxygakis *et al.*, 2002; Scherfer *et al.*, 2006). This suggests a possible link between melanization and systemic expression of AMP.

2.2.5 Adjusting the immune response

Extensive activation of the immune response is generally considered deleterious for the host in terms of both resource allocation and potential damage to host tissues. Indeed, aberrant activation of the Imd or the Toll pathways results in lethality (Georgel *et al.*, 2001). Recent studies in *Drosophila* have revealed that multiple levels of regulation are employed to suppress Imd pathway activity and prevent excessive or prolonged immune activation (Figure 2.5).

2.2.5.1 Catalytic downregulators of the Imd pathway

In contrast to recognition PGRPs, proteins referred to as catalytic PGRPs have demonstrated (PGRP-SC1A/B, -LB, -SB1/2) or predicted (PGRP-SC2)

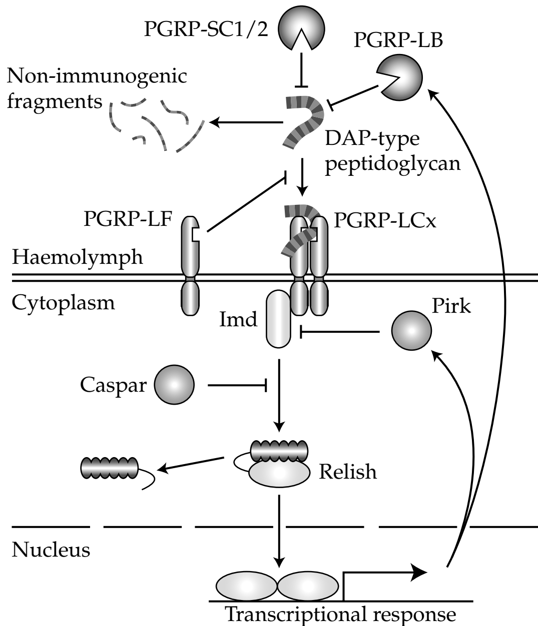


Figure 2.5 Negative regulation of Imd signalling. Prolonged or constitutive activity of the Imd pathway is likely to be deleterious for *Drosophila*, so various mechanisms have evolved to limit activation and downregulate it following an immune challenge. Detection of small quantities of DAP-type peptidoglycan is prevented by the amidases PGRP-SC1/2, which cleave peptidoglycan into non-immunogenic fragments (Bischoff *et al.*, 2006; Zaidman-Rémy *et al.*, 2006), and PGRP-LF, which binds DAP-type peptidoglycan and may sequester it, preventing Imd activation (Maillet *et al.*, 2008). Intracellularly, Caspar, a homologue of human FAF1, blocks the cleavage and nuclear translocation of Relish (Kim *et al.*, 2006). Immune activation of the Imd pathway leads to upregulated expression of the amidase PRGP-LB, which cleaves peptidoglycan, and a novel protein, Pirk, which binds to PGRP-LC (Aggarwal *et al.*, 2008; Lhocine *et al.*, 2008; Kleino *et al.*, 2008). These proteins downregulate the Imd pathway, limiting the duration of the immune response.

zinc-dependent amidase activity that removes peptides from the glycan chains, thereby eliminating the immuno-stimulatory activity of peptidoglycan. Secreted amidase PGRPs, such as PGRP-SC1A and PGRP-LB, scavenge extracellular peptidoglycan and prevent its binding to PGRP-LC (Bischoff *et al.*, 2006; Zaidman-Remy *et al.*, 2006). Interestingly, PGRP-LB is a target of the Imd pathway, establishing a negative-feedback mechanism capable of adjusting the level of the immune response to the severity of infection.

2.2.5.2 Non-catalytic downregulators of the Imd pathway

Scavenging of immune-activating peptidoglycan by amidases is only one approach used by *Drosophila* to downregulate Imd pathway activation. Recently, PGRP-LF, a membrane-bound non-catalytic PGRP with two PGRP domains, was demonstrated to be a key negative regulator of PGRP-LC signalling (Maillet *et al.*, 2008). Specifically, PGRP-LF prevents PGRP-LC activation in the absence of infection, but in contrast to PGRP-SC and -LB does not affect the Imd pathway after immune challenge. The mechanism of action of PGRP-LF is not known, but may prevent aberrant activation of both the Imd and c-Jun N-terminal kinase (JNK) pathways by residual peptidoglycan fragments ingested with food or released by indigenous microbes. Additionally, *Drosophila* deficient in PGRP-LF exhibit defects (abnormal wings) associated with the constitutive activation of these pathways in developmental tissues such as imaginal discs. Pirk (Poor Imd response upon knock-in) a protein interacting with PGRP-LC and regulated by the Imd pathway, has been shown to regulate the Imd pathway receptor and thus, participate in the precise control of Imd pathway induction (Aggarwal *et al.*, 2008; Kleino *et al.*, 2008; Lhocine *et al.*, 2008).

2.2.6 Relevance of Toll and Imd pathway to host defence

Mutations affecting the Toll and Imd pathways have their greatest impact on resistance to systemic infections by septic injury, and death is always associated with excessive bacterial or fungal proliferation (Lemaitre *et al.*, 1996; Rutschmann *et al.*, 2002). For this reason, it has been proposed that the Toll- and Imd-mediated systemic response plays an important role against opportunist infections that can occur upon host injury (Hultmark, 2003). Nevertheless, flies carrying mutations affecting the Toll pathway are more susceptible to natural infection with spores of *Beauveria bassiana* (Lemaitre *et al.*, 1997). This demonstrates a clear role of the Toll-mediated systemic immune response for resistance against entomopathogenic fungi. To date, there is no evidence demonstrating a role of the Imd pathway in the fat body during bacterial

natural infection, although this pathway has been shown to be critical for local defence against ingested bacteria in the gut (Liehl *et al.*, 2006; Ryu *et al.*, 2006; Nehme *et al.*, 2007).

2.2.7 Contribution of additional pathways to Drosophila immune response

While Toll and Imd are the main regulators of the *Drosophila* immune response, additional pathways have emerged as important participants in the systemic immune response (Figure 2.2). Gene expression profiling has identified a subset of *Drosophila* immune-response genes that are regulated by the JAK/STAT pathway, namely genes encoding the complement-like protein TEP2 and Turandot stress proteins (Agaisse *et al.*, 2003). It has been proposed that upon tissue damage haemocytes release a cytokine, Unpaired-3, that activates Domeless, the receptor of the JAK/STAT pathway, in the fat body. This pathway does not regulate AMPs during the systemic response but has been associated with the response to stress and tissue damage (Pastor–Pareja *et al.*, 2008).

The JNK pathway regulates many cellular processes in *Drosophila* and is required for proper healing of the epidermis following injury (Rämet *et al.*, 2001). In cell culture, JNK-dependent immune genes encode many proteins involved in cytoskeleton remodeling (Boutros *et al.*, 2002). Imd activates the JNK pathway through TAK1 that is thought to phosphorylate the JNK kinase *basket* (Silverman *et al.*, 2003). Additionally, some negative feedbacks between the Imd-Relish and Imd-JNK branches have been reported (Park *et al.*, 2004; Kim *et al.*, 2005). The exact contribution of the JNK pathway to the host defence is still a matter of debate, but it has been suggested that JNK is required for AMP gene expression by the fat body (Kallio *et al.*, 2005; Delaney *et al.*, 2006).

2.3 Epithelial immunity: the local immune response

The systemic immune response has been studied through the direct introduction of pathogens into the body cavity. This approach has limited the study of the immune response to the steps involved in recognition and antimicrobial response in the fat body. While these studies have revealed insights on the molecular basis of the *Drosophila* immune response, they may not reflect the most common mode of host interaction with potential pathogens. In metazoans, the epithelia of the digestive, respiratory, and genital tracts are constantly exposed to microbes of both indigenous and environmental origin. Thus, these routes have the potential to be the major routes of infection for a host. The next section of this chapter will focus on the immune epithelial response.

2.3.1 A gut-associated immune system

The systemic mode of infection bypasses the layered steps of a given host-microbe interaction by directly activating the immune response. The mere presence of a microbial elicitor is sufficient to induce an immune response. However, the circulatory system of animals is generally sterile, which is not the case for epithelia, especially that of the digestive tract that is frequently associated with an indigenous microbiota. In contrast to the systemic response, epithelia must tolerate the presence of some microbes while responding to potential pathogens. This implies a tight and specific regulation of the immune response in epithelia, balancing between immune activation and bacterial tolerance. Physical attributes of the host and host epithelial environment are one factor that can prevent either colonization or immune activation, thereby reducing the number of microbes that truly interact with the host. For this reason, only a handful of bacteria have been described as being infectious to *Drosophila* via oral ingestion (*Serratia marcescens*, *Enterococcus faecalis*, *Vibrio cholerae*, *Erwinia carotovora*, *Pseudomonas entomophila*) (Basset *et al.*, 2000; Vodovar *et al.*, 2005; Nehme *et al.*, 2007; reviewed in Vallet-Gely *et al.*, 2008). These bacteria are capable of persisting in the gut and/or inducing a local immune response (Figure 2.1). Among them, *E. carotovora* is able to induce a strong local and systemic immune response, but differs in that infection does not kill the host (Basset *et al.*, 2000). The use of these bacteria has demonstrated that two complementary effector mechanisms are key in controlling bacterial persistence and infection

in the gut: generation of ROS and local production of AMPs.

2.3.1.1 ROS production

In *Drosophila*, oral ingestion of bacteria induces rapid ROS synthesis in the gut by a NADPH oxidase enzyme, called dDuox. Adult flies in which dDuox expression is silenced by RNA interference (RNAi) show a marked increase in mortality following ingestion of microbe-contaminated food (Ha *et al*., 2005a). Ingested bacteria were shown to persist and proliferate throughout the intestinal tract of *dDuox* RNAi flies. To maintain the homeostatic redox balance perturbed by the ingestion of microbes, wild-type flies also express an antioxidant system composed of an extracellular immune-regulated catalase (IRC) (Ha *et al*., 2005a, 2005b). This ROS-dependent gut immunity is not affected by the Imd pathway and provides an initial barrier against ingested microbes. However, it has been shown that ROS-resistant bacteria are still controlled by local AMP expression (Ryu *et al*., 2006).

2.3.1.2 Local AMP expression

As observed in systemic infection, several AMP genes under the control of the Imd pathway (e.g. *Diptericin* and *Attacin)* are expressed in the digestive tract upon oral infection by Gram-negative bacteria (Tzou *et al*., 2000). This local AMP production is critical in the host response to control oral infection by Gram-negative bacteria; flies lacking a functional Imd pathway in the gut are more susceptible upon oral infection with *P. entomophila* and *S. marcescens* (Liehl *et al*., 2006; Nehme *et al*., 2007). The relevance of the local production of AMP is also revealed by the strategy of some entomopathogens that produce abundant extracellular metalloproteases that are capable of degrading AMPs (Liehl *et al*., 2006).

Similarly, as in the systemic response, the local immune response is inducible and triggered by the recognition of Gram-negative peptidoglycan by PGRP-LC activating the Imd pathway (Zaidman-Remy *et al*., 2006). AMP genes are also expressed in specific domains along the digestive tract (Figure 2.1), revealing that the gut is a complex and compartmentalized organ with distinct immunoreactive domains (Senger *et al*., 2006; Buchon *et al*., 2009). Additionally, transcription factors involved in defining cell identity, like the homeobox gene *caudal*, have been identified in controlling AMP expression in the gut. Caudal represses the expression of AMP in the posterior part of the midgut despite the presence of a functional Imd pathway in this location (Ryu *et al*., 2008). It was proposed that the suppression of AMP by Caudal is essential for the establishment of a beneficial microbiota in this segment of the gut (see below).

2.3.2 Gut microbiota and bacterial tolerance

Host ingestion of microbes typically results in benign interactions. Examination of gut-associated microbes of animals has demonstrated tolerance to a diverse and complex consortium of bacteria. Molecular analysis of the *Drosophila*-associated microbiota has revealed a relatively low species diversity, dominated by *Acetobacter*, *Gluconobacter*, and *Lactobacillus* spp. (Corby-Harris *et al*., 2007; Cox and Gilmore, 2007; Ren *et al*., 2007; Ryu *et al*., 2008). The constant presence of this microbiota raises the question as to why they do not generate a state of permanent immune activation in the gut and why this microbiota is not eliminated by the gut immune defence. This topic is also discussed in Chapter 7 in this volume.

2.3.2.1 Host factors that scavenge peptidoglycan and promote microbiota tolerance

A central role in bacterial tolerance of the gut has been attributed to amidase PGRPs, as they are proposed to scavenge peptidoglycan released by gut microbes (Bischoff *et al*., 2006; Zaidman-Remy *et al*., 2006). Resident bacteria may have a low rate of growth in the gut and therefore would release only low amounts of peptidoglycan that can be readily hydrolysed by amidase PGRPs, whereas infectious bacteria release large amounts of peptidoglycan while proliferating (Zaidman-Remy *et al*., 2006). This implies a threshold response for local immune activation to differentiate between indigenous micro-organisms and invading pathogens. This is supported by experiments showing that RNAi extinction of amidase-encoding genes *PGRP-SC1/2* or *PGRP-LB,* induces higher *Diptericin* expression after oral bacterial infection compared to wild-type flies (Bischoff *et al*., 2006; Zaidman-Remy *et al*., 2006).

Thus, amidase PGRPs downregulate the immune response and modulate the immune reactivity of the fly. Reduction of the immune response by amidase PGRPs may also prevent damage to host tissues from prolonged immune activity as demonstrated by increased lethality and developmental defects of *PGRP-SC1/2* RNAi larvae orally infected with bacteria (Bischoff *et al*., 2006). In addition to amidase PGRPs, negative regulators of the Imd pathway, such as Pirk, also prevent activation of the Imd pathway by the gut microbiota, thus promoting tolerance (Lhocine *et al*., 2008).

2.3.2.2 Host transcription factors that promote the establishment of the gut microbiota

However, despite this role of amidase PGRPs, the microbiota still activates the Imd pathway as reflected by the permanent nuclear translocation of Relish along the gut. The homeobox transcription factor Caudal has been shown to downregulate AMP expression at the transcriptional level in the posterior part of the midgut. This appears to be critical for the establishment of a normal gut microbiota. RNAi inhibition of *caudal* leads to increased expression of antimicrobial peptides in the posterior part of the gut and long-term mortality. This increased mortality and loss of gut immune regulation is associated with an imbalance of the gut microbiota (Ryu *et al*., 2008). Both the lack of Caudal or artificial over-expression of AMPs increased the representation of an AMP-resistant minor constituent of the microbiota, resulting in the observed pathology. This study identified one mode of interaction between the microbiota and host immunity and suggests a complex interplay between the host, gut microbiota, and innate immune response.

2.3.3 Other epithelial responses

Use of *Drosophila* green fluorescent protein (GFP) reporter genes has revealed that all epithelia in communication with the external environment (e.g. gut) express a subset of AMPs (Ferrandon *et al*., 1998; Tzou *et al*., 2000). Expression of AMPs in epithelia is inducible and regulated by the Imd pathway. In some tissues, AMPs are constitutively expressed and in this case their expression is thought to be governed by developmental genes such as Caudal (Ryu *et al*., 2008; reviewed by Uvell and Engstrom, 2007).

In *Drosophila*, tracheae are formed by invaginations of the ectoderm and thus, are lined by a cuticular intima that is continuous with the external cuticle. Tracheae are likely to be exposed constantly to microbes, although little is known about pathogens that can infect this tissue or the specifics of tracheal immune defense (Wagner *et al*., 2008). Natural infection with Gram-negative bacteria such as *Erwinia carotovora* induces the expression of AMPs in the trachea (Tzou *et al*., 2000). Interestingly, the antifungal peptide gene *Drosomycin* is induced in the trachea by the Imd pathway in contrast to the regulation observed during the systemic response. Additionally, infection induces local activation of the phenoloxidase cascade leading to melanization that may confine the spread of infection (Tang *et al*., 2008).

Another epithelium that risks injury and infection is the genital epithelia of females following copulation. In order to prevent infection, many AMPs are expressed in this tissue. This is the case for Drosomycin which is constitutively expressed in spermathecae, receptacles that store spermatozoids (Ferrandon *et al*., 1998). Interestingly, it has been shown that sex peptide, a short peptide present in the sperm of males, induces local AMP expression in females (Peng *et al*., 2005). This induction appears to be independent of any microbial elicitors and may limit the entry of potential infectious agents just after copulation (see also Chapter 15 in this volume).

2.4 Interaction between the local and systemic immune response

The local immune response in diverse epithelia and the systemic immune response of the fat body are independent immune mechanisms, as each can be selectively induced depending on the mode of infection. However, some infections may require coordination of both systems to fully control pathogens. Recently, a number of recent studies have identified potential mechanisms that facilitate communication between the different immune compartments of *Drosophila* (Figure 2.6).

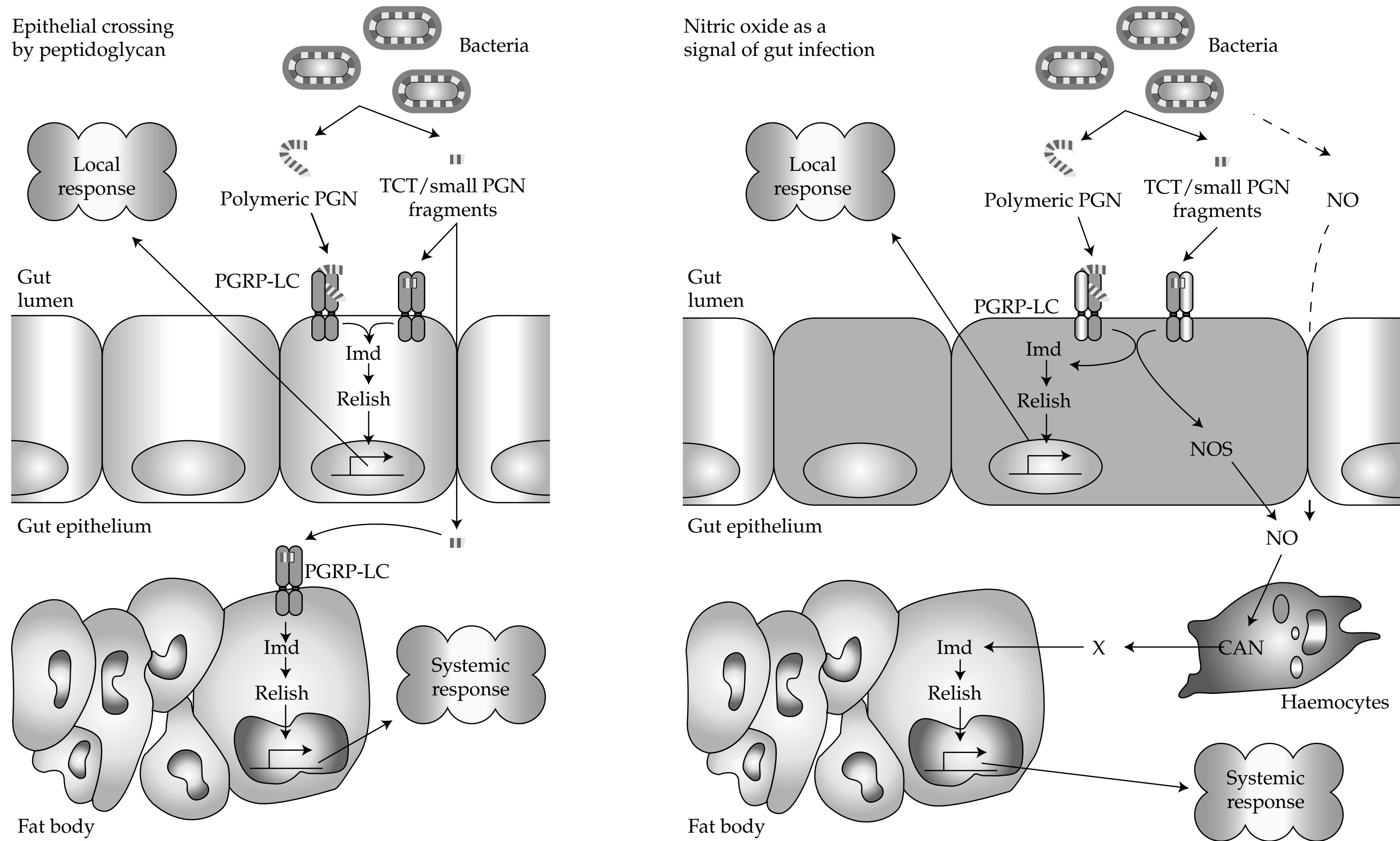


Figure 2.6 Models for fat body sensing of gut infections. Oral infection with certain bacteria (e.g. *P. entomophila*) or ingestion of TCT results in both a local and systemic immune response. Small fragments of peptidoglycan (PGN) from bacteria in the gut could cross the gut epithelium and enter the haemolymph, where they can be detected by the fat body (Zaidman-Rémy *et al.*, 2006). At low bacterial densities, amidase PGRP degradation of peptidoglycan in the gut would limit this peptidoglycan crossing. Alternatively, a second messenger system, potentially involving NO, could signal to fat body. The local immune response of the gut increases the activity of nitric oxide synthase (NOS), potentially leading to secretion of NO into the haemolymph (Foley and O'Farrell, 2003). Circulating haemocytes are proposed to detect NO in a calcineurin (CAN)-dependent manner and generate an unidentified signal (X) which stimulates the Imd pathway in the fat body, leading to a systemic immune response (Dijkers and O'Farrell, 2007). An additional facet of this model is that haemocytes could also detect NO generated directly by bacteria and translocated across the gut epithelium.

2.4.1 Gut infection and fat body immune response

Both *Ecc15* and *P. entomophila* are able to trigger a strong systemic immune response in *Drosophila* larvae following oral ingestion, pointing to an integration of both responses at the whole-organism level and the existence of a signalling mechanism between the gut and the fat body (Basset *et al*., 2000; Vodovar *et al*., 2005). This immune response correlates with the capacity of these bacterial species to persist and multiply inside the gut and does not appear to rely on physical crossing of the gut wall. Two mechanisms have been proposed to explain the capacity of bacteria that infect through the oral route to induce the systemic immune response.

2.4.1.1 A role for nitric oxide

Nitric oxide (NO) is a signalling molecule implicated in multiple physiological processes in animals, including innate immunity. In vertebrates, NO possesses direct effector functions and is an important signalling molecule that regulates gene expression and influences cell differentiation. NO has also been implicated in gastrointestinal motility, mucosal permeability after bacterial infection, and epithelial-associated pathologies such as colon cancer (Bogdan, 2001). In *Drosophila*, biochemical modulation of NO signalling has demonstrated its requirement in immune signalling between the gut and fat body. Reduction of nitric oxide synthase (NOS) activity in *Drosophila* using the inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), led to increased larval lethality after oral ingestion of *Ecc15*. In addition, this study demonstrated that exogenous NO in the gut triggers *Diptericin* expression in the fat body even in the absence of a pathogen (Nappi *et al*., 2000; Foley and O'Farrell, 2003). According to this model, ingestion of bacteria induces NO in sentinel tissues like the gut, and activates a signalling cascade in haemocytes that leads to induction of the Imd pathway in the fat body by an unknown mechanism. Additionally, mutations in *Drosophila* calcineurin have shown that NO signalling acts in a calcium-dependent manner (Dijkers and O'Farrell, 2007). Interestingly, NO production is also produced in response to bacterial infection in the Malpighian tubules, and induces AMP production in the same tissue in an autocrine fashion (McGettigan *et al*., 2005).

2.4.1.2 Translocation of peptidoglycan

Alternatively, it has been proposed that this systemic immune response is mediated by the translocation of small peptidoglycan fragments from the gut lumen to the haemolymph. This view is supported by the observation that ingestion of monomeric peptidoglycan can stimulate a strong systemic immune response in PGRP-LB RNAi flies that have reduced amidase activity and are unable to degrade peptidoglycan to its non-immunogenic form (Zaidman-Remy *et al*., 2006). Transfer of peptidoglycan would provide an indirect mechanism for recognition of Gram-negative bacteria that may explain the existence of different PGRP-LC isoforms devoted to the detection of monomeric peptidoglycan that are small enough to efficiently cross the gut barrier.

2.4.2 Other examples of immune response integration

Additional studies support the existence of dialogue between other immune tissues. Larvae mutated for Serpin 77BE have melanized trachea and this local melanization is sufficient to activate the Toll pathway in the fat body (Tang *et al*., 2008). Therefore, it can be speculated that induction of the Toll pathway by tracheal melanization indicates signalling between the local and systemic immune responses. This communication would serve to alert and prepare the host for potential invasion of internal tissues by pathogens. Such an alarm system could be advantageous for organisms in which pathogens are naturally first encountered at epithelial surfaces. Finally, haemocytes have also been implicated in activation of the systemic response, although this remains an area of active investigation. A previous study using larvae without haemocytes suggested that AMPs are not induced in the fat body in the absence of haemocytes (Basset *et al*., 2000). Likewise, a more recent study suggests that phagocytosis by haemocytes is required for *Defensin* expression in the fat body upon septic injury (Brennan *et al*., 2007).

2.5 Concluding remarks

Using *Drosophila* as a model, the systemic immune response has been the paradigm of invertebrate responses to microbial infection. This body of research has demonstrated that this response is not adaptive, as seen in invertebrate immunity, but is adapted to specific pathogens through the regulation of the Toll and Imd pathways. The specificity of this response is based on the use of a large array of effectors, although the mechanisms these effectors employ to control microbes remain unclear.

Recent studies focusing on the local response to pathogens are starting to reveal the emerging complexity of the epithelial immune response. Further study of epithelial infections will assist in identifying features specific to local immunity and clarify the growing link between the stress and immune responses. Additionally, increased understanding of the role of microbiota in the development and function of the innate immune response will help elucidate the complex mechanisms underlying gut homeostasis.

Finally, emerging studies in *Drosophila* support the consideration of the innate immune response as a holistic process. The interplay between different tissues and the integration of these responses probably contributes to each host–microbe interaction (Lemaitre and Hoffmann, 2007; Shirasu-Hiza and Schneider, 2007). Thus, signalling between the local and systemic response, while not required in every interaction, may dictate whether microbial infection leads to tolerance, resolution, or host lethality. The role of this signalling and its in-depth characterization are some of the current challenges facing the field of innate immunity.

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CHAPTER 3

Roles of haemolymph proteins in antimicrobial defences of *Manduca sexta*

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3.1 Introduction

Research on lepidopteran insects has been instrumental in the discovery and the biochemical understanding of many haemolymph plasma proteins with immune functions. After the identification of inducible bactericidal activity in the haemolymph of the wax moth *Galleria mellonella* (Stevens, 1962), the first well-characterized insect antimicrobial peptides and proteins (Faye *et al.*, 1975; Hoffmann *et al.*, 1981; Hultmark *et al.*, 1980, 1983; Powning and Davidson, 1973; Steiner *et al.*, 1981) as well as pattern-recognition proteins that function in immune surveillance (Yoshida *et al.*, 1986, 1996; Ochiai and Ashida, 1988; Kang *et al.*, 1998) were initially studied in silkmoths and other caterpillars. Biochemical characterization of the prophenoloxidase (proPO) activation system was also pioneered in work with *Bombyx mori* (Ashida and Brey, 1998).

Insect plasma proteins, including those involved in immune responses, are synthesized primarily by the fat body, with some contribution from haemocytes and other tissues (Dunn, 1990; Kanost *et al.*, 1990). The functions of many plasma immune proteins and the regulation of their gene expression have been characterized in *Drosophila melanogaster* through extensive genetic analyses (Chapter 2; Ferrandon *et al.*, 2007; Lemaitre and Hoffmann, 2007). Details of immune mechanisms from other arthropod species have also been reviewed (Iwanaga and Lee, 2005; Jiravanichpaisal *et al.*, 2006). In this chapter, we will focus on recent advances in our understanding of plasma proteins in the immune responses of the tobacco hornworm, *Manduca sexta*, and will point out some related work done with other insect species for background and comparison. Additional reviews on aspects of the *M. sexta* immune response are available (Kanost *et al.*, 2004; Jiang, 2008; Kanost and Nardi, 2008).

3.2 Antimicrobial peptides and proteins

Microbial infection or exposure to bacterial peptidoglycan induces synthesis of lysozyme and antibacterial peptides that are secreted into *M. sexta* plasma (Dunn *et al.*, 1985; Kanost *et al.*, 1988). Lysozyme (Mulnix and Dunn, 1994; López-Zavala *et al.*, 2004) and antibacterial peptides from the cecropin, attacin, moricin, gloverin, and lebocin families (Dickinson *et al.*, 1988; Kanost *et al.*, 1990; Zhu *et al.*, 2003a; Dai *et al.*, 2008) from *M. sexta* plasma have been investigated by biochemical and DNA cloning methods. They are synthesized by the fat body within a few hours of bacteria being injected, and can remain at high concentrations in plasma for 2–3 days, providing strong protection against bacterial growth in the haemocoel (Tzou *et al.*, 2002; Eleftherianos *et al.*, 2006a). Lysozyme hydrolyses peptidoglycan in bacterial cell walls, which causes bacterial lysis. In addition, the liberated

peptidoglycan fragments serve as signals to promote induced expression of immune genes and phenoloxidase activation (Kanost *et al.*, 1988; Wang *et al.*, 2006; Park *et al.*, 2007; Kim *et al.*, 2008). These antibacterial factors are also present in *M. sexta* eggs, which can synthesize antimicrobial peptides in response to bacteria (Gorman *et al.*, 2004). High concentrations of lysozyme and other antibacterial proteins are also secreted into the midgut lumen during metamorphosis (Dunn *et al.*, 1994).

3.3 Pattern-recognition proteins

An essential early step in an immune response is recognition of an invading pathogen as dangerous non-self (Janeway and Medzhitov, 2002; Matzinger, 2002; Sansonetti, 2006). Surface molecules of potential pathogens such as bacteria and fungi include β-1,3-glucan, peptidoglycan, lipopolysaccharide (LPS), and lipoteichoic acid (LTA), which can be recognized by insect pattern-recognition proteins (Yu *et al.*, 2002; Kurata *et al.*, 2006). Binding of these pattern-recognition proteins in turn leads to activation of other immune responses. Endogenous danger signals, molecules that are only released from cells under conditions like injury or infection (Matzinger, 2002), are starting to be recognized in insects. Fragments of collagen and nucleic acids can elicit immune responses in *G. mellonella* (Altincicek and Vilcinskas, 2006; Altincicek *et al.*, 2008). Our discussion will focus on known pattern-recognition receptors.

3.3.1 Hemolin

Hemolin is a 48 kDa protein found so far only in lepidopteran species (Faye and Kanost, 1998). It contains four immunoglobulin domains, which form a horseshoe-like three-dimensional structure (Su *et al.*, 1998). The sequence of hemolin is most similar to the four immunoglobulin-domains at the N-terminal end of the cell-adhesion protein, neuroglian, which is expressed in neural cells and also in a population of plasmatocytes in *M. sexta* (Zhuang *et al.*, 2007). *M. sexta* hemolin can bind to many different bacterial surface components including LPS and LTA (Yu and Kanost, 2002), as well as to haemocytes (Ladendorff and Kanost, 1991). We speculate that the effects of hemolin on haemocyte adhesion (Ladendorff and Kanost, 1991; Bettencourt *et al.*, 1997) may be through binding to neuroglian, or to neuroglian ligands such as integrin, on haemocyte surfaces (Zhuang *et al.*, 2007). A haemocyte membrane-associated form of hemolin has also been observed in *Hyalophora cecropia* (Bettencourt *et al.*, 1997). Hemolin is present at low levels in naïve haemolymph, but is upregulated in response to bacterial challenge. In naïve insects, hemolin expression increases in both fat body and midgut during the wandering stage (Yu and Kanost, 1999). Recent RNA interference (RNAi) studies showed that knockdown of hemolin expression led to decreased phagocytosis and nodulation of *Escherichia coli* (Eleftherianos *et al.*, 2007). Hemolin has also been proposed to be involved in antiviral defence (Terenius, 2008).

3.3.2 C-type lectins

Another class of proteins that binds both haemocytes and microbial polysaccharides are calcium-dependent (C-type) lectins. In *M. sexta*, four soluble C-type lectins with two carbohydrate-recognition domains have been characterized and named immulectins 1–4 (IML-1–4) (Yu *et al.*, 1999, 2005, 2006; Yu and Kanost, 2000). Similar tandem-domain C-type lectins are found in other lepidopterans and are involved in binding bacteria and haemocyte aggregation (Koizumi *et al.*, 1999; Shin *et al.*, 2000; Watanabe *et al.*, 2006; Chai *et al.*, 2008). Expression of all four *M. sexta* IMLs is upregulated in the fat body upon immune challenge (Yu *et al.*, 2002, 2005, 2006). IML-1 and IML-4 agglutinate Gram-positive and Gram-negative bacteria and yeast (Yu *et al.*, 1999, 2006). IML-2 causes aggregation of Gram-negative bacteria (Yu and Kanost, 2000) and binds to haemocytes (granular cells and oenocytoids), as well as nematodes (Yu and Kanost, 2004). IML-2 can bind to a wide range of microbial surface molecules, including LTA, laminarin (branched β-1,3-glucan), mannose, and LPS (Yu and Ma, 2006). Depletion of IML-2 by addition of IML-2 antibodies decreases bacterial clearance and makes larvae more susceptible to bacterial infection with *Serratia marcesens* (Yu and Kanost, 2003). The importance of IML-2 during an immune response was also shown

by RNAi against IML-2, which led to a decrease in the length of *M. sexta* survival after infection by either of two *Photorhabdus* species (Eleftherianos *et al*., 2006a, 2006b).

IML-3 and IML-4 both bind to LPS, LTA, and laminarin (Yu *et al*., 2005, 2006). Coating agarose beads with carbohydrate-recognition domain 2 of IML-2 or full-length IML-3 or IML-4 stimulated increased haemocytic encapsulation of those beads (Yu and Kanost, 2004; Yu *et al*., 2005, 2006). IML-2 and IML-4 also enhanced melanization of encapsulated beads (Yu and Kanost, 2004; Yu *et al*., 2006). Immulectins bind microbial surfaces and haemocytes and participate in aggregation and encapsulation responses; this evidence indicates that immulectins are pattern-recognition proteins important for cellular responses. IML-1 and -2 also have a role in the melanization response, as they increase phenoloxidase activation in haemolymph (Yu *et al*., 1999; Yu and Kanost, 2000), and IML-2 binds to cleaved serine protease homologues (Yu *et al*., 2003).

3.3.3 β-1,3-Glucan-recognition proteins/ Gram-negative-binding proteins

β-1,3-Glucan-recognition proteins (βGRPs) and Gram-negative-bacteria-binding proteins (GNBPs) form a family of pattern-recognition proteins that bind β-1,3-glucan, LPS, or both, and trigger proPO activation and antimicrobial peptide synthesis (Beschin *et al*., 1998; Lee *et al*., 2000; Ma and Kanost, 2000; Ochiai and Ashida, 2000; Kim *et al*., 2000b; Fabrick *et al*., 2003; Jiang *et al*., 2004). βGRPs were first identified in *B. mori* (Ochiai and Ashida, 1988, 2000) and have an N-terminal glucan-binding domain and a catalytically inactive glucanase domain at the C-terminus (Kanost *et al*., 2004). βGRPs stimulate proPO activation in the presence of β-1,3-glucans (Jiang *et al*., 2004; Ma and Kanost, 2000). The functions of the two individual domains from βGRP from the moth *Plodia interpunctella* have been investigated (Fabrick *et al*., 2004). The N-terminal domain alone can bind to β-1,3-glucans (curdlan or laminarin), LPS, and LTA, and the C-terminal domain binds to branched β-1,3-glucans (laminarin). However, full-length *P. interpunctella* βGRP is required for agglutination of bacteria.

Two βGRPs have been characterized in *M. sexta.* Both bind and aggregate yeast, Gram-negative bacteria, and Gram-positive bacteria. However, their expression patterns differ. βGRP1 is not induced by immune challenges and is constitutively expressed during the feeding and wandering stages (Jiang *et al*., 2004; Ma and Kanost, 2000). βGRP2 is only expressed in feeding-stage larvae after wounding or immune challenge, yet is strongly expressed in naïve insects starting at the wandering stage (Jiang *et al*., 2004). βGRP-mediated recognition of fungi stimulates autoactivation of a serine protease that triggers proPO activation in *M. sexta* (Wang and Jiang, 2006).

Lee *et al.* (1996) isolated a *B. mori* GNBP from plasma by taking advantage of its binding to Gram-negative bacteria. Related proteins, which form a branch of the βGRP family, have been identified by DNA sequencing in a number of insect species (Fabrick *et al*., 2003). The *D. melanogaster* GNBP1 binds LPS and β-1,3-glucan (Kim *et al*., 2000b) and is required for Toll activation in response to Gram-positive bacteria (Pili-Floury *et al*., 2004). *D. melanogaster* GNBP1 forms complexes with peptidoglycan-recognition proteins PGRP-SA and PGRP-SD during Toll activation (Wang *et al*., 2006, 2008). GNBP1 from the beetle *Tenebrio molitor* also interacts with a PGPR-SA and triggers proPO activation (Kim *et al*., 2008).

3.3.4 Peptidoglycan-recognition proteins (PGRPs)

Peptidoglycan, a component of bacterial cell walls, is a polymer of a repeating disaccharide unit, β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid. These polymer chains are cross-linked by short peptides in which the third residue is a *meso*-diaminopimelic acid (DAP) group, as in Gram-negative bacteria and *Bacillus* species, or another amino acid, often a lysine, in many Gram-positive bacteria. Gram-negative bacteria and most *Bacillus* species have a direct cross-link between the DAP group in position three and a D-Ala in position four of the other chain. However, Gram-positive bacteria show diversity in the length and composition of the interpeptide bridge that links the two peptidoglycan chains (Schleifer and Kandler, 1972).

Peptidoglycan can be detected by PGRPs, which are present in invertebrates and vertebrates (Dziarski and Gupta, 2006). PGRP was discovered in *B. mori* and found to activate the proPO cascade in the presence of peptidoglycan (Yoshida *et al.*, 1996; Ochiai and Ashida, 1999). Studies initiated using plasma proteins from caterpillars also demonstrated that PGRP sequences are related to bacteriophage lysozymes and are conserved in mammals, as well as in insects (Kang *et al.*, 1998).

Thirteen PGRP genes are present in the *D. melanogaster* genome. Different PGRPs can recognize different types of peptidoglycan (Kurata *et al.*, 2006; Wang and Ligoxygakis, 2006). Many of the long forms of PGRPs (PGRP-Ls) are transmembrane proteins with an extracellular PGRP domain, whereas short PGRPs (PGRP-Ss) are secreted (Werner *et al.*, 2000). PGRP-LE appears to function in proPO activation (Takehana *et al.*, 2002). PGRP-LC and PGRP-LE stimulate activation of the IMD pathway leading to synthesis of antimicrobial peptides in response to DAP-type peptidoglycan (Choe *et al.*, 2002; Gottar *et al.*, 2002; Takehana *et al.*, 2004). PGRP-SA binds Gram-positive bacteria and activates the Toll pathway (Michel *et al.*, 2001) through a mechanism that involves GNBP1 (Gobert *et al.*, 2003). PGRP-SD also activates the Toll pathway in response to some Gram-positive bacteria (Bischoff *et al.*, 2004) and, to a lesser degree, Gram-negative bacteria (Leone *et al.*, 2008). PGRP-SD interacts with GNBP1 and PGRP-SA (Wang *et al.*, 2008).

Recently it was determined that *B. mori* contains six short and six long PGRPs (Tanaka *et al.*, 2008). There are at least three short, secreted PGRPs in *M. sexta*, of which PGRP-1 has been most studied. We expect additional PGRPs will be found in *M. sexta*, including long PGRPs. PGRP-1 expression is immune-inducible (Zhu *et al.*, 2003a), and it increases from 2 μg/ml in naïve larval plasma to 60 μg/ml in plasma from larvae 24 h after injection of bacteria (Yu *et al.*, 2002). RNAi knockdown of *M. sexta* PGRP-1 increased susceptibility to *Photorhabdus* species (Eleftherianos *et al.*, 2006a, 2006b). PGRP-1 binds to the surface of Gram-negative bacteria and *Bacillus thuringiensis*, suggesting that it binds to DAP-type peptidoglycan (Ragan, 2008). PGRP-A from *Samia cynthia ricini*, a wild silkworm, has high sequence similarity to *M. sexta* PGRP-1 and was purified based on binding to *Bacillus subtilis* cell walls. It binds to DAP-type cross-linked peptidoglycan (from *Bacillus* cell wall) and to non-cross-linked Lys-type peptidoglycan (from *Micrococcus luteus*) but not very strongly to cross-linked Lys-type peptidoglycan (from *M. luteus*) (Onoe *et al.*, 2007). Additional studies of *M. sexta* PGRP-1, -2, and -3 underway have indicated that all three PGRPs bind *E. coli* and to purified peptidoglycan from *M. luteus* (H. Jiang and J. Sumathipala, unpublished results). Non-cross-linked peptidoglycan has been shown to be a powerful inducer of lysozyme and bactericidal activity in *M. sexta* and *B. mori* (Kanost *et al.*, 1988; Iketani and Morishima, 1993; Iketani *et al.*, 1999). In *T. molitor*, partial lysozyme digestion of peptidoglycan from *M. luteus* and *Staphyloccocus aureus* enhanced binding of PGRP-SA (Park *et al.*, 2007). These results suggest that the interpeptide bridges found in peptidoglycan from many Gram-positive bacteria prevent effective PGRP binding and that *M. sexta* PGPR-1 and *Samia* PGPR-A may recognize peptidoglycan regardless of the presence of DAP or Lys in the third position of the peptide.

3.4 Protease cascades in immune responses

Extracellular serine protease cascades have evolved in animals for signalling that stimulates rapid responses to infection or wounding. Mammalian blood coagulation, fibrinolysis, and complement activation are prominent examples of such pathways. Haemolymph coagulation in horseshoe crabs in response to LPS or β-1,3-glucan is a well-characterized serine protease cascade in arthropods (Iwanaga, 2007). Melanization and activation of the Toll pathway are immune responses of insects, which implement a cascade of serine proteases to generate an effector response. The horseshoe crab and insect cascades have in common the involvement of clip-domain serine proteases, which have a C-terminal protease domain from the S1 (chymotrypsin) family and an N-terminal clip domain thought to function in localization or regulation of the proteases (Jiang and Kanost, 2000). Clip-domain proteases are synthesized and secreted into haemolymph as inactive zymogens, which are

activated by a specific proteolytic cleavage carried out by another protease. These pathways also often involve serine protease homologues (SPHs), which contain a domain with sequence similarity to serine proteases, but with the active-site serine changed to an inactive residue, most often glycine. SPHs may also contain N-terminal clip domains and are fairly abundant in insect genomes (Ross *et al*., 2003; Waterhouse *et al*., 2007). Although their functions are not yet well understood, SPHs appear to interact with clip-domain proteases and their substrates to modulate or regulate immune cascade pathways. SPHs in *M. sexta* and *Holotrichia diomphalia* bind phenoloxidase (PO) and form high-molecular-weight complexes (Yu *et al*., 2003; Wang and Jiang, 2004a; Gupta *et al*., 2005; Piao *et al*., 2005). We have identified more than 25 clip-domain proteases and four SPHs expressed in *M. sexta* fat body or haemocytes (Jiang *et al*., 2005). Described below are the results of efforts to understand the functions of these haemolymph proteases (HPs) in innate immune responses.

3.4.1 Proteases and stimulation of antimicrobial peptide synthesis

The Toll pathway in *D. melanogaster* triggers synthesis of drosomycin and other antimicrobial peptides in response to fungi and many Gram-positive bacteria (Lemaitre and Hoffmann, 2007). The response is initiated by recognition proteins that bind pathogens or microbial patterns, as discussed briefly above. Such binding triggers an extracellular serine protease cascade involving multiple serine proteases, terminating in cleavage and activation of the cytokine Spätzle by a clip-domain protease called Spätzle-processing enzyme (SPE) (Jang *et al*., 2006). Upstream of SPE in this pathway are clip-domain proteases called Persephone (Ligoxygakis *et al*., 2002), Spirit, and Grass (Kambris *et al*., 2006; El Chamy *et al*., 2008) (Figure 3.1). At this point it is not known what protease activates SPE in *D. melanogaster*, although a SPE-activating enzyme (SAE) has been identified in the beetle *T. mollitor* (Kim *et al*., 2008). Activated Spätzle binds to the Toll membrane receptor in the fat body and haemocytes, and triggers an intracellular signal transduction cascade that activates Rel-family transcription factors. The Toll pathway was initially discovered for its role in dorsal–ventral patterning during embryogenesis. However, the embryonic pathway leading to Spätzle processing involves different serine proteases (Dissing *et al*., 2001; LeMosy *et al*., 2001; Rose *et al*., 2003). The last two proteases in this pathway, Snake and Easter, each contain a clip domain. The initiation of this embryonic serine protease cascade is poorly understood, but seems to be triggered by a product of Pipe, a heparin sulphate 2-O-sulfotransferase that is specifically expressed on the ventral side of the follicular epithelium (Sen *et al*., 1998).

D. melanogaster Spätzle can be activated in response to fungi in at least two ways. GNBP3 (≈65% similar in its N-terminal domain to that of *M. sexta* βGRPs) binds to β-1,3-glucan and induces expression of drosomycin in response to *Candida albicans*. However, certain entomopathogenic fungi, which secrete serine proteases to penetrate the cuticle, activate the Toll pathway by directly cleaving the protease Persephone (Gottar *et al*., 2006). A complete pathway for Spätzle activation in response to Lys-peptidoglycan containing Gram-positive bacteria has been worked out in *T. molitor*. It requires the recognition proteins PGRP-SA and GNBP1, modular serine protease (which has 36% sequence identity with *M. sexta* HP14), SAE, and SPE (Kim *et al*., 2008).

A Toll pathway also appears to function in stimulating antimicrobial peptide synthesis in lepidopteran insects, including *M. sexta*. A Toll receptor is constitutively expressed in several tissues of *M. sexta* and is present on the surface of haemocytes. Its mRNA is upregulated in response to yeast and bacteria (Ao *et al*., 2008). *B. mori* Spätzle-1 has been shown to upregulate antimicrobial gene expression in both *B. mori* and *M. sexta* (Wang *et al*., 2007). We recently isolated a cDNA for a *M. sexta* homologue of Spätzle (C. An, H. Jiang, and M.R. Kanost, unpublished results) and are investigating its activation and biological function. *M. sexta* HP8, a clip-domain protease most similar to *Drosophila* Easter and SPE (the known Spätzle activators), has been identified as a protease that cleaves and activates *M. sexta* Spätzle. HP6 is a clip-domain protease that appears to be the *M. sexta* orthologue of *Drosophila* Persephone. Biochemical analysis using purified recombinant proteins indicates that HP6 is

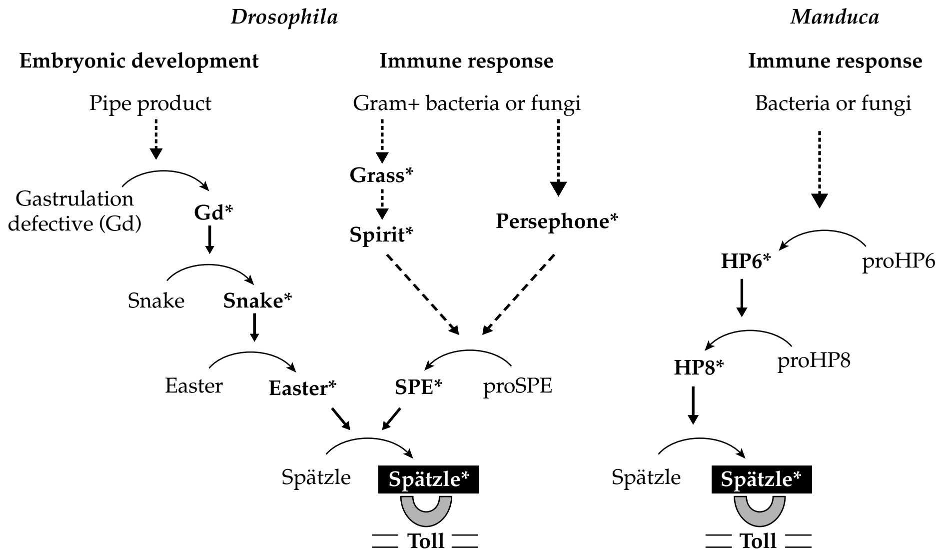

Figure 3.1 A model of Toll-activation pathways in *D. melanogaster* and *M. sexta*. *D. melanogaster* Spätzle activation occurs during embryonic development and in haemolymph during immune challenge. During embryonic development, gastrulation defective (Gd) becomes active (*) in the ventral region of the perivitelline space in response to Pipe expression via an unknown mechanism. Activation of Snake by Gd leads to activation of Easter, which cleaves Spätzle to form an active Toll ligand. In immune-related Toll activation, pattern-recognition proteins recognize Gram-positive bacteria or fungi and activate, through an unknown mechanism, serine protease cascades that involve serine proteases Grass and Spirit, and the terminal protease, Spätzle-processing enzyme (SPE). The serine protease Persephone is also involved upstream of SPE and may be activated directly by fungal or bacterial proteases. How these proteases are activated or what their targets are remains unknown, with the exception of SPE cleavage of Spätzle. In *M. sexta* we identified the two terminal proteases in Spätzle activation. Haemolymph proteinase 6 (HP6) activates proHP8 and HP8 cleaves Spätzle, creating the active Toll ligand. HP6 also is involved in a cascade leading to PO activation and the melanization response.

an activator of HP8, which in turn activates Spätzle (Figure 3.1). ProHP6 and proHP8 in plasma both become activated after exposure to bacteria, and injection of either HP6 or HP8 into *M. sexta* larvae results in induced expression of several antimicrobial peptides (C. An, H. Jiang, and M.R. Kanost, unpublished results).

3.4.2 ProPO activation

M. sexta pathways for proPO activation are now relatively well understood. PO catalyses oxidative reactions involved in melanin synthesis, which has been implicated in microbial killing through the generation of toxic compounds (Cerenius *et al.*, 2008). PO activation and melanization in response to microbial exposure can occur faster than antimicrobial peptide synthesis. Oxidation reactions catalysed by PO lead to the formation of reactive quinone intermediates, cytotoxic molecules like 5,6-dihydroxyindole (DHI), and reactive oxygen or nitrogen intermediates that may contribute to the killing of invading pathogens (Nappi and Christensen, 2005; Zhao *et al.*, 2007). Melanization can also occur in haemocyte nodules or on the surface of encapsulated objects or parasites (Cerenius and Söderhäll, 2004).

PO hydroxylates tyrosine to form an *o*-diphenol, 3,4-dihydroxyphenylalanine (DOPA), using its monophenol monooxygenase (tyrosinase) activity. PO also oxidizes *o*-diphenols such as DOPA to their corresponding quinones (Kanost and Gorman, 2008). The orthoquinones resulting from this process polymerize to form melanin. Specifically, dopaquinone can non-enzymically cyclize to form dopachrome. Dopachrome-converting enzyme catalyses the decarboxylation of dopachrome to DHI, which can be converted by PO to indole-5,6-quinone, which

then polymerizes to form DHI eumelanin (Nappi and Christensen, 2005).

Tyrosine hydroxylase is an intracellular enzyme with monophenoloxidase activity that also can convert tyrosine to DOPA. Tyrosine hydroxylase expression is upregulated in *M. sexta* upon immune challenge and may have a significant role in melanin synthesis in immune responses (Gorman *et al.*, 2007a). Another intracellular, immune-induced enzyme important in the early stages of melanin synthesis is Dopa decarboxylase (Kim *et al.*, 2000a; Zhu *et al.*, 2003a), which converts DOPA to dopamine. Dopamine is a better PO substrate than DOPA and is present at higher concentration in *M. sexta* haemolymph, and thus probably contributes significantly as a precursor for quinone and melanin synthesis.

Two proPO genes are expressed constitutively in *M. sexta* oenocytoids, each producing an inactive, approximately 80 kDa zymogen (Hall *et al.*, 1995; Jiang *et al*; 1997, Gorman *et al.*, 2007a). The two *M. sexta* proPOs are approximately 50% identical in sequence and form heterodimers. *M. sexta* proPOs lack signal peptides, which is common among arthropod proPOs. They are apparently released into plasma by lysis of oenocytoids, a process whose regulation needs investigation. PPO is activated through specific proteolytic cleavage. In *M. sexta,* three proPO-activating proteases (PAPs) have been discovered: PAP1, PAP2, and PAP3 (Jiang *et al.*, 1998, 2003a, 2003b). Each of the three PAPs is synthesized as a proPAP zymogen, and they must be activated by specific proteolytic cleavage. PAP1 contains a single N-terminal clip domain and is activated by cleavage after Arg-127. PAP2 and PAP3 each contain two N-terminal clip domains and are activated by cleavage after Lys-153 and Lys-146, respectively. The solution structure of the dual clip domains from PAP-2 has been solved (Huang *et al.*, 2007). Each clip domain adopts a fold in which a three-stranded antiparallel β-sheet is flanked by two α-helices. This structure should be helpful in designing experiments to examine functions of the clip domains in formation of protein complexes in proPO activation. The *M. sexta* PAPs require a cofactor composed of SPHs assembled in a high M_r form (Yu *et al.*, 2003; Gupta *et al.*, 2005). The SPHs must also be activated by limited proteolysis at a specific Arg or Lys residue. ProPO activation includes a complex network of proteins that are sequentially activated by recognition of damaged tissue or invading microbes (Figure 3.2) (Jiang, 2008).

Recently, serine protease cascades that lead to proPO activation in *M. sexta* have been elucidated. At the top of one identified pathway is HP14, a complex protein that contains five low-density lipoprotein receptor class A domains, a Sushi domain, a cysteine-rich region, and a C-terminal protease domain (Ji *et al.*, 2004). HP14 can autoactivate in the presence of peptidoglycan or β-1,3-glucan and βGRP2 (Wang and Jiang, 2006). HP14 then activates proHP21, and HP21 cleaves and activates proPAP2 or proPAP3 (Gorman *et al.*, 2007b; Wang and Jiang, 2007), which then activates proPO in the presence

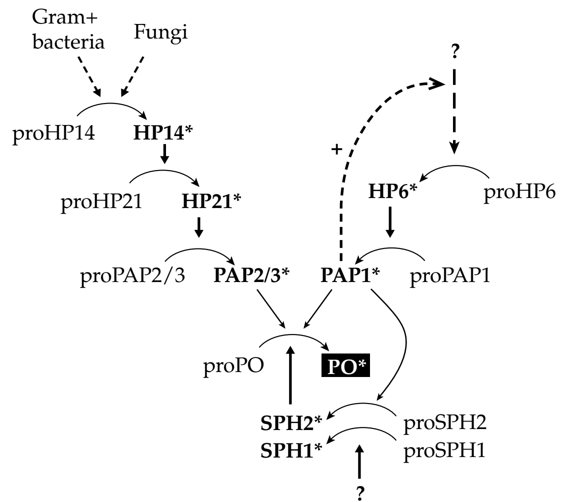

Figure 3.2 Protease cascades involved in *M. sexta* proPO activation. An initiator protease, proHP14, is activated subsequent to interactions between haemolymph plasma pattern-recognition proteins and microbial patterns. HP14 activates proHP21 and HP21 then activates proPAP2 and proPAP3. In another branch, HP6 activates proPAP1. HP6 is also involved in *M. sexta* Toll activation (see Figure 3.1). The activator(s) of proHP6 are not yet known but proHP6 activation is stimulated by PAP1 in a positive-feedback loop. SPHs are not catalytically active proteases but still must be cleaved to function in PO activation. PAP1 can cleave SPH2; other SPH activators are unknown. Any one of the active PAPs can interact with cleaved SPHs to form a functional PO activation complex, which cleaves proPO to active PO. PO oxidizes catechols in the haemolymph that, after further reactions, form melanin. HP, haemolymph proteinase; PAP, prophenoloxidase-activating proteinase; PO, phenoloxidase; SPH, serine proteinase homologue.

of cleaved SPHs (Figure 3.2). ProPAP1 is not activated by HP21 (Wang and Jiang, 2007). However, HP6 can cleave and activate proPAP1 (C. An, H. Jiang, and M.R. Kanost, unpublished results), indicating at least two protease cascades which can lead to proPO activation, as well as potential cross-talk between pathways for melanization and Toll activation, both involving HP6. As an additional positive regulatory mechanism, active PAP1 can cleave SPH2 and also stimulates activation of proHP6, suggesting that a self-reinforcing, positive feedback mechanism helps to promote rapid proPO activation (Wang and Jiang, 2008). In *T. molitor*, active SPE can cleave proPO and SPH1, which together are sufficient for melanization (Kan *et al*., 2008).

3.5 Regulation of *Manduca* immune responses by serine protease inhibitors

Extracelluar serine protease cascades in animals are often regulated by proteins of approximately 45 kDa known as serpins, which are specific serine protease inhibitors (Silverman *et al*., 2001). Serpins form covalent complexes with target proteases. The C-terminal region of the serpin is an extended loop that serves as bait for the target protease. Inhibitory selectivity depends on the sequence and conformation of the reactive site loop. The protease binds to the loop and cleaves a specific bond between residues designated P1 and P1′. The P1 residue of the serpin fits into the primary substrate-specificity pocket of the protease and is particularly important in determining the selectivity of a serpin for protease inhibition (Yu *et al*., 2001). Upon cleavage, the serpin undergoes a major rearrangement, inserting its reactive-site loop into one of its β-sheets and moving the protease with it about 70 Å, distorting the protease active site (Whisstock and Bottomley, 2006). The covalent ester linkage between the protease and serpin remains intact, because it is not accessible to water for completion of the hydrolysis reaction (Dementiev *et al*., 2006).

Serpins from insects were first identified in the lepidopterans, *B. mori* and *M. sexta* (Kanost, 1999). They regulate melanization and Toll cascades in *D. melanogaster* (Reichhart, 2005) and influence activation of melanization in *Anopheles gambiae* (Michel *et al*, 2006). Serpins in *M. sexta* have been reviewed recently (Kanost, 2007). *M. sexta* has at least seven serpin genes, but the overall number of functional serpins is higher due to alternative splicing of the ninth exon of serpin-1, yielding 12 serpin-1 isoforms with different reactive-site loops and therefore different protease selectivities (Jiang *et al*., 1996; Jiang and Kanost, 1997). Serpin-1 is expressed in feeding larvae, but not during larval or pupal moults, and overall protein level is unchanged after immune challenge (Kanost *et al*., 1995). In response to bacterial challenge, serpins-3, -4, -5, and -6 are upregulated in the fat body; serpins-4, -5, and -6 are also upregulated in haemocytes (Tong and Kanost, 2005; Zhu *et al*., 2003b; Zou and Jiang, 2005). Serpin-3 inhibits all three PAPs, while serpin-6 specifically inhibits PAP3 (Zhu *et al*., 2003b; Wang and Jiang, 2004b). Serpin-1 isoform J also inhibits PAP3 (Jiang and Kanost, 1997). Serpin-protease complexes in plasma have been identified: serpin-4 complexes with HP1, HP6, and HP21, while serpin-5 complexes with HP1 and HP6 (Tong *et al*., 2005). Addition of recombinant serpins-1J, 3, 4, 5, and 6 to plasma can diminish proPO activation, indicating that a protease they inhibit functions involved in proPO activation. Serpin-4 and serpin-5 inhibit proPO activation but do not inhibit PAPs, consistent with the conclusion that HP6 is involved in a serine protease cascade that leads to proPO activation. Another target of serpin-4, HP21, has a known function in proPO activation through activation of PAP2 and PAP3, as discussed above. HP1 and/or HP6 are apparently part of an additional PAP-activation pathway or a pathway involved in SPH cleavage. Functions for the remaining serpin-1 isoforms and alternative functions for the other extracellular serpins are topics of current investigation.

3.6 Conclusions and future prospects

Although there has been significant progress in identifying and characterizing functions of plasma proteins that take part in innate immune responses of *M. sexta*, significant gaps remain in our knowledge, and important questions require further experimentation. There is a need to re-examine the mixture of antimicrobial peptides in plasma using

modern purification and proteomics methods. It is likely that undiscovered peptides and proteins exist, with potentially useful and interesting functions. In this regard, little is known about antifungal gene products from *Manduca*, and future bioassays should include searches for such activities. Gene-discovery efforts will require sequencing of the *M. sexta* genome at some point, to permit a thorough examination, and this will certainly yield a wealth of prospective immune system genes to be investigated experimentally.

Future research may yield answers to intriguing questions about the functions of clip domains in protease cascades, as well as the mechanisms by which the SPHs act as cofactors. Comparison of the pathways for intracellular signalling and transcriptional regulation in lepidopteran insects with the well-characterized *Drosophila* systems, will likely yield important insights regarding the evolution of immune systems in insects. Protease pathways and their regulation in insect immunity are very complex and are just beginning to be understood. It is likely that further study of immunity in *M. sexta* can serve as a model to direct and promote investigation of these pathways in other species.

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CHAPTER 4

Drosophila as a model for studying antiviral defences

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4.1 Introduction

As obligate intracellular pathogens, viruses are the simplest and most intimate of the various life forms (bacteria, fungi, worms, etc.) that are programmed to live in, or on, other organisms. Although some viruses can survive for a time in the external environment, they all ultimately rely on strategies requiring further infection and replication in naïve hosts that allow high levels of virus production to facilitate transmission. At the same time, they must at all times cope with the host immune response and therefore develop counter-mechanisms to guarantee their survival.

Insect viruses have probably existed for as long as insects themselves, and have long been of interest to humans. Viral infection of insects can have serious consequences. For example, yellow fever virus, West Nile, and Dengue viruses are mosquito-borne and cause severe illness, such as encephalitis in humans (Mackenzie *et al.*, 2004). As exemplified in recent years for the West Nile virus in the USA, these viruses provide a representative example of emerging diseases of global significance. In addition, many insect or invertebrate viruses are of great concern, as they cause substantial damage to the environment or agriculture. For example, some of these viruses threaten beneficial insects, such as honey bees, or human enterprises, such as the silkworm industry. White spot syndrome virus is also one of the most virulent pathogens of cultured shrimps and causes massive loss of this commercial product worldwide. Therefore, a genetic model for studying host–virus interactions in insects would be beneficial to society in many ways.

The fruit fly, *Drosophila melanogaster* has been established as a powerful model for the mechanistic studies of innate immunity (see Chapter 2). Most studies so far have focused on the response of *Drosophila* to extracellular pathogens, such as bacteria and fungi, and have uncovered two complementary pathways, Toll and Imd, regulating different members of the nuclear factor κB (NF-κB) family of transcription factors, and expression of genes encoding antimicrobial peptides (Lemaitre and Hoffmann, 2007). In contrast to the wealth of information now available for fungal and bacterial infections, the interaction of *Drosophila* with viruses has only recently started to be addressed. Here we review the progress made in the past few years on antiviral defence mechanisms in *Drosophila,* and discuss the relevance of these findings for our understanding of the complex interaction of viruses with their invertebrate or mammalian hosts.

4.2 Models of *Drosophila* virus infection

Insects are susceptible to highly diverse families of RNA and DNA viruses (Friesen and Miller, 2001). Interestingly, many members of these insect virus families have counterparts in vertebrates. This is the case for insect viruses that belong to the families Poxviridae, Reoviridae, Picornaviridae, or Parvoviridae, and this observation suggests that viruses of vertebrates and invertebrates have common origins. The unusual conservation of specific genes found in baculoviruses, entomopoxviruses,

and orthomyxoviruses also suggests an evolutionary link between these diverse virus families. More than 25 viruses have been documented to infect *Drosophila*, and the effects of few of these have been studied recently (Huszar and Imler, 2008). Below we review briefly the main characteristics of the viruses that naturally infect *Drosophila* or which were isolated from other insects, but can infect *Drosophila* cells and be used to study antiviral reactions in flies.

4.2.1 *Drosophila* viruses

4.2.1.1 Sigma virus

Sigma virus (SIGMAV) is widespread in natural populations of *Drosophila* and is one of the best characterized of the viruses infecting fruit flies. SIGMAV is a member of the Rhabdoviridae, an important family of RNA viruses infecting both animals and plants. Insects play a central role in the horizontal transmission of these viruses to either plants or insects (Hogenhout *et al*., 2003). SIGMAV is atypical, in that it has no known vertebrate or plant hosts, and only infects *Drosophila* in which it is maintained through vertical transmission via germ cells (Table 4.1). Rhabdoviruses are enveloped RNA viruses with a single-stranded RNA genome of negative polarity (ss(–)RNA), meaning that it has to be transcribed in infected cells before viral proteins can be translated. Virions have a characteristic bullet shape, with a length of 180–200 nm and a diameter of 60–80 nm.

Flies infected with SIGMAV suffer few adverse effects, which include reduced viability of infected eggs and lower survival over winter. In fact, the parameter used in the laboratory to monitor infection is the sensitivity of exposure to pure CO_2, a treatment used routinely for brief anaesthesia of the flies. This artificial treatment has a dramatic effect on SIGMAV-infected flies, which become irreversibly paralysed, possibly as a result of viral proliferation in the central nervous system (Tsai *et al*., 2008). Five host loci have been proposed to be involved in the control of SIGMAV infection. The best characterized is *ref(2)P*, a strongly polymorphic gene from the second chromosome (see below).

4.2.1.2 Drosophila *C virus (DCV)*

DCV is the best-studied *Drosophila* virus. It was first reported in 1972 in a laboratory stock that exhibited unusually high and unexplained lethality. DCV is a non-enveloped RNA virus that resembles picornaviruses by many aspects of its structural properties and replication cycle, and belongs to a new family of RNA viruses, the Dicistroviridae, order Picornavirales (Le Gall *et al*., 2008) (Figure 4.1). The virion is a non-enveloped, icosaedral particle with a diameter of 30 nm containing a single-stranded

Table 4.1 Characteristics of viruses infecting insects.

Characteristics	Family	Representative member
dsDNA, enveloped	Baculoviridae	*Autographa californica* nuclear polyhedrosis virus (AcNMPV)
	Polydnaviridae	*Campoletis sonorensis* virus (CsV)
	Poxviridae	*Amsacta moorei* entomopoxvirus (AmEPV)
dsDNA, non-enveloped	Iridoviridae	*Chilo* iridescent virus (CIV)
ssDNA, non-enveloped	Parvoviridae	*Galleria mellonella* densovirus (GmDNV)
dsRNA, non-enveloped	Reoviridae	Bluetongue virus (BTV), *Drosophila* F virus (DFV)
	Birnaviridae	*Drosophila* X virus (DXV)
ssRNA, enveloped	Togaviridae	Sindbis virus (SINV)
	Flaviviridae	Yellow fever virus (YFV), West Nile virus (WNV)
	Rhabdoviridae	Sigma virus (SIGMAV)
	Bunyaviridae	Sandfly fever virus (SFSV)
	Errantiviridae	Gypsy virus (GypV)
ssRNA, non-enveloped	Nodaviridae	Flock House virus (FHV)
	Dicistroviridae	*Drosophila* C virus (DCV)

ds, double-stranded; ss, single-stranded.

positive strand RNA genome (ss(+)RNA). The viral replication cycle of DCV has been studied extensively *in vitro* and *in vivo*. Viral particles are internalized by clathrin-mediated endocytosis (Cherry and Perrimon, 2004). DCV then replicates on cellular vesicles derived from the Golgi apparatus, and translation of viral proteins is highly sensitive to the levels of ribosomes in the cells (Cherry *et al.*, 2006). The mechanism of assembly of the viral particles after replication and translation is still poorly characterized.

The outcome of the infection varies strikingly depending on the infection route. DCV is extremely pathogenic when injected intrathoracically into adult flies, replicating to high levels in multiple tissues. By contrast, natural infection (by the oral route or also possibly the respiratory tract) does not lead to major symptoms of infection, and is almost non-pathogenic. DCV is not transmitted vertically and infection occurs exclusively between individuals, either at the larval or adult stages. Overall, these findings point to the existence of a complex network of interactions between DCV and its host (reviewed in Huszar and Imler, 2008).

4.2.1.3 Drosophila *X virus (DXV)*

DXV was first identified as a contaminant in a series of experiments with SIGMAV. It was later found in many *Drosophila* cell lines, although it has never been found in wild populations of flies. The name DXV reflects the enigmatic origin of this virus.

DXV belongs to the Birnaviridae family. These viruses are characterized by a double-stranded (ds) RNA genome, and owe their name to their bipartite genome. The virions are non-enveloped, icosahedral particles with a diameter of 70 nm. *In vivo* interactions between DXV and *Drosophila* are poorly characterized. Adult flies injected with a suspension of DXV die 10–20 days after the injection, depending on the inoculum's concentration

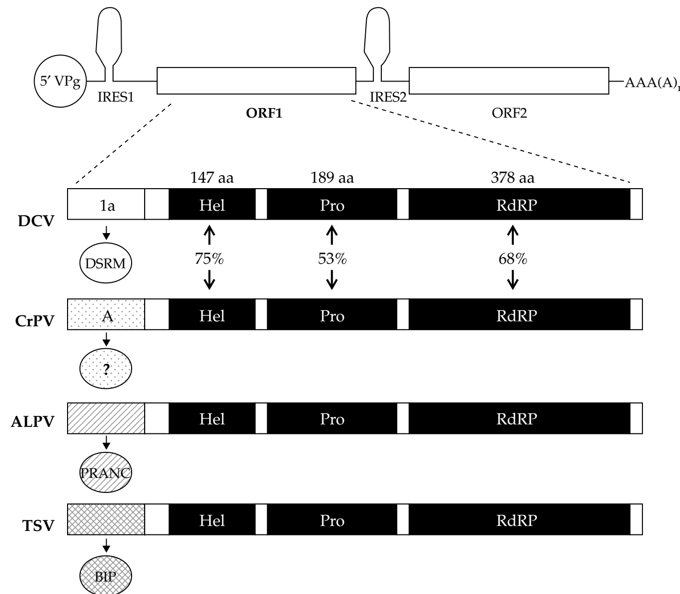

Figure 4.1 Schematic representation of the genome structure of members of the Dicistroviridae family. The genome is a single-stranded RNA molecule of positive polarity, with a covalently attached viral protein (VPg) at the 5′ end and a poly(A) tail at the 3′ end. It contains two open reading frames (ORFs) encoding non-structural (ORF1) and capsid (ORF2) proteins. Translation of viral proteins is regulated by two internal ribosome entry sites (IRES). The size of the three non-structural proteins encoded by DCV ORF1 is indicated, as well as the percentage of identity with the corresponding sequences in cricket paralysis virus (CrPV) ORF1. The variable domain at the N-terminal end of the polyprotein encoded by ORF1 in *Drosophila* C virus (DCV) and cricket paralysis virus suppresses host defence. APV, avian polyoma virus; BIR, baculovirus inhibitor of apoptosis repeat; DSRM, double-stranded RNA-binding motif; Hel, helicase; PRANC, Pox proteins repeats of ankyrin-C-terminal; Pro, protease; RdRP, RNA-dependent RNA polymerase; TSV, Taura syndrome virus.

(Zambon *et al.*, 2005). As described above for SIGMAV, one symptom of infection by DXV is sensitivity to anoxia, which becomes apparent 5–7 days after infection. Viral particles in dead flies are found in the brain but also in several other organs (reviewed in Huszar and Imler, 2008).

4.2.1.4 Other Drosophila *RNA viruses*

Other RNA viruses, belonging to the Reoviridae family, or as yet unclassified, have been reported in *Drosophila*. *Drosophila* F virus (DFV) has been identified as a latent virus in laboratory stocks and natural populations of *D. melanogaster*, and is also present in *Drosophila* tissue-culture cells. As a typical Reovirus, it is a non-enveloped virus with dsRNA genome. DFV virions are spherical particles of 60–70 nm diameter, with a capsid composed of two layers comprising eight polypeptides. These polypeptides are encoded by 10 segments of dsRNA. The replication cycle of DFV in *Drosophila* cells has not been studied.

Drosophila P virus (DPV) has been recovered from laboratory stocks and wild populations of flies, mostly from tropical areas. It has a single-stranded coding (positive-polarity) RNA genome and small (27–30 nm diameter) non-enveloped capsids, but differs significantly from DCV on the basis of serology, pathogenesis, and/or physicochemical properties. The virus is much less virulent than DCV, and can be transmitted vertically through the female germ line. DPV has not been characterized molecularly at this stage, nor is its replicative mechanism known (reviewed in Huszar and Imler, 2008).

Finally, Nora virus causes persistent infection in *D. melanogaster* and is present in both laboratory stocks and wild populations, at a titre varying between 10^4 and 10^{10} genome copies per fly. It does not cause any obvious pathological effect. The viral particles are non-enveloped, with a diameter of about 30 nm, and contain a polyadenylated positive-sense single-stranded RNA genome. Unlike other picorna-like viruses, the 11 879 nt RNA genome of Nora virus exhibits four open reading frames (ORFs) instead of one or two. Only the largest of these, ORF2, bears significant sequence similarity with picornavirus-like genes, and includes sequences coding an RNA helicase, a protease, and an RNA-dependent RNA polymerase (Habayeb *et al.*, 2006).

4.2.2 Non-*Drosophila* viruses

These are viruses not so far isolated from natural populations or routine laboratory cultures of flies, which can be used as research tools to investigate the basic mechanisms of virus–host-cell interactions in *Drosophila*.

4.2.2.1 Flock House virus (FHV)

FHV belongs to the Nodaviridae family. These are small (≈30 nm diameter), non-enveloped riboviruses with a genome composed of two single-stranded, positive-sense RNAs. Both RNAs are capped at the 5′ end, but not polyadenylated. This bipartite genome is packaged in an icosahedral capsid assembled from 180 copies of a single type of coat protein. The genome organization and replication strategy of the nodaviruses is among the simplest of known viruses (reviewed in Venter and Schneemann, 2008). RNA1 (3.1 kb) encodes the 112 kDa replicase, whereas RNA2 (1.4 kb) encodes the 43 kDa capsid-protein precursor. In infected cells, the subgenomic RNA3 (0.4 kb) is produced from RNA1. It encodes the protein B2, a potent suppressor of RNA interference (Li *et al.*, 2002).

FHV was originally isolated from the grass grub *Costelytra zealandica* near the Flock House Agricultural research station in New Zealand in 1983. Although isolated from a Coleopteran insect, FHV replicates efficiently in cultured *Drosophila* cells and *in vivo*, providing a valuable model to study host–virus interactions (Li *et al.*, 2002; Galiana-Arnoux *et al.*, 2006; Wang *et al.*, 2006).

4.2.2.2 Sindbis virus (SINV)

SINV is the type-specific member of alphaviruses, a widely distributed group of significant human and animal pathogens that belong to the family of Togaviridae. Among them, Venezuelan equine encephalitis and O'nyong nyong viruses are an important public health threat. Alphaviruses circulate in nature by continuous transmission between mosquitoes and susceptible vertebrate hosts. In insect vectors, they cause lifelong chronic infections, whereas vertebrate hosts show acute disease

characterized by a high viraemia. SINV was originally collected in 1952 from the mosquito *Culex univittatus* near the town of Sindbis in Egypt. SINV is one of the least pathogenic alphaviruses, but its study provided highly valuable information about the mechanism of RNA replication and virus interaction with host cells. SINV has a single-stranded RNA genome of 11 703 nt. The RNA is capped at the 5′ end, and polyadenylated at the 3′ end. It is packaged in an icosahedral nucleocapsid surrounded by a lipid envelope embedded with glycoprotein components. Virions are about 70 nm in diameter. The 5′ two-thirds of the genomic RNA encode four non-structural proteins, nsP1 to nsP4, that form, together with cellular factors, an RNA-dependent RNA polymerase complex required for transcription and replication of the RNA. The 3′ one-third codes for the structural proteins C, E1, E2, E3, and 6K. In nature, the basic maintenance cycle of SINV is between mosquitoes and birds, although other vertebrates, including humans, may also be infected, causing only subclinical disease fever. SINV can also replicate in *Drosophila*, but does not cause overt pathology upon infection in wild-type flies (Galiana-Arnoux *et al*., 2006).

4.2.2.3 *Cricket paralysis virus (CrPV)*

CrPV was isolated from laboratory colonies of Australian field crickets (*Teleogryllus oceanicus* and *Teleogryllus commodus*) containing some early-instar nymphs which developed a paralysis of the hind legs, became uncoordinated, and died. Electron microscopic sections of paralysed insects revealed many virus-like particles in crystalline arrays reminiscent of those observed in picornavirus-infected cells. CrPV belongs to the Dicistroviridae family and is closely related to DCV (Figure 4.1). Although originally isolated from crickets, CrPV has a wide host range, infecting insects belonging to the orders Diptera, Lepidoptera, Orthoptera, and Heteroptera. Importantly, it also replicates efficiently in *Drosophila* SL2 cells and is pathogenic when injected into flies (Wang *et al*., 2006).

4.2.2.4 *Vesicular stomatitis virus (VSV)*

VSV is a member of the family Rhabdoviridae, genus *Vesiculovirus*. Virions are 70 nm in diameter and 170 nm long, and consist of an envelope within which is a helically coiled cylindrical nucleocapsid. The precise cylindrical form of the nucleocapsid is what gives the viruses their distinctive bullet or conical shape. The genome is a single, linear, negative-sense 11.2 kb molecule of single-stranded RNA. Virus replication is restricted to the cytoplasm of the host cell. VSV infects biting insects (e.g. sand flies, mosquitoes) and can be transmitted to mammalian hosts, in particular cattle, horses, and swine, by these insects. In cultured *Drosophila* cells, VSV establishes a persistent, non-cytopathic infection (Dezélée *et al*., 1987).

4.2.2.5 *Insect DNA viruses*

Insects can also be infected by DNA viruses. These include baculoviruses, which are large rod-shaped (250–300 nm long, 30–60 nm in diameter) enveloped viruses (Friesen and Miller, 2001). The baculovirus genome is a large covalently closed circle of dsDNA. The *Autographa californica* nucleopolyhedrovirus (AcMNPV) genome (≈134 kb) encodes more than 300 ORF. Baculoviruses have been isolated from many insect species. In general they have distinct and relatively narrow host ranges. However, certain baculoviruses, including AcMNPV, have broader host ranges. Interestingly, AcMNPV can efficiently enter *Drosophila* cells, but viral entry or early gene expression triggers apoptosis and as a consequence, *Drosophila* is not permissive for this DNA virus (Lannan *et al*., 2007). Iridoviridae, Ascoviridae, Poxviridae, and Parvoviridae are also found in insects. Curiously however, no DNA viruses have been isolated from *Drosophila* so far.

4.2.2.6 *Infectious retrotransposons and endogenous retrovirus*

Retrotransposons are transposable elements that replicate by reverse transcription of an RNA intermediate, followed by integration of the resulting DNA into the genome of host cells. Retrotransposons are widespread in *Drosophila*, and belong to different classes (Kaminker *et al*., 2002). Some *Drosophila* long terminal repeat (LTR) retrotransposons contain an *env* gene and are classified in the family Errantiviridae. The *env* gene encodes a transmembrane glycoprotein, which can mediate binding to a host-cell receptor. However, whereas vertebrate

retroviruses are predominantly transmitted horizontally by cell-to-cell infection, Errantiviridae, also known as endogenous retroviruses, are mainly transmitted vertically from mother to offspring as integrated copies in the host-cell genome. The genome of *D. melanogaster* contains a large number of LTR retrotransposable elements (up to 304, belonging to 49 families), of which only a few contain an *env*-like gene. The endogenous retroviruses from *Drosophila* include *gypsy*, the best-characterized Errantivirus, which has a genetic organization reminiscent of that from classical vertebrate gammaretroviruses. Importantly, *gypsy* is the only endogenous errantivirus that has been demonstrated to be capable of exogenous infection in *Drosophila* (Bucheton, 1995).

4.3 *Drosophila* immune response against viruses

Although still preliminary, recent studies in *Drosophila* suggest the existence of two types of response to virus infection: degradation of viral RNA by RNA interference (RNAi) and an inducible response involving the induction of a large number of genes, which may counter viral infection.

4.3.1 RNAi as a nucleic-acid-based immune defence

RNAi is a form of highly specific defence reaction, based on the specific base-pairing between small RNAs and invading nucleic acids. Different types of small RNAs, called microRNAs (miRNAs), small interfering RNAs (siRNAs), or Piwi-associated RNAs (piRNAs), have been described in insects and other multicellular organisms.

4.3.1.1 RNAi pathways

miRNAs are produced from nuclear genes, which are transcribed in pri-miRNAs. These precursors, which contain stem loops, are processed in the nucleus by the RNaseIII Drosha, to generate pre-miRNAs. Pre-miRNAs exit the nucleus through the exportin-5 system, to access the cytosol, where they are processed by another RNaseIII enzyme, Dicer-1, to generate miRNAs (Lee *et al.*, 2004). miRNA duplexes are dissociated with the help of the dsRNA-binding protein R3D1, and incorporated into the miRNA-dependent RNAi-silencing complex (miRISC) (Jiang *et al.*, 2005). The miRISC contains the RNaseH-like enzyme Argonaute-1 (AGO-1) that will be guided by the miRNA towards complementary RNA sequences (Okamura *et al.*, 2004). Binding of the miRISC to an RNA molecule can result either in translation inhibition if the complementarity between the miRNA and the RNA molecule is not perfect, or in RNA cleavage if the complementarity is complete (Brodersen *et al.*, 2008). miRNAs can also affect the chromatin structure, and affect transcription of the gene encoding the corresponding mRNA. miRNAs, which also exist in vertebrates and in plants, play important roles in development. As a result, Dicer-1 and AGO-1 mutant flies are embryonic lethal (Lee *et al.*, 2004; Okamura *et al.*, 2004).

Small interfering RNAs (siRNAs) are produced from dsRNA molecules, which are recognized by the second Dicer enzyme encoded by the *Drosophila* genome, Dicer-2, and processed into 22 bp fragments (Lee *et al.*, 2004). The dsRNA molecules can have an endogenous origin, resulting from annealing of RNAs generated by bidirectional transcription of overlapping genes, from transcription of palindromic sequences generating hairpin structures, or from inverted-repeat pseudogenes. In this case, Dicer-2 generates endo-siRNAs, which are then incorporated in an siRNA-dependent RNAi-silencing complex (siRISC) containing the AGO-2 enzyme. R3D1 is also required for this step (reviewed in Obbard and Finnegan, 2008). Importantly, dsRNA can also have an exogenous origin, and betray the presence of foreign nucleic acids in the *Drosophila* cells. Indeed, many viruses have dsRNA genomes (e.g. Reoviridae, Birnaviridae), or generate dsRNA replicative intermediates in the cytosol of infected cells. These exo-siRNAs are generated by Dicer-2, and incorporated into an AGO-2-containing RISC complex. However, this incorporation requires R2D2 instead of R3D1, revealing differences in the processing of siRNAs of endogenous or exogenous origin (Figure 4.2).

Finally, Piwi-associated RNAs (piRNAs) are substantially longer (24–30 nt) than miRNAs and siRNAs; they are involved in heterochromatin maintenance. As their name indicates, piRNAs are

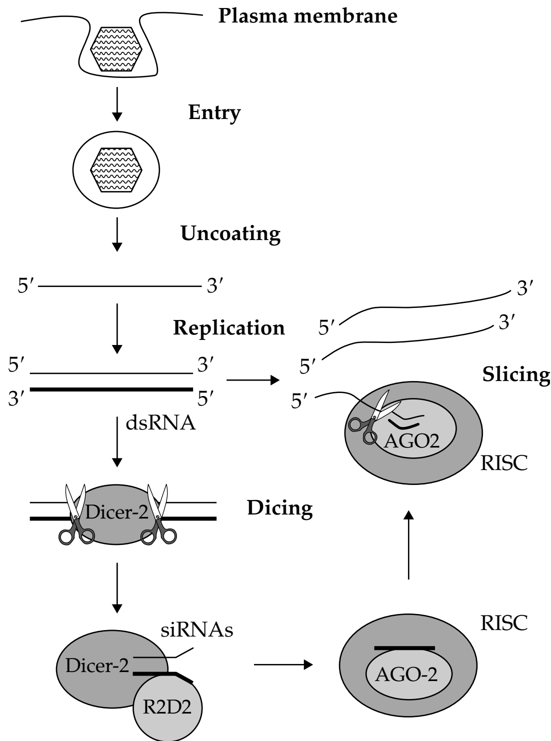

Figure 4.2 siRNA-mediated antiviral defence in *Drosophila*. Double-stranded RNA (dsRNA) molecules produced during replication of a (+) single-stranded RNA virus are recognized by the RNaseIII enzyme Dicer-2 in the cytosol of infected cells and are processed into 21–22 nt short interfering RNA (siRNA) duplexes (Dicing). R2D2 separates the two siRNA strands and loads the guide strand on to the Argonaute protein AGO-2 in the RNAi-silencing complex (RISC), which can then target single-stranded RNA molecules of complementary sequence. Viral RNA molecules are then cleaved by AGO-2 (Slicing).

associated with Piwi proteins, which form a distinct subfamily in the AGO superfamily. In total, the *Drosophila* genome encodes five AGO proteins, two of which (AGO-1 and AGO-2) belong to the Argonaute subfamily and have been mentioned above, whereas the three remaining members belong to the Piwi subfamily (Piwi, Aubergine, and AGO-3) and are involved in the production and function of piRNAs. Unlike miRNAs and siRNAs, piRNAs are not generated from a dsRNA precursor, and do not require Dicer enzymes. Rather, these small RNAs are produced by an original amplification mechanism involving Piwi, Aubergine, and AGO-3 (Girard and Hannon, 2007) (Figure 4.3).

4.3.1.2 RNAi and virus control in Drosophila

RNAi was first shown to be an important antiviral defence mechanisms in plants. Plants have expanded the Dicer family to four members, with Dicer-like (DCL)1 primarily dedicated to the production of miRNAs. DCL2, 3, and 4 process long dsRNA molecules and participate in the control of RNA (DCL2, 4) or DNA (DCL3) virus infections (reviewed in Ding and Voinnet, 2007). In insects, Dicer-2 plays an important role in the resistance to viral infections, as shown by the increased susceptibility of *Dicer-2* mutant flies to infection by the RNA viruses DCV, CrPV, FHV, and SINV (Galiana-Arnoux *et al*., 2006; van Rij *et al*., 2006; Wang *et al*., 2006). Curiously, however, *Dicer-2* mutant flies are as resistant as wild-type controls to infection with the dsRNA virus DXV (Zambon *et al*., 2006). *AGO-2* and *r2d2* mutant flies have also been shown to be more susceptible to DCV, CrPV, FHV, and DXV infections. Increased lethality of virus towards mutant flies correlates with increased viral load and high levels of viral RNAs in infected flies, in good agreement with the mode of action of RNA interference. West Nile virus (WNV), an arbovirus of the Flavoviridae family that can readily infect *Drosophila*, also replicates to higher titres in *AGO-2* mutant flies than in wild-type controls (Chotkowski *et al*., 2008). However, as in the case of DXV, a similar increase in viral titres is not observed in *Dicer-2* mutant flies. Curiously, *piwi* appears to be involved in the control of the viral load in both DXV- and WNV-infected flies, even though this gene is reportedly essentially expressed in the germ line (Zambon *et al*., 2006; Chotkowski *et al*., 2008).

In addition to genetic evidence, the role of RNA interference as an antiviral defence in *Drosophila* is confirmed by the detection of siRNAs corresponding to viral sequences in infected flies. Altogether, these data point to a mechanism whereby dSRNA corresponding either to the viral genome or to replication intermediate forms is detected by Dicer-2 and cleaved into siRNAs. The guide strand of the siRNAs is then incorporated in a R2D2-dependent manner into AGO-2-containing RISC complexes, which will degrade viral RNAs in the cytosol of

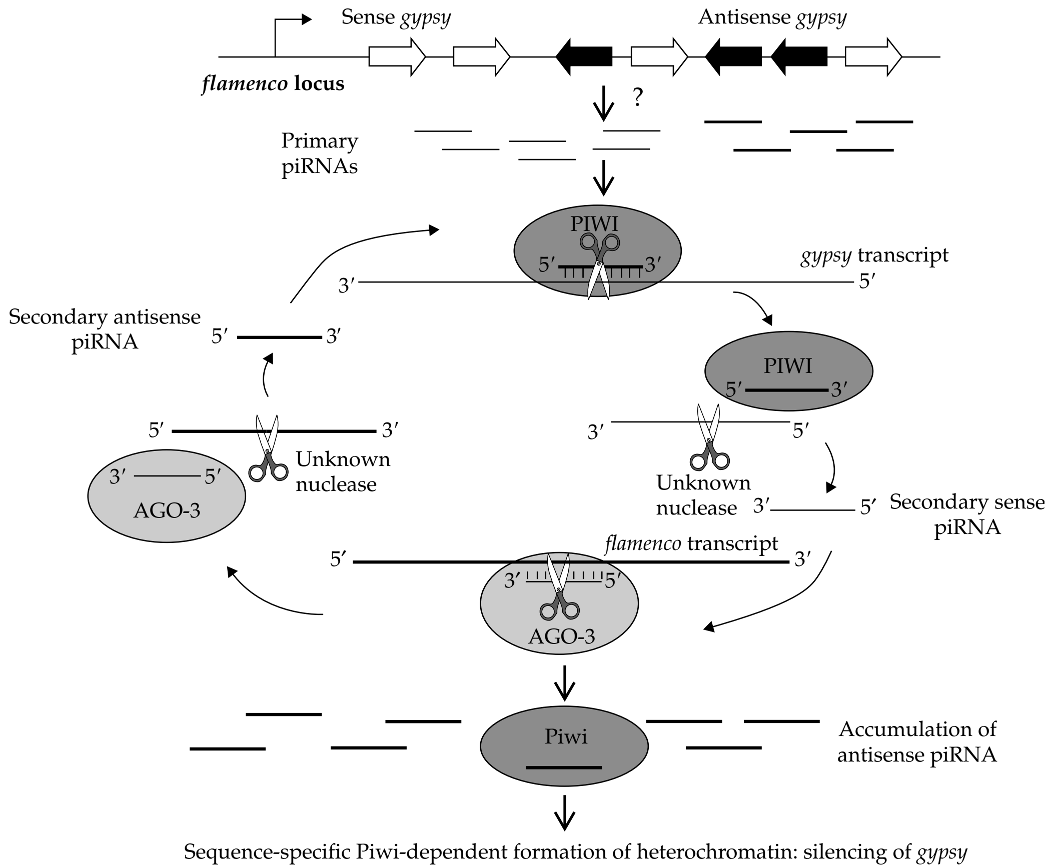

Figure 4.3 Model for synthesis of piRNAs and silencing of *gypsy* expression in *Drosophila*. The *flamenco* locus, which consists of defective integrated copies of *gypsy*, produces primary Piwi-associated RNA molecules (piRNAs). Antisense primary piRNAs guide Piwi (or Aubergine) to complementary sequences, which are cleaved by the slicer protein Piwi to generate the 5′ end of secondary piRNAs. The 3′ end of these piRNAs is generated by an unidentified mechanism. Secondary piRNAs of sense polarity then associate with AGO-3, and guide it to *flamenco* transcripts, which are cleaved to generate more antisense piRNAs.

infected cells (Figure 4.2). Consistent with this idea, transgenic flies expressing FHV dsRNA are protected against a challenge with FHV, but not with the unrelated DCV (Galiana-Arnoux *et al*., 2006). Thus, the siRNA pathway provides flies with a highly specific antiviral defence system, based on the pairing of complementary nucleic acids.

Another RNAi pathway, the piRNA pathway, plays an important role in the control of nucleic acid parasites like transposons or endogenous retroviruses in *Drosophila*. Genetic studies have shown that two loci located in heterochromatic regions of the X chromosome and known as *flamenco* and *X-TAS* restrict the ability of endogenous retroviruses of the Gypsy, Idefix, or ZAM family (*flamenco*) or transposons of the P element family (*X-TAS*) to translocate within the genome. *Flamenco* and *X-TAS* correspond to insertion hotspots for different types of mobile genetic elements, which will lead to the generation of large quantities of distinct piRNAs. These piRNAs associate with Piwi proteins and guide them to silence transposons or endogenous retroviruses dispersed all over the genome (Figure 4.3). Interestingly, a similar mechanism is present in mammalian genomes to control transposon mobility in the germ line (reviewed in Girard and Hannon, 2007). Expression of transposons is also

controlled in somatic tissues, by endo-siRNAs (Obbard and Finnegan, 2008).

4.3.1.3 Viral suppressors of RNAi

RNAi is an efficient antiviral defence mechanism in plants, and as a result viruses that successfully replicate in plant cells express suppressors of RNAi or viral suppressors of RNAi (VSRs). A few dozen VSRs have so far been identified from plant viruses. Interestingly, these molecules are extremely diverse both structurally and in terms of mode of action, suggesting that they have evolved independently to provide a variety of solutions to counter the host's RNAi-based defence (Ding and Voinnet, 2007). While some plant VSRs encode dsRNA-binding proteins that prevent interaction of dsRNA with Dicer enzymes (e.g. turnip crinkle virus p38), others prevent assembly of the RISC complex (e.g. gemini virus AC4), interfere with slicing (e.g. cauliflower mosaic virus 2b), or even promote ubiquitin-dependent proteolysis of key molecules of the RNAi machinery such as AGO-1 (e.g. Polerovirus P0).

In agreement with the proposed critical role of RNAi in antiviral defence in insects, insect viruses have also been shown to express VSRs. Further, as would be expected if RNAi were a crucial antiviral defence, *Dicer-2*, *AGO-2*, and *r2d2* are among the fastest-evolving genes in the *Drosophila* genome (Obbard *et al.*, 2006). The best-characterized VSR is the B2 protein from FHV. B2 is a 106 amino acid molecule, which dimerizes and forms a four-helix bundle. It is synthesized at high levels in infected cells, and binds dsRNA with nanomolar affinity. By contrast, it does not bind DNA, single-stranded RNA, or DNA–RNA hybrids. Interaction with dsRNA is sequence-independent, and B2 binds with affinity to dsRNAs as short as 17 bp. Hence, B2 interacts with both long dsRNA and siRNA, and can interfere with RNAi both before and after cleavage by Dicer enzymes (Chao *et al.*, 2005). Multiple B2 proteins may associate with a dsRNA molecule, thus coating the FHV replication intermediates, and preventing interaction with Dicer or other molecules of the RNAi machinery. In agreement with the proposed function of B2, B2-deficient viruses are not virulent, and are barely detected in injected wild-type flies. These viruses can, however, replicate in Dicer-2 or AGO-2 mutant flies, proving that B2 acts *in vivo* as a suppressor of RNAi (Galiana-Arnoux *et al.*, 2006; Wang *et al.*, 2006).

The Dicistroviruses DCV and CrPV have also been shown to encode VSRs. Interestingly, even though the two viruses are closely related and the two VSRs map to the N-terminus of the ORF1, the two suppressor proteins do not show any sequence similarity, and appear to function differently (Figure 4.1). Indeed, sequence analysis of the first 100 amino acids of DCV ORF1 reveals the presence of a canonical dsRNA-binding domain, whereas the N-terminus of CrPV ORF1 does not contain any known structural motifs. *In vitro* experiments with recombinant protein confirmed that the DCV VSR, known as DCV-1A, binds long dsRNAs with high affinity in a sequence-independent manner, and prevents processing by Dicer-2 (van Rij *et al.*, 2006). Unlike B2, DCV-1A does not bind to siRNAs, and seems to act only upstream of Dicer. Globally however, both FHV B2 and DCV-1A act by sequestering dsRNA, even though they do not share any sequence similarity, providing a nice example of convergent evolution in two insect viruses to counteract RNAi. The mode of action of the VSR of CrPV remains mysterious (Wang *et al.*, 2006). It is fascinating that DCV and CrPV, which are two closely related members of *Dicistroviridae* family, sharing high sequence similarity throughout their genomes, are completely divergent in the N-terminus of their ORF1. Examination of the N-terminus of ORF1 from the other sequenced Dicistroviruses reveals that they are not related, suggesting that they may all have evolved original strategies to evade host defences. Interestingly, two of these viruses have motifs at the N-terminus of ORF1 that may hint to the function of the suppressors (Figure 4.1). In the case of aphid lethal paralysis virus (ALPV), a discrete Pox protein repeats of ankyrin-C-terminal (PRANC) motif is observed. This motif is present at the C-terminus of a variety of poxvirus proteins, and is related to F-box proteins. Thus, this protein may function in a manner similar to P0 from plant poleroviruses and target one component of the RNAi pathway for degradation. The second case is the shrimp virus Taura syndrome virus (TSV), which encodes a baculovirus inhibitor of apoptosis repeat (BIR) domain.

As indicated by its name, this domain is present in some viral proteins that interfere with the apoptotic pathway. Thus, for some Dicistroviruses, the N-terminus of ORF1 may be used to interfere with antiviral defences other than RNAi.

4.3.2 Inducible response to virus infection

A hallmark of the immune antiviral response in mammals is the production of interferons, which are induced upon recognition of viral pathogens by innate immunity receptors (Toll-like receptors (TLRs) and RIG-like receptors). Interferons then trigger the production of antiviral molecules and establishment of an antiviral state in the infected cells (Beutler *et al.*, 2007). There is now also evidence for an inducible innate antiviral defence system in *Drosophila*.

4.3.2.1 Imd and Toll pathways

One major characteristic of the response to bacterial or fungal infections is the inducible secretion into the haemolymph of a cocktail of antimicrobial peptides (Lemaitre and Hoffmann, 2007). In a first step to characterize the response of *Drosophila* to virus infection, an attempt was made to identify molecules induced by DCV infection that could serve as markers of the antiviral response. Proteomic analysis of the haemolymph of DCV-infected flies revealed that antimicrobial peptides regulated by the Toll or Imd pathways are not induced upon infection by this virus (Sabatier *et al.*, 2003). An independent proteomic analysis more recently identified some 150 proteins upregulated in FHV-infected *Drosophila* tissue-culture cells, but, again, the known *Drosophila* antimicrobial peptides were not induced (Go *et al.*, 2006). Genome-wide microarray analysis of the transcriptome of flies infected with DCV, either by injection or by ingestion, suggest that some genes encoding antimicrobial peptides regulated by the Toll or the Imd pathway are induced (Roxström-Lindquist *et al.*, 2004; Dostert *et al.*, 2005). However, at least in the case of the injection model, quantitative analysis by RNA blot hybridization or quantitative real-time PCR showed that these genes are only weakly upregulated compared to bacterial or fungal challenges (Dostert *et al.*, 2005). Thus, neither DCV nor FHV seem to activate the Toll or the Imd pathways.

Different results were obtained with DXV, which appears to induce several genes encoding antimicrobial peptides to the same levels as infection with the Gram-negative bacterium *Escherichia coli*. However, genetic analysis revealed that flies mutant for the NF-κB-related transcription factor Relish are not more susceptible to DXV infection than wild-type flies, ruling out a role for the Imd pathway in the resistance to this virus. By contrast, flies mutant for Dif, the NF-κB-like transcription factor regulated by the Toll pathway, are more susceptible to DXV infection than wild-type flies. Surprisingly, loss-of-function mutants for other components of the Toll pathway (*Toll*, *Spätzle*, *Tube*, and *Pelle*) succumb to DXV infection at a similar rate as wild-type controls (Zambon *et al.*, 2005), suggesting that Dif mediates resistance to DXV infection independently of the classical Toll pathway. Of note, the gene *ref(2)P*, which mediates refractoriness to SIGMAV infection (see above), has been proposed to encode a component of the Toll pathway, suggesting a connection between SIGMAV infection and the activity of Dif and Dorsal (Avila *et al.*, 2002). However, flies containing a permissive allele of *ref(2)P* are more susceptible to SIGMAV infection than flies that are deficient for the gene, indicating that SIGMAV uses the permissive allele to infect flies. This recent finding implies that the control of SIGMAV by *ref(2)P* may not result from a host defence mechanism (Carre-Mlouka *et al.*, 2007).

SIGMAV was also shown to induce expression of antimicrobial peptides, at least at the mRNA level. Of note, SIGMAV infection leads to upregulation of the genes encoding the antibacterial peptides diptericin and drosocin, regulated by the Imd pathway, but not the antifungal peptide drosomycin, suggesting that the Imd pathway, rather than the Toll pathway, is involved (Tsai *et al.*, 2008). It should be noted, however, that induction of antimicrobial peptides was not shown to confer protection against SIGMAV infection. Finally, a last indication for the possible participation of NF-κB pathways in the control of viral infections in *Drosophila* comes from the fact that some insect DNA viruses, such as the insect-specific polydnaviruses, inhibit this signalling pathway by encoding inhibitors

structurally homologous to the inhibitory κB (IκB) proteins known to inhibit Dif and Relish expression in the cytoplasm (e.g. vankyrins). These viral proteins probably function in a way analogous to IκB in infected tissues and compromise the insect immune response by repressing NF-κB-like transcription factors (Thoetkiattikul *et al.*, 2005; Kroemer and Webb, 2006).

In summary, there are at present no clear experimental data connecting the Toll and Imd NF-κB pathways to antiviral immunity. One should, however, remain open-minded until a larger number of viruses have been tested, and additional features other than antimicrobial peptides have been examined. The Imd pathway, for example, regulates apoptosis, a well-known antiviral response, and can be suppressed by expression in transgenic *Drosophila* of the baculovirus anti-apoptotic protein p35 (Georgel *et al.*, 2001). Clearly, the involvement of the Toll and Imd pathways in the resistance to viral infections deserves further investigation.

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4.3.2.2 vir-1 *and the JAK/STAT pathway*

Genome-wide microarray analysis of the transcriptome of flies 24 or 48 h after injection of DCV identified some 140 genes induced by a factor of at least two. Only one-third of these genes are also upregulated following bacterial or fungal infections, confirming that pathways different from Toll and Imd are activated in response to DCV infection. Since the list of genes induced did not provide any hints pointing to the pathway activated, the regulation of the gene *vir-1* (*virus-induced RNA 1*) was studied. This gene is not expressed in adult flies, and is strongly induced by DCV and FHV infection, but not by bacteria or fungi (Dostert *et al.*, 2005; Hedges and Johnson, 2008). *vir-1* is a previously unrecognized transcript of the gene *CG31764*, which is produced from an inducible promoter. Promoter truncation experiments in transgenic flies led to the demonstration that the virus-response element maps to a 190 bp fragment, which contains a consensus binding site for the transcription factor STAT92E. Introduction of point mutations in this STAT-binding site strongly reduces induction of the *vir-1* promoter, and DCV infection triggers induction of STAT DNA-binding activity in fly nuclear extracts (Dostert *et al.*, 2005; C. Dostert and J.L. Imler, unpublished results). Altogether, these data suggest that the Janus kinase/signal transduction and activators of transcription (JAK/STAT) pathway is involved in the induction of *vir-1*. In *Drosophila*, this pathway is composed of a single JAK kinase, encoded by the gene *hopscotch*, and a single STAT factor (known as STAT92E) encoded by the gene *marelle*. The kinase is regulated by the cytokine receptor Domeless, which bears some similarity with the gp130 subunit of the interleukin-6 receptor in mammals (Figure 4.4). The *Drosophila* JAK/STAT pathway controls cell multiplication and differentiation in multiple tissues and developmental stages (Arbouzova and Zeidler, 2006). Most mutants of the pathway therefore exhibit developmental phenotypes, making genetic experiments in adult flies a difficult task. However, analysis of the flies carrying a viable combination of a null and a hypomorph allele of *hopscotch* (Agaisse *et al.*, 2003) revealed that the JAK kinase is required for induction of *vir-1*. This result was confirmed in flies overexpressing either a dominant-negative version of the Domeless receptor, or the negative regulator of the pathway dPIAS (Dostert *et al.*, 2005). These data are consistent with a model in which one cytokine of the Unpaired (Upd) family (Upd-1, -2, and -3) is induced by DCV infection, and triggers an antiviral state in cells through activation of the JAK/STAT pathway.

Of note, several other genes induced by DCV were found to contain consensus binding sites for STAT92E in their proximal promoter, and to be dependent on Hopscotch for full induction in virus-infected flies. Importantly, this response is associated with protection against infection, as shown by the fact that *hopscotch* mutant flies contain higher viral load than wild-type controls, and succumb more rapidly. Thus, at least some of the genes induced by DCV participate in the control of the viral amplification, by mechanisms that remain to be identified. Some of these genes may encode antiviral molecules targeting the virions or interfering with one step of the viral replication cycle. These antiviral mechanisms are probably distinct from RNA interference, since the list of DCV-induced genes does not contain any genes of the RNAi pathway. Interestingly, there is some

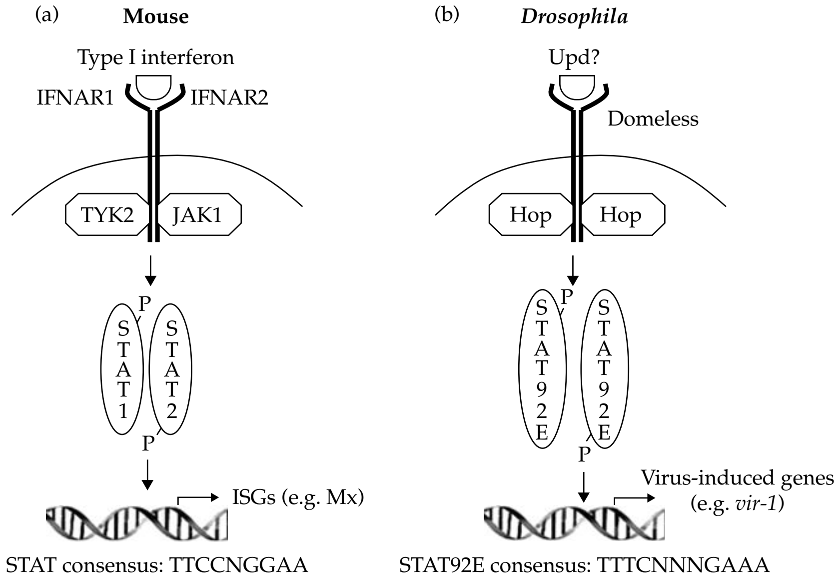

Figure 4.4 The JAK/STAT signalling pathway and antiviral immunity in mammals and flies. In mice (and humans) stimulation of the interferon receptor (IFNAR) by type I interferon produced in virus-infected cells leads to activation of the JAKs TYK2 and JAK1. These tyrosine kinases then phosphorylate (P) the transcription factors STAT1 and STAT2, triggering SH2-domain-mediated dimerization and nuclear translocation. Once in the nucleus, STAT transcription factors induce expression of interferon-stimulated genes (ISGs), such as those encoding the Mx proteins. In *Drosophila*, the JAK encoded by the gene *hopscotch* activates the STAT transcription factor STAT92E and induces expression of several virus-induced genes, such as *vir-1*. The Domeless cytokine receptor is required for the induction of the pathway in response to viral infection, but the cytokine involved, which is most likely one of the three members of the Unpaired (Upd) family, has not been identified yet. The consensus recognition DNA sequences for STAT1 and STAT3 in mammals and STAT92E in *Drosophila* are indicated.

evidence that the inducible response contributes to the pathogenesis of DCV-infected flies, as *hopscotch* mutant flies succumb more rapidly to DCV infection than wild-type flies when they are challenged with a low dose of virus, but not when they are challenged with a high dose of virus (Dostert *et al.*, 2005). One interpretation for this result is that *hopscotch* mutant flies, which make an attenuated inducible response, can cope with a higher viral load. A similar type of observation was made when the sensitivity of *Dicer-2* mutant flies to FHV infection was studied: *Dicer-2* mutant flies resist infection only poorly when infected with a high dose of FHV, even though they do not contain more viral RNA than wild-type flies (because of the expression of B2, which suppresses RNAi; see above). This strong susceptibility to viral infection correlates with an increased induction of *vir-1* in *Dicer-2* mutant flies (Galiana-Arnoux *et al.*, 2006). Altogether, these data indicate that the inducible response to virus infection is more complex than the inducible response to bacteria or fungi, where detection of the infection primarily triggers the production of effector molecules. It appears that some of the virus-induced genes alter the physiology of the host, a situation reminiscent of inflammation in mammals, which is well known to be associated with adverse effects in severe cases of sepsis.

4.3.2.3 The JAK/STAT pathway is necessary, but not sufficient for the inducible antiviral response

Although playing an important role in the control of at least some viral infections, the inducible antiviral response is not limited to the JAK/STAT pathway. Indeed, genes from the *Turandot* (*Tot*) family, which are regulated by the JAK/STAT pathway, are not induced by DCV infection, at least in the first 2–3 days of infection. *Tot* genes are, however, strongly induced following FHV infection. The *TotA* gene was initially identified in a screen for

genes differentially expressed in bacteria-infected flies (Ekengren *et al.*, 2001). *TotA* belongs to a family of eight genes in *D. melanogaster* that are upregulated by bacterial challenge, but also by a number of other stresses, such as extreme heat shock (temperatures above 37°C), mechanical pressure, or ultraviolet irradiation in larvae. The induction by the Gram-negative bacterium *E. coli* was later shown to involve the JAK/STAT pathway, since (1) *TotA* and *TotM* induction is abolished in flies carrying loss-of-function mutations in the *hopscotch* gene and (2) *Tot* genes are constitutively expressed in flies carrying the constitutively active allele of *hopscotch*, $hop^{Tum\text{-}l}$ (Agaisse *et al.*, 2003). These data suggest that the JAK/STAT pathway is both necessary and sufficient for the regulation of *Tot* genes, implying that if the pathway is activated during DCV infection, *Tot* genes should be induced. Experimental data demonstrate that things are more complex, however, and the regulation of *vir-1*, and probably also that of *Tot* genes, cannot be narrowed down to a single pathway. The fact that *vir-1* is not constitutively expressed in flies containing the $hop^{Tum\text{-}l}$ allele clearly points out that activation of the JAK/STAT pathway is not sufficient to trigger expression of this gene. In mammals, STAT transcription factors have long been known to function with cofactors. For example, the antiviral effects of type I interferon are not mediated by STAT-1 only, but by the association of STAT-1 with the IRF9 transcription factor. Both STAT-1 and IRF9 bind DNA, and the juxtaposition of STAT-1- and IRF9-binding sites defines the subset of STAT-1-regulated promoters that are induced by type I interferon. In *Drosophila*, the Bcl6-related factor encoded by the gene *Ken & Barbie* (*Ken*) provides an example of a transcription factor that selectively modulates the activity of STAT92E on some promoters *in vivo* (Arbouzova *et al.*, 2006). Induction of *Tot* genes also requires the *Relish* transcription factor (Agaisse *et al.*, 2003), and the MEKK-1 (mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) kinase kinase 1) pathway (Brun *et al.*, 2006), providing a further possible explanation for the differential regulation of *vir-1* and *Tot* genes.

The fact that some virus-response genes remain fully inducible in *hopscotch* mutant flies provides an independent line of evidence that other signalling pathways contribute to the inducible antiviral response. The gene *Vago* (CG2081), which remains inducible in *hopscotch* mutant flies, provides a good model to identify these alternative pathways (Dostert *et al.*, 2005). Unlike *vir-1*, *Vago* is induced in DCV-infected cells of the fat body, suggesting that it may be induced directly upon sensing the presence of virus in the cells, rather than by a cytokine produced from virus-infected cells. *Vago* is induced following infection by DCV and SINV, but not by FHV. The protein B2 accounts for this difference, and acts as a suppressor of *Vago* induction (S. Deddouche and J.L. Imler, unpublished results). Because this viral protein is a dsRNA-binding protein, these findings strongly suggest that *Drosophila* cells, like their mammalian counterparts, sense dsRNA as a molecular pattern betraying the presence of virus in the cell. It will be particularly interesting to understand how the expression of *Vago* is regulated, and compare this signalling mechanism to the one leading to interferon production in mammalian cells.

4.3.3 ATP-sensitive potassium channels and resistance to virus infection

How does the inducible antiviral response contribute to the pathogenesis of the infection? In mammals, inflammatory cytokines, such as tumour necrosis factor (TNF), trigger inflammation by acting on blood vessels (i.e. vasodilatation) and the systemic effects of TNF can lead to septic shock. Could there be a similar situation in flies? Recent genetic data suggest that this might be the case. These studies were initiated by the identification of the *mayday* mutation in mice, which leads to increased sensitivity to infection with the DNA virus mouse cytomegalovirus (MCMV). Strikingly, *mayday* mutant mice die abruptly 2–3 days after infection, without showing any signs of overt disease, and when viral titres are still fairly low. Induction of interferons and other inflammatory cytokines is not affected in *mayday* mutant mice, indicating that these animals do not appear to be conventionally immunocompromised, and viral titres in mutant and wild-type mice are comparable at the time of death of *mayday* mutant mice. Chromosomal mapping and positional cloning pinpointed the *Kcnj8*

gene as the target of the *mayday* mutation. *Kcnj8* encodes Kir6.1, the pore-forming component of an inwardly rectifying ATP-sensitive potassium channel (K_{ATP}) that is expressed in coronary artery smooth muscle cells. This channel also incorporates the regulatory subunit SUR2 (sulphonylurea receptor 2). Altogether these data highlight the important role of K_{ATP} channels in modulation of cardiovascular stress during the immune response to infections (Croker *et al*., 2007) (Figure 4.5).

K_{ATP} channels are evolutionary conserved, and *Drosophila* has two orthologues (*Ir* and *Irk2*) of the gene encoding the potassium channel pore, but only a single orthologue of the gene encoding the regulatory subunit (SUR2) of the mammalian potassium channel. Of note, this gene, called *Drosophila* SUR (*dSUR*), is mainly expressed in the contractile dorsal vessel that forms the *Drosophila* heart. Moreover, dSUR has a protective role against hypoxic stress and heart failure induced by electrical pacing (Akasaka *et al*., 2006). This may have relevance for resistance to viral infection, since sensitivity to hypoxia is a readout for the susceptibility to two *Drosophila* viruses, SIGMAV and DXV. These findings led us to test the role of dSUR in the resistance to virus infection in flies. Silencing of dSUR expression in the *Drosophila* heart, but not in other tissues, was found to result in increased susceptibility to challenge with FHV. By contrast, dSUR knock-down flies experienced mortality similar to that of wild-type controls when infected with DCV or the bacterial pathogens *Enterococcus*

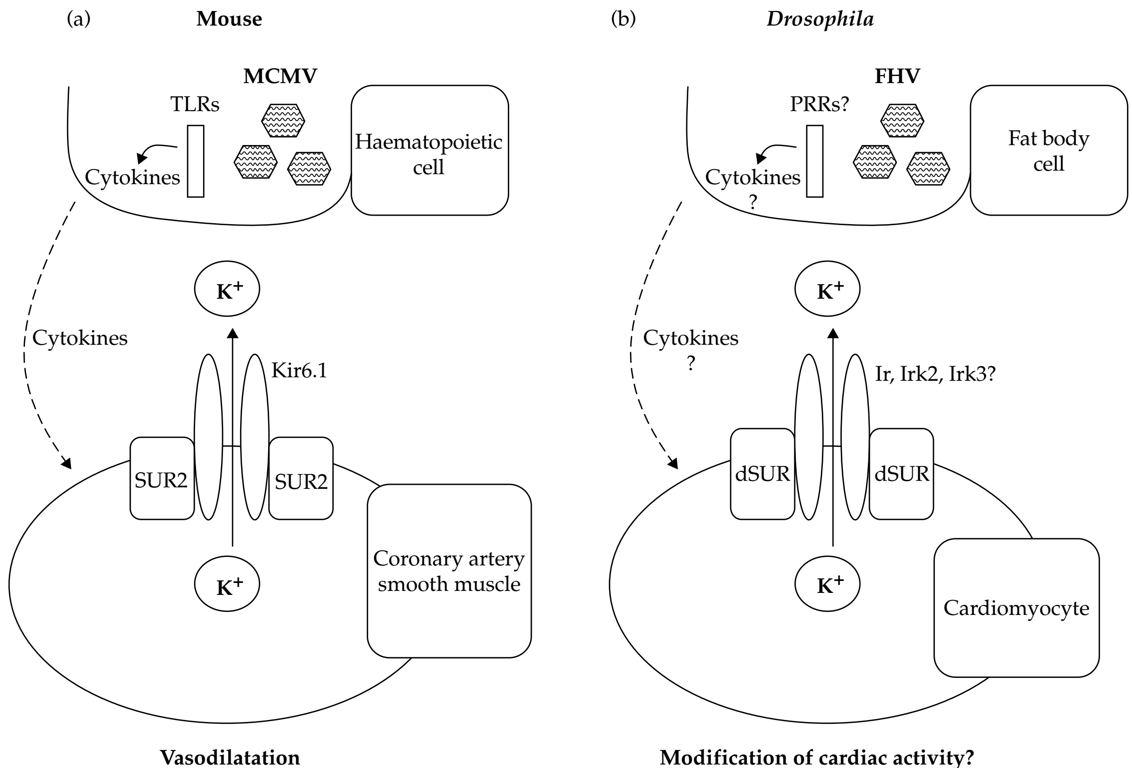

Figure 4.5 Model for the role of ATP-sensitive potassium channels (K_{ATP}) in the resistance to virus infection. In mice, cytokines produced in virus-infected cells act on blood vessels and trigger vasoconstriction. This effect is opposed by K_{ATP} channels, which are composed of a pore subunit and a regulatory subunit (Kir6.1 and SUR2 in coronary arteries). By analogy, K_{ATP} channels in *Drosophila* may counteract cytokine-mediated effects on the dorsal vessel of FHV-infected cells, thus explaining why *Drosophila* SUR (dSUR) expression in cardiomyocytes is required to resist infection by this virus. FHV, Flock House virus; Kir6.1, inwardly rectifying K_{ATP}; MCMV, mouse cytomegalovirus; PRRs, pattern-recognition receptors; SUR2, sulphonylurea receptor 2; TLRs, Toll-like receptors.

faecelis, *Enterobacter cloacae*, or the fungus *Beauveria bassiana*. In addition, treatment of wild-type flies with tolbutamide, an antagonist of the channel, also affects resistance to FHV (Croker *et al*., 2007).

These findings provide further evidence that both insects and mammals rely on a common homeostatic mechanism for protection against viruses, and open the way for future studies aimed at elucidating the exact role of K_{ATP} channels in the physiology of viral infections.

4.4 Relevance to other insects and invertebrates

Although we have focused here on *Drosophila*, antiviral defences have also been studied in other insect or invertebrate species, either because of their medical interest as vectors for viral diseases or because of their economic importance. These studies, which highlight the relevance of the *Drosophila* model, are summarized below.

4.4.1 RNAi in mosquitoes

The recognition and destruction of dsRNA has recently been recognized as an intracellular antiviral response against insect viruses of medical importance, and is the subject of a number of arbovirus-vector competence studies. In addition, mosquito cells support RNAi and homologues of the *Drosophila* RNAi components are encoded by the genome of *Anopheles gambiae*, which transmits the O'nyong nyong alphavirus and *Aedes aegypti*, the yellow fever virus vector (Waterhouse *et al*., 2007). It has been shown that replicating viral RNAs are naturally targeted for destruction by RNAi in mosquito cells (Li *et al*., 2004), and increased viral loads have been reported in *A. gambiae* upon silencing of the gene encoding the AGO-2 protein (Keene *et al*., 2004).

Previous experiments indicated that RNA-based strategies could be used to control vector-borne viruses of public health importance. For example, expression of dengue virus genome fragments in mosquito cells or *in vivo* inhibited later dengue virus infection and replication (Olson *et al*., 1996; Adelman *et al*., 2001). Similarly, *A. aegypti* were genetically modified to express an inverted repeat RNA that derives from the dengue type 2 virus (DENV2) *prM* gene, under the control of a midgut-specific promoter. These transgenic mosquitoes express a dsRNA product corresponding to prM sequences after they imbibe a bloodmeal, thus triggering anti-DENV2 RNAi and thereby keeping the virus from disseminating to the host salivary glands (Franz *et al*., 2006). Thus, by genetically manipulating *A. aegypti* to enhance RNAi, the competence of such mosquitoes to serve as vectors for dengue viruses becomes greatly reduced.

4.4.2 Inducible response

Evidence for the existence of an inducible antiviral response has also been obtained in several other invertebrate species. For example, injection of dsRNA in shrimps or sandflies can induce an antiviral response (Robalino *et al*., 2005; Pitaluga *et al*., 2008). In the crayfish *Pacifastacus leniusculus*, the large DNA virus white spot syndrome virus (WSSV family Nimaviridae) induces expression of the antilipopolysaccharide factor (ALF), which interferes with WSSV replication *in vitro* and *in vivo* (Liu *et al*., 2006). The mode of action and the mechanism of regulation of ALF remain to be characterized. Finally and of great potential interest, infection of the vector insect *A. aegypti* by arboviruses also leads to altered gene expression. For example SINV infection leads to changes in expression in 135 genes, some of which are strongly upregulated in the midgut (Sanders *et al*., 2005). Interestingly, one of these strongly induced genes is the orthologue of Unc93b, which plays a critical role in TLR-mediated antiviral defences in mammals (Beutler *et al*., 2007). Infection with dengue virus also leads to induction of many genes, including antimicrobial peptides, and a bias towards the Toll and JAK/STAT pathways. Furthermore, silencing of MyD88, a key component of the Toll pathway, resulted in a small but significant increase in dengue viral load in the midgut of infected mosquitoes, supporting the concept that upon infection the Toll pathway regulates expression of antiviral molecules (Xi *et al*., 2008). An evolutionarily conserved antiviral role of the JAK/STAT pathway is also supported by independent studies in other invertebrate models. The induction of STAT DNA-binding activity in the

mosquito cell line C6/36 has been reported following infection by the flavivirus Japanese encephalitis virus (Lin *et al.*, 2004). In addition, WSSV, which infects shrimps, also induces STAT-binding activity in infected animals, and subverts it to enhance the expression of its immediate-early genes (Liu *et al.*, 2006).

4.4.3 Baculoviruses and apoptosis

Apoptosis is a conserved mechanism of programmed cell death that is essential for development and homeostasis of animals. In addition, apoptosis is an effective strategy used by vertebrate and invertebrate hosts to counter viral infection, since the induction of programmed cell death in virus-infected cells efficiently prevents viral amplification and spreading. Curiously, apoptosis has not been involved in antiviral defences in *Drosophila* so far. This may reflect the limited number of viruses that have been tested. Indeed, apoptosis has mostly been associated as an antiviral defence against DNA virus infections, and these viruses have not yet been identified in *Drosophila*.

Insect DNA viruses, such as baculoviruses, have evolved methods to bypass this antiviral defence by expressing suppressors of apoptosis. A characteristic example of such a suppressor is the caspase inhibitor p35, encoded by the baculovirus AcMNPV. Expression of p35 protein in infected cells prevents apoptosis, increases virus titre in the insect tissues and allows successful infection of the host (Clarke and Clem, 2003). Infectivity of p35 mutant viruses is greatly reduced, thus revealing the important role of apoptosis as an antiviral defence mechanism in insects. Subsequent efforts to identify other suppressors of apoptosis led to the identification of the family of inhibitors of apoptosis (IAP) (Srinivasula and Ashwell, 2008). Unlike p35, baculoviral IAPs have cellular orthologues, which interact with caspases and inhibit them. Of note, the *Drosophila* genome encodes two IAP molecules, DIAP1 and DIAP2, one of which (DIAP2) is a component of the Imd pathway.

Microarray and proteomic analysis suggest that apoptosis could be involved in antiviral defences in flies. For example, the gene encoding the caspase Damm appears to be upregulated following DCV infection in flies (Dostert *et al.*, 2005). On the other hand, experiments in tissue culture *Drosophila* Schneider's DL1 cells revealed that FHV infection induces expression of croquemort (involved in the uptake of apoptotic bodies) and represses expression of DIAP1 (Go *et al.*, 2006). Interestingly, FHV was indeed shown to trigger apoptosis in DL1 cells. FHV inhibits host-cell protein synthesis, which leads to the rapid depletion of the short-lived DIAP1 protein, and activation of the caspases DRONC and DrICE, leading to apoptosis. Importantly, however, apoptosis seems to be of little consequence for FHV multiplication, which questions the importance of programmed cell death in the control of FHV infection (Settles and Friesen, 2008).

4.5 Comparison with mammalian antiviral defences

Based on its critical role in the control of viral infection in plants and invertebrates, RNAi is often presented as an evolutionarily conserved antiviral defence. But is RNAi involved in the control of viral infections in mammals? While there is no doubt that RNAi can be used to fight viral infection, when cells are supplied with exogenous siRNAs or small hairpin (sh) RNAs, vertebrate cells apparently cannot process viral RNAs into siRNAs. This probably reflects the fact that vertebrates only have a single *Dicer* gene, mediating the production of miRNAs, whereas insects and plants have respectively one or three additional Dicer enzymes producing siRNAs. There are several examples in the literature demonstrating that miRNAs can modulate the replication cycle of viruses in mammalian cells (reviewed in Müller and Imler, 2007). The situation closest to that found in insects and plants is that of cellular miRNAs targeting viral sequences. The role of these miRNAs has been best described in the case of the Indiana strain of VSV, where two miRs, miR24 and miR93, target the *L* and *P* genes from the virus. The relevance of this control has been attested by studies using mice containing a hypomorphic mutation of *Dicer*, which exhibit a higher sensitivity to VSV infection. Importantly, while *Dicer* mutant macrophages produce five- to 10-fold more VSV than wild-type macrophages, they do not have a general antiviral defect, and produce viral titres similar to the wild-type when challenged

with several other viruses (Otsuka *et al.*, 2007). One key question—if cells can use miRNAs to target viral genomes—is why the viruses do not change the target sequence to escape recognition. Viruses are indeed known to rapidly mutate and evolve to adapt to their host. One possible answer is that it is not the miRNA that targets the viral genome, but rather the virus that targets the miRNA, and uses it to modulate its replication, to avoid causing too much damage to its host. In support of this explanation, the New Jersey strain of VSV, which is known to induce a stronger interferon response in cattle than the Indiana strain, cannot be recognized by miR24 and miR93 (Müller and Imler, 2007). A similar situation may occur for hepatitis C virus, where viral genomic RNA is recognized by several cellular miRNAs that have antiviral effects. Interestingly, expression of these miRNAs appears to be regulated by interferons, providing a way for the virus to modulate its effects on the host (Pedersen *et al.*, 2007). In summary, even though there is no question that viruses can be targeted by miRNA in mammalian cells, and even in extreme cases use the immune system to upregulate some miRNAs, this probably reflects more an adaptation of viruses to their hosts than a *bona fide* immune response, associated with protection of the host against infections. In this respect, the situation in mammals is different from that in insects and plants.

More similarities between insects and mammals are apparent for the inducible response to viral infection, since the JAK/STAT pathway mediates signalling downstream of many cytokine receptors, including the interferons. In mammals, expression of interferons is mediated by the transcription factors IRF3 and IRF7, which are activated upon sensing viral RNAs in infected cells by the cytosolic DExD/H-box helicases RIG-I or MDA5 (reviewed in Beutler *et al.*, 2007). The fly genome does not encode orthologues of these receptors, nor of IRF transcription factors, suggesting that other mechanisms are involved in the sensing of viruses. Viral nucleic acids are also detected in mammalian cells by the Toll-like receptors TLR3, TLR7, and TLR9. As we have seen above, there is some evidence that the Toll pathway participates in the resistance to DXV infection in flies, suggesting that Toll may detect viral components and regulate expression of cytokines activating the JAK/STAT pathway. However, one must keep in mind that, up to now, Toll in flies has been shown to function like a receptor for the cytokine Spätzle (Weber *et al.*, 2003), rather than like a pattern-recognition receptor.

Another interesting point of comparison between mammals and insects is the function of the induced genes. In mammals, interferon regulates the expression of more than 300 interferon-stimulated genes (ISGs), but most of them remain poorly characterized. In fact, most attention has focused on four major effector molecules that help to contain the viral infection (reviewed in Sadler and Williams, 2008): (1) protein kinase R, which phosphorylates the translation factor eIF2α, and inhibits translation, thus blocking the synthesis of viral proteins; (2) 2′-5′ oligoadenylate synthase (OAS), which regulates the activity of RNaseL; (3) the Mx GTPases, which regulate membrane trafficking and can trap essential viral components; and (4) the 15 kDa ubiquitin-like molecule ISG15, which can be conjugated to protein substrates (for example IRF3), resulting in their activation. Importantly, none of these effector molecules are present in *Drosophila*. Thus, flies appear to rely on specific antiviral mechanisms. The identification of these mechanisms may therefore reveal novel ways to interfere with the viral cycles that could lead to novel ideas for therapeutic intervention.

In summary, even though there appears to be striking conservation of the mechanisms operating during the inducible antiviral response in flies and mammals at the conceptual level (induction of several hundred genes; involvement of a cytokine activating the JAK/STAT pathway), important differences are apparent in the sensing of viral infection, and the effector molecules induced by infection. Whether the similarities reflect a common ancestry, or convergent evolution, will become apparent when the mechanisms of the inducible antiviral immunity in flies have been better characterized, and the extent of the similarities/differences is more apparent.

4.6 Conclusions and perspectives

Over the past few years, insect immunologists working with *Drosophila*, exploiting its unparalleled

power as a model system (forward genetic screening in adult flies, genome-wide RNAi screening of *Drosophila* cell lines, and sequenced genome of 12 *Drosophila* genomes, providing a unique opportunity for comparative genomics), have made significant progress towards deciphering many aspects of antiviral immune defence mechanisms in insects. Even though we are still far from having a complete picture of the mechanisms of resistance to viral infections in flies, it is clear at this stage that flies can defend themselves against virus infections, and that the mechanisms involved are mostly different from those triggering the humoral response activated by bacterial and fungal infections. As has already been appreciated in previous studies on bacterial and fungal infections, the mechanisms involved are evolutionarily conserved, at least in part. Of note, it appears that there may be more than one type of antiviral immune response, as exemplified by the importance of Dicer-2 to control infection by DCV, FHV, and SINV, but not DXV; the induction of *Tot* genes by FHV but not by DCV; and the role of dSUR in the resistance to FHV, but not DCV infection. It will take some time to fully appreciate the complexity of antiviral immunity in *Drosophila*. In addition, our data with K_{ATP} channels indicate that, both in flies and mammals, antiviral immunity is only one of the parameters that affect the outcome of the pathogenesis associated with viral infection. *Drosophila* provides an ideal system to address experimentally the complex issue of homeostasis in virus-infected animals, and to provide an integrative view, at the organism level, of the complex interaction between viruses and their hosts. One can hope that this understanding will lead to new concepts that will be helpful to unravel the genetic mechanisms of antiviral resistance in more complex organisms (i.e. mammals). These studies are also likely to be of relevance for the understanding of the interactions between vector insects and arboviruses, a major challenge for the years to come.

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CHAPTER 5

Specificity of the innate immune system: a closer look at the mosquito pattern-recognition receptor repertoire

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5.1 Introduction

Broadly speaking, the insect innate immune system is encoded by three major functional categories of genes that are involved in (1) recognition of invading microbes, (2) immune-signal amplification and transduction, and (3) effector mechanisms that mediate the killing and clearance of infectious micro-organisms. Despite its lack of adaptive immune mechanisms and antibody-mediated defences similar to those found in vertebrates, the innate immune system in insects is quite specific in its antimicrobial action. Once invading microbes are recognized through specific interaction between pattern-recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs), a variety of defence reactions can be activated (Medzhitov and Janeway, 2002). The activation of immune responses can either occur directly, as in the case of phagocytosis and melanization, or indirectly, through intracellular immune-signalling pathways that initiate the transcriptional activation of appropriate antimicrobial peptides and other immune effector genes (Dimopoulos, 2003; Christophides *et al.*, 2004; Osta *et al.*, 2004). In this chapter, we will specifically focus on the specificity of the innate immune responses at the level of the PRRs, with a major focus on the mosquito *Anopheles gambiae* as a model system. We will first provide a general overview of the insects' PRR repertoire and highlight some of its most interesting features with regard to antimicrobial defence (section 5.2). We will then provide detailed molecular and functional descriptions of some of the best-characterized PRR families (sections 5.3–5.7).

5.2 The mosquito PRR repertoire

Our discussion here is focused on the general features of the PRR repertoire in the mosquito *A. gambiae*, as representative of an insect that is exposed to a particularly broad range of microbes. The genome of the *A. gambiae* mosquito harbours approximately 150 germ-line-encoded PRR genes. The majority of these genes encode secreted proteins with adhesive domains that can interact with PAMPs, which are commonly lipopolysaccharides or peptidoglycans. Most mosquito PRRs have a single pattern-recognition domain, but some of them have a more complex pattern of gene organization that includes multiple domains with other functional roles, such as catalysis or signal transduction. All known mosquito PRRs belong to larger gene families, most of which have expanded significantly when compared to their homologues in the fruit fly *Drosophila melanogaster*. Quite a few *A. gambiae* PRRs have been functionally implicated

in the mosquito's anti-*Plasmodium* defence and presumably participate in recognition of the parasite.

One of the most well-characterized PRRs is the thioester-containing protein 1 (TEP1), a complement-like phagocytic factor that also recognizes and associates with the malaria parasite (Levashina *et al*., 2001). TEP1 is produced by haemocytes and is found to colocalize with the surface of *Plasmodium* ookinetes in the midgut epithelium. The ligand of TEP1 appears to be ookinete-specific, since the later oocyst stages are only weakly recognized, and no association with the late sporozoite stage has been documented. Once it has interacted with the parasite, TEP1 appears to activate a powerful killing mechanism that eliminates the parasite (Blandin *et al*., 2004).

Another anti-*Plasmodium* PRR is the leucine-rich-repeat-containing protein LRIM1, which is specifically involved in killing the rodent *Plasmodium berghei* parasite but has no apparent effect on the human parasite, *Plasmodium falciparum* (Osta *et al*., 2004). The mechanism of action of LRIM1 is unknown, and its association with the parasite has yet to be demonstrated. The differential effect of this protein on the two parasite species is likely to reflect a high degree of pattern-recognition specificity.

Interestingly, not all PRRs are negative effectors with regard to pathogen development. For example, some PRRs have been shown to act as agonists with regard to infection by the rodent *Plasmodium* parasite. The infection-inducible C-type lectins, CTL4 and CTLMA2, have a protective effect on the parasite and prevent its melanization (Osta *et al*., 2004). It is likely that the *P. berghei* parasite associates with these lectins and uses them to camouflage itself from the mosquito's defence system. Interestingly, the protective capacity of these C-type lectins is specific for *P. berghei* and is not seen in the case of infection with the human pathogen, *P. falciparum* (Cohuet *et al*., 2006).

A plethora of other PRRs have also been shown to modulate *Plasmodium* infection in the mosquito, but their specificities and mechanisms of action are still under investigation. For instance, AgMDL1 is an *A. gambiae* PRR that is specific for immune defence against the human pathogen *P. falciparum* (Dong *et al*., 2006a). The mammalian homologue of AgMDL1, MD-2, recognizes lipopolysaccharide (LPS), and then acts as an adaptor protein that activates the Toll-like receptor 4 to initiate immune response activation. The role of AgMDL1 in either recognizing and/or defending against *Plasmodium* is not yet clear. Other key players in the anti-*Plasmodium* defence are two leucine-rich-repeat-domain-containing PRRs, APL1/LRRD19 and APL2/LRRD7, which have been shown by gene expression analysis and quantitative trait locus linkage mapping to be induced upon *Plasmodium* infection. These genes map to a chromosomal region that has been shown to contribute to *P. falciparum* resistance in certain natural mosquito populations (Dong *et al*., 2006a; Riehle *et al*., 2006).

Numerous other PRRs have been linked to the anti-*Plasmodium* and antimicrobial defence in the mosquito, and we will more specifically focus on some of the better-studied and potentially more interesting mosquito PRR gene families. The Gram-negative-bacteria-binding protein (GNBP) gene family was one of the first mosquito PRRs to be studied, together with the peptidoglycan-recognition protein (PGRP) gene family. Members of these two PRR gene families have been implicated in immune-signalling pathway activation and will be discussed in this review in greater detail. We will also address a very large receptor gene family that includes many members with PRR function, the immunoglobulin gene superfamily (IgSF). We will finally look at two PRRs that significantly contribute to the expansion of the mosquito PRR repertoire: the Down syndrome cell-adhesion molecule (Dscam) and the members of the fibrinogen domain immunolectin (FBN) gene family.

5.3 The GNBP gene family

The first GNBP was identified in *Bombyx mori* through a search for insect haemolymph proteins with properties similar to those of the mammalian LPS-binding protein CD14. This GNBP has significant homology to polysaccharide-binding motifs of bacterial β-1,3–1,4-glucanases, has been shown to have strong affinity for the cell wall of Gram-negative bacteria (Lee *et al*., 1996), and has been found to be upregulated following bacterial challenge. Although GNBP was isolated as a soluble

protein from haemolymph, the hydrophobic nature of the C-terminal portion of the molecule, together with the existence of a putative glycosylphosphatidylinositol anchor site, suggest that a membrane-bound form of this protein may exist.

Other *GNBP* homologues with a conserved β-1,3-glucan-binding domain were later identified in *D. melanogaster*, *A. gambiae*, and *Aedes aegypti* (Dimopoulos *et al.*, 1997; Richman *et al.*, 1997; Kim *et al.*, 2000; Christophides *et al.*, 2002; Waterhouse *et al.*, 2007). The *A. gambiae* GNBPs were shown to be responsive not only to bacterial infection but also to *Plasmodium* infections (Dimopoulos *et al.*, 1998; Tahar *et al.*, 2002; Rosinski-Chupin *et al.*, 2007; Warr *et al.*, 2008).

Of the three *D. melanogaster* GNBPs, DGNBP1 has been shown to have a high affinity for microbial immune elicitors such as LPS and β-1,3-glucan, suggesting that it functions as a PRR (Kim *et al.*, 2000). Over-expression of DGNBP1 in *Drosophila* immunocompetent cells led to an enhanced LPS- and β-1,3-glucan-induced expression of nuclear factor κB-dependent antimicrobial peptide genes, and this induction could be specifically blocked by an anti-DGNBP1 antibody. These results pointed to a role for DGNBP1 as a PRR for LPS and β-1,3-glucan that could activate the Toll immune-signalling pathway, leading to the induction of antimicrobial peptide genes (Kim *et al.*, 2000).

The *A. gambiae* genome harbours six *GNBP* genes that fall into two distinct sequence groups, together with their known moth and fruit fly homologues (see Figure 5.1 for GNBP domain organization) (Christophides *et al.*, 2002; Waterhouse *et al.*, 2007). Subfamily A includes all known fruit fly and moth *GNBP*s, as well as two mosquito *GNBP*s (*AgGNBPA1* and *A2*). The *GNBPA2* gene of *Anopheles* and the *GNBP3 Drosophila* gene are orthologues. The new subfamily B, which is mosquito-specific (*AgGNBPB1–B4*), has three of its four members tightly clustered in chromosomal subdivision 13E (Christophides *et al.*, 2002). All six members of the *A. gambiae* GNBP protein family have a signal peptide sequence at the N-terminal end. Three *A. gambiae* GNBPs (AgGNBPB1, B2, and B4) contain putative glycosylphosphatidylinositol (GPI)-anchor sequences, and three other *A. gambiae* GNBPs (AgGNBPA1, B1, and B3) have several potential N-linked glycosylation sites, suggesting that GNBPs are cell-surface molecules or secretory proteins that are involved in cell–cell adhesion or recognition (Warr *et al.*, 2008). However, it is to be noted that AgGNBPA2 has neither the GPI-anchor nor N-linked glycosylation sites. Several studies have provided detailed information on the transcription of mosquito *GNBP* in various tissues: *AgGNBPB1* is mainly expressed in the thorax and salivary gland and to a lesser extent in other tissues (Dimopoulos *et al.*, 1997, 1998, 2000). All six *AgGNBP* transcripts display higher levels of expression in the posterior region of the female midgut than in the cardia itself (Warr *et al.*, 2008). At the protein level, the AgGNBPB4 protein is more abundant in thorax, fat-body tissue, and abdomen than in the head or midgut compartments (Warr *et al.*, 2008).

AgGNBP mRNAs have been shown to be induced in the midgut, carcass, abdomen, and salivary gland in response to infection with various bacteria

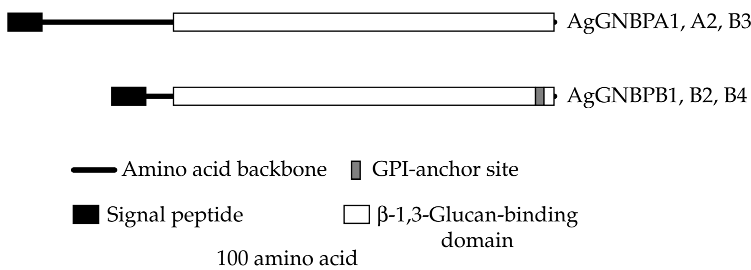

Figure 5.1 The domain organization of the GNBP protein family of *A. gambiae* is shown; the thin horizontal black bar indicates the length of each protein while boxes indicate specific domains. Subfamily A includes three GNBPs (AgGNBPA1, A2, and B3) and subfamily B also includes three GNBPs (AgGNBPB1, B2, and B4). GPI, glycosylphosphatidylinositol.

(Gram-positive and Gram-negative) or *Plasmodium* (Richman *et al*., 1997; Dimopoulos *et al*., 1998; Tahar *et al*., 2002; Dong *et al*., 2006a; Rosinski-Chupin *et al*., 2007; Warr *et al*., 2008). Gene silencing of *GNBPA2* had the strongest effect on *P. falciparum* infection, while *GNBPB3* and *GNBPB4* silencing had the strongest effect on permissiveness with regard to *P. berghei* infection (Warr *et al*., 2008). These results suggest that the GNBP family members show differences in the specificity of their interactions with various microbes and in their defensive activity against those microbes.

As has previously been shown for the *D. melanogaster* DGNBP1, the mosquito GNBPB4 regulates the expression of a number of immune genes (*Gambicin*, *Defensin1*, *Cecropin 3*, *LRIM1*, *CLIPB14*, and *PGRPLC3*) (Warr *et al*., 2008). The bias in this immune gene regulation toward the Imd pathway has suggested that GNBPs may be playing a dual role in activating both the Toll and Imd pathways in response to different microbial challenges.

5.4 The PGRPs

Peptidoglycan, which is mainly found in the Gram-positive bacteria cell wall, is a highly potent target for recognition by eukaryotic cells. The term peptidoglycan-recognition protein was first introduced by Ashida's group (Yoshida *et al*., 1996); they isolated a 19 kDa protein from the haemolymph of the silkworm *B. mori* that showed a high affinity for Gram-positive bacteria and peptidoglycan and activated the downstream prophenoloxidase cascade that leads to melanization of microbial substances (Ochiai and Ashida, 1999). Subsequently, genes encoding PGRP-related structures were identified in other organisms, from *Drosophila* to *Anopheles* to humans (Kang *et al*., 1998; Liu *et al*., 2001; Christophides *et al*., 2002; Waterhouse *et al*., 2007), all having a peptidoglycan-recognition domain of approximately 165 amino acids with structural similarity to the peptidoglycan-binding region of lysozyme (Kim *et al*., 2003).

Based on the length of their gene products, insect *PGRP*s have been grouped into two classes: short PGRPs (PGRP-S), which are small extracellular proteins (19–20 kDa) similar to the originally described PGRP, and long PGRPs (PGRP-L), which are either intracellular or membrane-spanning proteins (Christophides *et al*., 2002). The C-terminal region of the *PGRP-L*s, which is the most highly conserved and is a homologue of *PGRP-S*s, has three domains (I, II, and III) (Werner *et al*., 2000). *Drosophila* has 13 *PGRP* genes that are transcribed into at least 17 PGRP proteins (Werner *et al*., 2000; Christophides *et al*., 2002). Of these 17, seven are short DPGRPs (SA, SB1, SB2, SC1a, SC1b, SC2, and SD), and 10 are long DPGRPs (LAa, LAb, LAc, LB, LCa, LCx, LCy, LD, LE, and LF) that either have a signal peptide and a predicted transmembrane domain or are intracellular proteins that are secreted by unknown mechanisms. Separate reduplication of two adjacent PGRP-LC domains in *Drosophila* has generated a novel gene, *PGRP-LF*, which is absent from mosquitoes (Christophides *et al*., 2002).

Anopheles has seven PGRP genes (Christophides *et al*., 2002; Waterhouse *et al*., 2007), three encoding short *AgPGRP*s (*S1*, *S2*, and *S3*) and the remaining four encoding six long *AgPGRP*s (*LA1*, *LA2*, *LB*, *LC1*, *LC2* and *LC3*), some of which are splice variants (see Figure 5.2 for PRGP domain organization). Sequencing of the human genome has led to the identification of two additional PGRP homologues, *HPGRP-I*α and *HPGRP-I*β (Liu *et al*., 2001). The crystal structures of *Drosophila* PGRP-LB and -SA and human PGRP-Iα have been determined in separate studies (Kim *et al*., 2003; Guan *et al*., 2004; Reiser *et al*., 2004) and found to be very similar to bacteriophage T7 lysozyme.

Most PGRP genes are expressed in all post-embryonic stages, and insect *PGRP-S* and other short *PGRP*s are present in the haemolymph, cuticle, fat body, epidermal cells, gut, and, to a lesser extent, haemocytes. Long insect *PGRP*s are mainly expressed in haemocytes (Ochiai and Ashida, 1999; Werner *et al*., 2000; Christophides *et al*., 2002; Dimopoulos *et al*., 2002). The expression of several short and long *PGRP*s has been shown to be upregulated in *D. melanogaster* and in *A. gambiae* by exposure to various bacteria, to bacterial peptidoglycan, and by *P. berghei* challenge (Kang *et al*., 1998; Dimopoulos *et al*., 2002).

In *D. melanogaster DPGRP-SA* mutants, activation of the Toll pathway by Gram-positive bacteria has been shown to be blocked, and resistance to

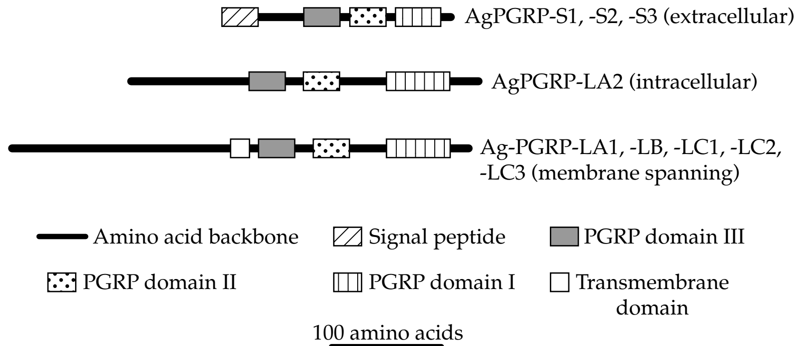

Figure 5.2 The domain organization (signal peptide and PGRP domains) of the PGRP protein family of *A. gambiae* is shown; the thin horizontal black line indicates the length of each protein and boxes indicate specific domains The short PGRPs (S1, S2, and S3) are extracellular and among the long ones PGRP-LA2 is intracellular while the other five (PGRPLA1, -LB, -LC1, -LC2, and -LC3) are membrane-spanning.

Gram-positive infection has been found to be decreased (Michel *et al*., 2001; Gobert *et al*., 2003). In another report, a mutation in the gene *PGRP-LC* was shown to reduce survival in Gram-negative sepsis but to have no effect on the response to Gram-positive bacteria or natural fungal infections (Gottar *et al*., 2002). Experiments using RNA interference (RNAi) in *Drosophila* mbn-2 cells have shown that *PGRP-LCx* is the only isoform that is required to mediate signals from Gram-positive bacteria and purified bacterial peptidoglycan. In contrast, the recognition of Gram-negative bacteria and bacterial lipopolysaccharide requires both *PGRP-LCa* and *-LCx*. The third isoform, *LCy*, is expressed at lower levels than the other two and may be partially redundant (Werner *et al*., 2003). In *Drosophila* larvae, the Imd-mediated antibacterial defence has been shown to be activated by *PGRP-LE*. The product of this gene binds to the diaminopimelic acid (DAP)-type peptidoglycan, a cell-wall component of the bacteria that is capable of activating the Imd pathway, but not to the lysine-type peptidoglycan (Takehana *et al*., 2002). In a later study, PGRP-SC1/-2-depleted flies demonstrated a specific over-activation of the Imd signalling pathway after bacterial challenge (Bischoff *et al*., 2006).

In summary, the *Drosophila PGRP-SA* is required to activate the Toll receptor in response to the cleavage of the Toll-ligand Spätzle in the protease cascade that occurs as a result of infection with a Gram-positive bacterium (Michel *et al*., 2001). *PGRP-LC* is required for the activation of the Imd receptor in response to fungal and Gram-negative bacterial infection (Choe *et al*., 2002). It is also presumably involved in the phagocytosis of Gram-negative bacteria, since inhibition of *PGRP-LC* expression in *Drosophila* cell lines results in a decreased phagocytosis of *Escherichia coli* (Rämet *et al*., 2002). PGRP-SC1b has been suggested to possess an amidase activity that can degrade peptidoglycan (Mellroth *et al*., 2003). Thus, the PGRPs play a multi-faceted, pivotal role in *D. melanogaster* innate immunity and in the mosquito's defences against Gram-positive bacteria.

5.5 The immunoglobulin domain PRR family

The immunoglobulin domain plays a primary role in pattern recognition in the mammalian immune system. As a building block of antibodies, major histocompatibility complexes (MHCs), and other proteins that are responsible for making direct contact with pathogens, the immunoglobulin domain is evolutionarily engineered for the specific recognition and binding that is required for pattern recognition. Different subcategories of immunoglobulin domain may be recognized on the basis of sequence similarity, but the structure of the various domains tends to remain conserved within the superfamily. Immunoglobulin domains are characterized by a region of approximately 100 amino

acids that fold to form two facing, anti-parallel β-sheets that interact with each other hydrophobically while residues between these sheets bend outward. These intervening loops are available for ligand binding and can accommodate amino acid sequence changes without changing the conserved structure, thus allowing for a high degree of interaction specificity and diversity. This property gives these domains a propensity for contact-dependent functions (Williams and Barclay, 1988) and makes the immunoglobulin superfamily (IgSF) members ideal proteins for any process requiring adhesion and recognition, especially when a domain is repeated often or found adjacent to other highly interactive domains.

According to the broadest definition, the IgSF of any species contains genes encoding at least one immunoglobulin domain, as defined by a typical conserved sequence and structure. Although most thoroughly studied in mammals, immunoglobulin-domain-containing proteins in a wide range of species are responsible for recognizing invading pathogens as non-self and promoting their elimination via a range of immune mechanisms, both cellular and humoral. Here, we outline what is known about the pattern recognition and immune involvement of IgSF members in invertebrates. Invertebrates do not have antibodies, a MHC, or other prototypical immunoglobulin-containing immune molecules, yet they do have proteins that contain immunoglobulin domains and possess either putative or confirmed pattern-recognition capabilities. In some cases, these invertebrate IgSF members also have catalytic or signalling domains that can initiate an immune response in response to a pathogen, as is true for mammalian PRRs (such as antibodies).

Several individual invertebrate IgSF members have been analysed in terms of their recognition properties and immune relevance. Studies in *Manduca sexta* and *Hyalophora cecropia* (as well as other moths) have described hemolin, a protein previously known as P4 that contains five immunoglobulin domains and which is present in the haemolymph of both insects (Sun *et al*., 1990). Hemolin is transcriptionally induced by bacteria and seems to play a role in regulating cellular immune responses, such as the prevention of haemocyte aggregation and initiation of phagocytosis (Ladendorff and Kanost, 1991; Lanz-Mendoza *et al*., 1996). Since hemolin can bind bacteria and is specific for the lipid A moiety of lipopolysaccharide, it is considered a true PRR (Daffre and Faye, 1997; Yu and Kanost, 2002). In addition to its effect on aggregation and phagocytosis, hemolin is also important for phenoloxidase activity, suggesting that insects are highly dependent on this PRR for immune defence (Terenius *et al*., 2007). This conclusion is supported by the increased susceptibility of *M. sexta* to entomopathogenic bacteria that is seen when the hemolin gene is silenced by RNAi prior to bacterial infection (Eleftherianos *et al*., 2006b). Further experiments have suggested that hemolin is broadly specific for Gram-negative bacteria (Eleftherianos *et al*., 2006a, 2007).

A putative PRR, the molluscan defence molecule (MDM) of *Lymnaea stagnalis*, shares a five-immunoglobulin domain structure with and is similar in sequence to hemolin. This molecule is gradually downregulated as the infection of the mollusc with a schistosomal parasite progresses. Hoek and colleagues hypothesize that the expression of MDM is manipulated by the schistosome as a method to avoid immune surveillance (Hoek *et al*., 1996).

The Dscam of *A. gambiae* comprises tandem immunoglobulin domains and has been shown to be involved in the insect's defence against bacteria and *Plasmodium* parasites (Dong *et al*., 2006b). This remarkable protein is discussed further in section 5.6.

Identified via a bioinformatic and transcriptomic screen of the entire *A. gambiae* IgSF, the proteins known as infection responsive with immunoglobulin domain 3, 5, and 6 (IRID3, IRID5, and IRID6) are major players in the mosquito's defence against bacteria and *Plasmodium* parasites (see Figure 5.3 for IRID domain organization). RNAi-mediated silencing of IRID3 and IRID5 increases the mosquitoes' susceptibility to Gram-positive and -negative bacteria, while IRID3 silencing disrupts the bacterial load in the haemocoel. IRID6-depleted mosquitoes become more amenable to infection by both rodent and human malaria parasites. All three molecules are transcriptionally influenced by bacterial or parasitic infection. The ability of these molecules

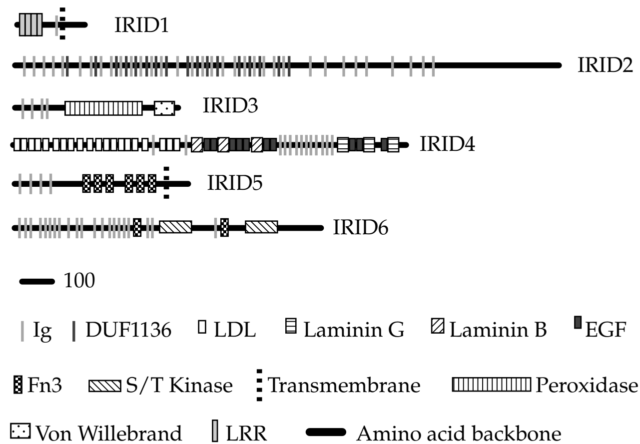

Figure 5.3 Domain organization of the IRID members of the IgSF family of *A. gambiae*. The domains of six IgSF members of IRID family are shown; the thin horizontal black line indicates the length of each protein whereas lines and boxes indicate specific domains based on similarity to amino acid sequences of domains with known functions as predicted according to the SMART database. These representations illustrate the diversity of domain architecture present in the IgSF. Some members are large and complex (such as IRID4) while others are short and quite simple, containing only a single immunoglobulin (Ig) with perhaps one other domain (such as IRID1). DUF, domain of unknown function; EGF, epidermal growth factor; Fn3, fibronectin 3; LDL, low-density lipoprotein; LRR, leucine-rich repeat; S/T kinase, serine/threonine kinase.

to bind pathogen surfaces has yet to be verified, but their domain structure suggests that such an interaction is likely (Garver *et al*., 2008).

IRID3 shows a remarkable similarity to peroxinectin, a PRR described primarily in crayfish and black shrimp. Peroxinectin's domain architecture combines the binding activity of immunoglobulin domains with the enzymic activity of a peroxidase domain. Since this protein has been shown to enhance both phagocytosis and encapsulation, it is thought to be opsonic; pathogens are bound by the immunoglobulin domains, and effector mechanisms are activated at the site of binding by the peroxidase domain (Johansson *et al*., 1995; Sritunyalucksana *et al*., 2001).

First identified and characterized in *Biomphalaria galabrata*, fibrinogen-related proteins 3 and 7 (FREP3 and FREP7) each have two immunoglobulin domains of variable sequence in addition to the fibrinogen domains that are characteristic of the FREP family. Transcription of both proteins is elevated during infection with the trematode *Echinostoma paraensei*, and both can bind and precipitate parasite surface antigens. Correspondingly, sequence analysis suggests that the positive selection is acting on the immunoglobulin domains of both FREP3 and FREP7, while the fibrinogen domains are relatively conserved. Taken together, their binding ability and sequence data make a strong case for considering these two FREPs to be innate PRRs (Adema *et al*., 1997; Zhang *et al*., 2001). The fact that homologues of the FREP genes are also encoded by the *Drosophila* and *Anopheles* genomes suggests that these PRRs may be widely represented throughout the invertebrates (Wang *et al*., 2005).

The concept of the immunoglobulin domain as the functional part of a PRR that binds pathogens in both vertebrates and invertebrates is not surprising, since the structure of this domain is ideal for this role. What is surprising and not well understood is the evolution of these domains and the immune molecules in which they are found. Although they perform similar functions using the same domain, antibodies and other vertebrate immune molecules are not closely related, according to phylogenic analyses. In fact, invertebrate immune factors such as those described here are more closely related to molecules of both the invertebrate and vertebrate nervous systems than they

are to molecules of vertebrate immune systems (Hughes, 1998). Conceptually, the requirements for neuronal wiring and pathogen recognition are similar, since both depend on the recognition of a specific pattern for protection and proper response (Parnes and Hunkapiller, 1987). This interrelationship is functionally evident in insects, in which such molecules as Dscam are involved in both immune-related and neuronal activity in *A. gambiae* and *D. melanogaster* (Schmucker *et al*., 2000; Watson *et al*., 2005; Dong *et al*., 2006b). Thus, pattern recognition is relevant not only to our understanding of the processes involved in immune defence but also to our understanding of the molecular evolution of multiple systems spanning diverse species.

5.6 The Dscam PRR

Unlike vertebrates, invertebrates lack the adaptive capacity and memory that allow the vertebrate immune surveillance system to distinguish a broad spectrum of micro-organisms by making use of a large and diverse collection of recognition receptors that are generated by somatic recombination of antibody immunoglobulin domains and clonal selection. The number of putative PRRs in the mosquito is limited, with only about 150 predicted PRR genes being identified in the *A. gambiae* genome and the total number of PRRs and effector genes numbering less than 1000 in most invertebrates (reviewed by Schulenburg *et al*., 2007).

One member of the immunoglobulin gene superfamily in *D. melanogaster*, the Down syndrome cell-adhesion molecule (*Dscam*) gene can potentially generate 38 016 different alternative splice isoforms through alternative splicing of 101 exons (Graveley, 2005). The *Drosophila* Dscam was named by analogy to the human protein DSCAM, which is a candidate disease gene for the mental retardation associated with Down's syndrome and which has been positionally cloned on chromosome 21 (Yamakawa *et al*., 1998). The remarkable molecular diversity of this *Drosophila* molecule is indicated by its gene organization. The molecule contains 10 immunoglobulin domains and six fibronectin type III domains as well as four arrays of alternative exon cassettes (the exon 4, 6, 9, and 17 cassettes) that have 12, 48, 33, and two variable exons, respectively. The analogue gene of *Dscam* in *A. gambiae* (*AgDscam*) has a similar number of alternative exons; it also includes exon cassettes of exons 4, 6, and 10, consisting of 14, 30, or 38 alternatively spliced immunoglobulin domain exons, respectively (see Figure 5.4 for Dscam domain organization). In theory, these opportunities for alternative splicing can result in 31 920 alternatively spliced forms in *A. gambiae*. Because of the existence of a mutually exclusive splicing mechanism in *Dscam* for generating sequence variability in its three immunoglobulin ecto-domains, D2, D3, and D7, the resulting protein isoforms all have the same domain architecture (Schmucker and Flanagan, 2004; Graveley, 2005). A regulator of *Dscam*'s mutually exclusive splicing, the heterogeneous nuclear ribonucleoprotein hrp36, has been shown to act specifically within the exon 6 cassette to prevent the inclusion of multiple exons or multiple exon 6 variants (Olson *et al*., 2007).

The majority of the constant exons are highly conserved between *Drosophila Dscam* and *AgDscam*

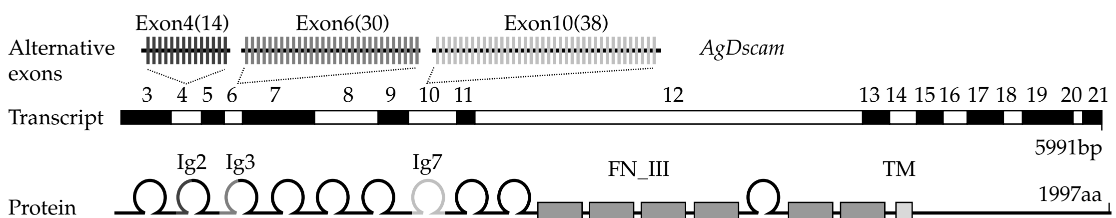

Figure 5.4 Gene and protein domain organization of AgDscam. *AgDscam* gene has three major arrays of alternative exons, with only one single exon from each array being incorporated into each mRNA molecule. The number above the transcript indicates the corresponding exon numbers. AgDscam protein has ten Ig-like domains, six fibronectin type III domains (FN_III), and a transmembrane domain (TM). Splicing variants are generated in the Ig2, Ig3, and Ig7 domains which are shaded correspondingly to splicing exons. aa, amino acids.

(70–95% sequence similarity at the amino acid level), while the alternative-splicing exons are more variable, with only 30–70% homology between these two arthropods. This exon-sequence divergence pattern suggests that constant and alternative exons are under different functional constraints, with the constitutive exons perhaps being involved in conserved functions and the hypervariable immunoglobulin domains reflecting the profound differences in the lifestyles and environmental exposure of the two insects.

Both the nervous system and the immune system require the extraordinary diversity and specificity characteristic of recognition receptors. A landmark study by Wojtowicz *et al.* (2004) has clearly demonstrated that the isoform diversity of *Dscam* involves a binding specificity that is mainly dependent on homophilic rather than heterophilic binding; this mechanism resembles that of many other immunoglobulin cell-adhesion molecules, which bind homophilically (Agarwala *et al.*, 2001; Wojtowicz *et al.*, 2004) and is consistent with a separate study showing that all three of the variable immunoglobulin domains (Ig2 (D2), Ig3 (D3), and Ig7 (D7)) contribute to the binding. In a more recent study, these researchers have provided evidence that more than 18 000 isoforms exhibit striking isoform-specific homophilic binding, and that through the binding of the same variable domain, self-binding domains can assemble in different combinations to generate an enormous repertoire of homophilic binding proteins (Wojtowicz *et al.*, 2007). *Drosophila* is likely using this vast repertoire to generate a unique identity and homotrophic binding specificity for each neuron as a means of helping neuronal processes to discriminate self and non-self more efficiently and specifically. The X-ray structure of the N-terminal four immunoglobulin domains (D1–D4) of two distinct Dscam isoforms (expressed using a baculovirus system) has revealed a horseshoe configuration in which the variable domains of D2 and D3 make up two independent surface epitopes (epitopes I and II) on either side of the receptor. Epitope I contributes to the homophilic binding specificity of full-length Dscam hypervariable receptors, as has been confirmed by mutagenesis studies and swapping of peptide segments (Meijers *et al.*, 2007).

Dscam has a dual role in both neural development and the immune system in insects. It contributes to axon guidance and neuron wiring in the nervous system (Schmucker and Flanagan, 2004; Chen *et al.*, 2006; Hattori *et al.*, 2007), while recent studies have established that Dscam also plays a role as a hypervariable PRR in the innate immune system of insects (Watson *et al.*, 2005; Dong *et al.*, 2006b). Transcriptional analysis based on microarray hybridization has indicated that *Dscam* repertoires are differentially expressed in haemocytes and in the nervous system, with the choice of splice variants being regulated both spatially and temporally (Neves *et al.*, 2004; Watson *et al.*, 2005). More interestingly, single-cell real-time PCR has demonstrated that individual cells belonging to the same cell type express diverse repertoires of *Dscam* isoforms, suggesting a mechanism for generating unique cell identity in both the nervous system and other tissues (Neves *et al.*, 2004). Tissue-specific expression profiling of brain, fat body, and haemocytes (based on a 50-mer oligo-microarray hybridization) has indicated that 59 of 60 alternative exon 4 and exon 6 sequences are expressed in all three different cell types, with only a subset of 14 being expressed in a tissue-specific manner, in either the fat body or haemocytes (Watson *et al.*, 2005).

Immunocompetent cells in *Drosophila* have the potential to express more than 18 000 isoforms of Dscam in the form of membrane-binding proteins that can serve as recognition receptors; these cells also express isoforms of Dscam that are secreted into the haemolymph. RNAi-mediated gene silencing of *Dscam* in larval haemocytes significantly decreases their efficiency in phagocytosing both Gram-negative and Gram-positive bacteria, suggesting that Dscam acts as either a recognition or signalling receptor during phagocytosis. Preliminary data obtained by expressing all of the extracellular domains, including all three variable immunoglobulin domains, has shown a strong binding of Dscam to bacteria, whereas binding by the isoform containing only the first two immunoglobulin domains was barely detectable, suggesting that specific homophilic binding and binding to bacteria utilize different immunoglobulin domains (Watson *et al.*, 2005; Meijers *et al.*, 2007). However, the molecular mechanisms by which Dscam binds

to bacteria remain unknown, and their elucidation requires detailed studies in the future.

Using quantitative real-time PCR and selecting exon cassette 4 as a proof of principle, Dong and colleagues have been able to show that alternative splicing of *AgDscam* is important for immune responsiveness to bacteria, malaria parasites, fungi, and the bacterial surface molecules LPS and peptidoglycan (Dong *et al.*, 2006b). Depletion of *AgDscam* by RNAi-mediated gene silencing of its constant domain caused a decrease in the phagocytic efficiency of the mosquito's immunocompetent cells with regard to both Gram-negative and Gram-positive bacteria. RNAi-mediated gene silencing and *in vitro* bacterial binding assays revealed that AgDscam was binding directly to the bacterial surfaces.

Interestingly, *AgDscam* was shown by exon-specific gene silencing to produce pathogen challenge-specific splice form repertoires that were enriched in receptor molecules with an increased affinity and defence-related specificity for the eliciting pathogen. *In vivo* gene silencing of *AgDscam* in *A. gambiae* increased the mosquitoes' susceptibility to infection with both bacteria and the malaria parasites. Depletion of certain isoforms that had been enriched by the bacterial challenge led to a decrease in the mosquitoes' survival rate when infected with the same micro-organism, suggesting that AgDscam responds to bacteria in a splice-form-specific manner. At the cellular level, AgDscam has been shown to colocalize with both Gram-positive and Gram-negative bacteria, as well as with rodent and human malaria parasites. Small interfering RNA-mediated gene silencing specifically targeting candidate alternative isoforms has shown a significant increase in parasite number in the mosquito's midgut epithelium, suggesting that AgDscam is also defending the insect against malaria parasites in a splice-form-specific manner (Y. Dong and G. Dimopoulos, unpublished results). The regulators of mosquito innate immune-signalling transduction pathways are also involved in regulating the alternative splicing of *AgDscam* (Y. Dong and G. Dimopoulos, unpublished results). Further analyses are currently addressing the mechanisms by which AgDscam binds to both bacteria and parasites and, more importantly, are attempting to determine whether the differential association of specific splice forms of AgDscam is the source of the binding specificity for the different pathogens' surface molecules.

The vertebrate Dscam molecule has been linked to Down's syndrome, but neither of the two human *Dscam* paralogues that have been studied has displayed a significant degree of alternative splicing (Agarwala *et al.*, 2001). The Dscam of zebrafish has been shown to be essential for cell migration (Yimlamai *et al.*, 2005). *Dscam*-like sequences have been identified in the Diptera and Hymenoptera; in four beetles, including *Tribolium castaneum* (Coleoptera); and in the silk moth *B. mori* (Lepidoptera); orthologous genes have been identified in all these species through comparative genomic analysis (Graveley, 2005; Watson *et al.*, 2005).

The *Dscam* gene with its alternatively spliced exons has evolved within several insect orders over 250 million years. However, homology in terms of the origin of the alternative spliced exons has not been found outside the Insecta (Crayton *et al.*, 2006). In a more recent study, comparative structural, expression, and evolutionary analyses of a *Dscam* homologue in two species of the crustacean *Daphnia* (Cladocera) has suggested that the diversification of Dscam is functioning outside the insect world and that more than 13 000 different transcripts can be produced through the alternative splicing of variable exons in *Daphnia Dscam* (Brites *et al.*, 2008). *Daphnia Dscam* is thought to function in both the nervous and immune systems, on the basis of the alternative expression of variable exons observed in brain cells and haemocytes.

It is possible that during evolution, different routes have been taken that have achieved functionally similar ends: Invertebrate immunity shows astounding analogies to the vertebrate adaptive immune system at the molecular level, with the alternative splicing of *Dscam* generating a massive quantity of recognition receptors (Kurtz and Armitage, 2006; Schulenburg *et al.*, 2007). However, many questions remain to be addressed regarding both recognition specificity and clonal selection (Du Pasquier, 2005). Considering that recognition diversity has been achieved through alternative splicing but not through the somatic DNA rearrangements seen during the development of

antibody diversity, selection likely occurs through the regulation of splicing, or it occurs after *Dscam* is expressed on the cell surface. How stable is the isoform repertoire after selection? How is the expression of *Dscam* regulated in a cell population as the cells are renewed? It has not yet been firmly established whether the different isoform repertoires mediate the specificity of the pattern recognition, or whether this recognition specificity has memory. Moreover, whether the *Dscam* diversification mechanisms that generate the massive expansion of receptors are active outside of the arthropod class is also as yet unknown (see reviews by Kurtz and Armitage, 2006; Schulenburg *et al*., 2007).

5.7 The fibrinogen domain immunolectin (FBN) gene family

Members of the FBN gene family, also known as fibrinogen-related proteins (FREPs), share a fibrinogen-like domain (FBG) that is evolutionarily conserved and extends from invertebrates to mammals (Gokudan *et al*., 1999; Fujita, 2002; Wang *et al*., 2005). In mammals, fibrinogen participates in both the cellular and fluid phases of coagulation. This soluble plasma protein is composed of six polypeptide chains, two each of the Aα, Bβ, and γ chains (Gorkun *et al*., 1997). The FBG domain consists of approximately 200 amino acid residues and shows high sequence similarity to the C-termini of the fibrinogen β and γ chains. FBG domains have been widely identified and well defined in mammals and invertebrates (Gorkun *et al*., 1997; Kairies *et al*., 2001; Fujita, 2002; Zhang *et al*., 2004).

In mammals, three distinct FBN proteins have been identified, Of these, the ficolins are the most important and have been identified in many vertebrate species, including human, rodent, pig, hedgehog, and *Xenopus*, as well as in the ascidians (Urochordata; reviewed by Fujita, 2002). Ficolins have been seen to participate in both phagocytosis and complement activation and to act as PRRs as part of the innate immune system (Erickson, 1993; Miller *et al*., 1993; Kobayashi *et al*., 1994; Lu and Le, 1998; Teh *et al*., 2000; Fujita, 2002; Lu *et al*., 2002; Matsushita and Fujita, 2002; Endo *et al*., 2007). Ficolins are a group of lectins that are each composed of an FBG domain attached to collagenous domain at the N-terminus. All of the mammalian FBG domain proteins contain a common pathogen-binding FBG domain at their C-terminus, while the N-terminal sequences vary from one organism to another; the FBG domain of ficolins is involved in binding *N*-acetyl-ᴅ-glucosamine (GlcNAc) and other sugars; this activity resembles that of the carbohydrate-recognition domain (CRD) of C-type lectins and provides evidence for a PRR role for these molecules (Miller *et al*., 1993; Lu and Le, 1998; Lu *et al*., 2002; Endo *et al*., 2007). ʟ-Ficolin has a globular structure similar to that of a CRD, a bouquet structure that is composed of 12 fibrinogen-like domain subunits that form a tetramer consisting of four triple helices produced through multimerization of collagen-like domains. Thus, multimerization of the N-termini of these molecules may help the FREPs to form multimeric protein bundles with potentially increased affinity and specificity for particular pathogens (Fujita, 2002).

Several different FREPs have been described in various species of invertebrates, with the earliest described being two tachylectins (TL5A and -5B) from the horseshoe crab *Tachypleus tridentatus* (Gokudan *et al*., 1999). Structural and functional characterization of TL5A has revealed its ability to specifically recognize acetyl-group-containing substances, such as GlcNAc, including non-carbohydrates; it is capable of agglutinating all types of human erythrocytes and Gram-positive and Gram-negative bacteria (Gokudan *et al*., 1999). Therefore, TL5A probably functions as a host defence protein on the front line. Both TL5A and -5B have similar fibrinogen-like structures, but they lack the typically collagen-like domain of ficolins at their N-terminus. Solution of the structure of TL5A within the GlcNAc–TL5A complex at 2.0 Å resolution has yielded insights into the lectin activity of TL5A and the evolutionary relationship of TL5A to fibrinogen γ chains (Kairies *et al*., 2001). Four aromatic side chains (Tyr-210, Tyr-236, Tyr-248, and His-220) form a funnel ligand-binding pocket specifically for the acetyl group.

Sequence and structural alignments of the fibrinogen γ fragment, ficolins, and TL5A have demonstrated that the overall three-dimensional structure, Ca^{2+}-binding site, and acetyl group ligand-binding pocket are essentially conserved.

Human ficolin is more closely related to TL5A and TL5B than to the fibrinogen γ chain, emphasizing the fact that tachylectins have functions that are more closely related to innate immunity than to anti-coagulation; moreover, this relationship indicates the high degree of similarity between mammalian and invertebrate innate immunity. Electron microscopic analysis of negatively stained TL5A and TL5B has provided high-resolution images of their oligomeric structures and has demonstrated that TL5A and TL5B form a three- or four-bladed and a two-bladed propeller structure, respectively. Each blade corresponds to a dimer formed through inter- and intrachain disulphide linkages involving conserved cysteine residues (Gokudan *et al*., 1999).

As a result of the availability of the full genomic sequences of *D. melanogaster*, *A. gambiae*, and *A. aegypti*, and more recently of 12 distinct species of *Drosophila* sequences, genome-wide comparisons of FBG domains from various species have identified FREP gene families in all these organisms (Waterhouse *et al*., 2007; Middha and Wang, 2008). There is a significant expansion in the FREP gene family in *A. gambiae*, which has 61 members; in contrast, the corresponding gene family in *A. aegypti* has 37 members, and that in *D. melanogaster* has only 14 members. The FREP gene family exhibits species-specific expansion in *A. gambiae*, with only three orthologous pairs having been identified in this species. This finding is consistent with a previous prediction that FBG domains may function by binding to pathogens as part of the host's immune response (Dimopoulos *et al*., 2000, 2001; Christophides *et al*., 2002; Wang *et al*., 2005; Waterhouse *et al*., 2007; Y. Dong and G. Dimopoulos, unpublished results).

Of the putative immune gene families that have been identified in *A. gambiae*, the FBN gene family is one of the largest, and phylogenetic analysis indicates that the gene expansion of the FBN gene family occurred after divergence of this species from others, in agreement with the hypothesis that recent gene duplications have occurred more often in *Anopheles* than in *D. melanogaster* (see Figure 5.5 for FBN domain organization) (Christophides *et al*., 2002). The predicted structures of the FBNs are closely related to that of TL5A, and the acetyl-group-binding sites, Ca^{2+}-binding sites, and cysteine residues involved in disulphide linkages are all conserved (Wang *et al*., 2005; Y. Dong and G. Dimopoulos, unpublished results). Correlation of sequence data with the chromosomal location of the FBG domains within the FBN gene family proteins has suggested that the expansion of the FBN gene family in *A. gambiae* is mainly accounted for by a major expansion of the FBG domains, and both tandem duplication and shuffling have been involved in this expansion. There is a strong correlation between phylogeny, chromosomal location, and the expression pattern of FBN genes in *A. gambiae*, pointing to conserved functions among the duplicated family members (Y. Dong and G. Dimopoulos, unpublished results).

Microarray-hybridization-based transcriptomic analysis has showed that FBN gene family members in *A. gambiae* are involved in the mosquito's

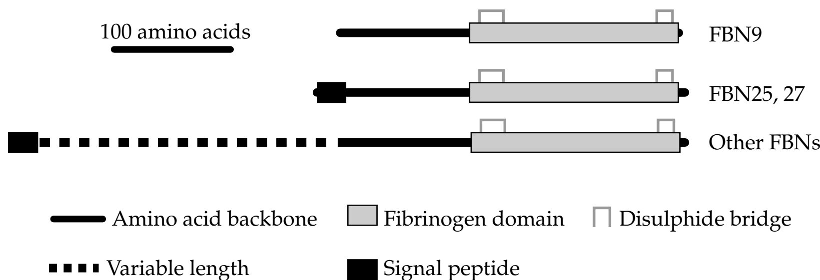

Figure 5.5 The domain organization of the fibrinogen domain immunolectin (FBN) gene family in *A. gambiae*. All the members of the FBN family contain a fibrinogen domain and the disulphide bridge is conserved in the majority of members. FBN9 represents the member without a signal peptide, and majority of the proteins, like FBN25, contain a signal peptide. The dotted line indicates the variation of the lengths of different FBN proteins.

immune response to both bacteria and malaria parasites. RNAi-mediated gene-silencing assays have indicated that FBN8, FBN9, and FBN39 are involved in the anti-*Plasmodium* defence, with FBN39 showing specificity in regulating the mosquito's resistance only to the human malaria parasite, *P. falciparum* (Dimopoulos *et al.*, 2000, 2001; Christophides *et al.*, 2002; Dong *et al.*, 2006a). In a more recent study, the functions of 38 members of the FBN gene family in *A. gambiae* have been characterized, and their involvement in antimicrobial and anti-*Plasmodium* activity has been corroborated by RNAi-mediated gene silencing, which has revealed that FBN has complementary and synergistic activities mediated by the association of different members of the gene family. FBN9 interacts with both Gram-negative and Gram-positive bacteria and is strongly colocalized with both rodent and human malaria parasites in the mosquitoes' midgut epithelium. Interestingly, *in vitro* bacterial binding assays with FBN9 antibody shows that FBN9 appears to form dimers and specifically bind to the bacterial surfaces with different affinity, suggesting that FBN might use a multimerization mechanism to form homo- or hetero-multimers as a means of increasing the mosquito's PRR repertoire, but the molecular basis for this mechanism still remains unknown (Y. Dong and G. Dimopoulos, unpublished results). Besides, the ability to FBN members to form homo- or hetero-multimers has not been investigated, and further detailed studies with more antibodies against different members of the FBN family will help to elucidate this mechanism.

FREPs have also been characterized in other invertebrates. FREPs from the snail *Biomphalaria glabrata* are composed of two functional domains, an N-terminal IgSF domain that may be repeated in tandem, and a C-terminal FBG domain (Adema *et al.*, 1997; Zhang and Loker, 2003; Zhang *et al.*, 2004). This gene family has at least 13 members; FREP2 is involved in immune responsiveness and plays a role in host–parasite interactions (Jiang *et al.*, 2006). FREPs have also been identified in the solitary ascidian *Halocynthia roretzi* (Kenjo *et al.*, 2001), a tachylectin-related protein in the sponge *Suerites domuncula* (Schroder *et al.*, 2003), and aslectin in the mosquito *Armigeres subalbatus* (Wang *et al.*, 2004). All of these FREPs contain a common C-terminal FBG domain, but their N-termini have no typical conserved IgSF structure like that seen in snails. These FREPs probably play an important role in the innate immune response against bacteria and parasites (Gokudan *et al.*, 1999; Schroder *et al.*, 2003; Wang *et al.*, 2004; Jiang *et al.*, 2006).

5.8 Conclusions

The evolutionary success of insects is largely attributed to their capacity to ward off a variety of pathogenic micro-organisms. Insect PRRs play a front-line, central role in this effective immune surveillance and defence. The insects' lack of hypersomatic mutation and recombination mechanisms as a source of adaptability is made up for by other mechanisms, such as alternative splicing and perhaps also combinatorial interactions between different receptors. While these PRRs show specificity in their interactions and spectrum of activity, a great deal of redundancy also seems to exist. For example, several mosquito PRRs have been shown to be involved in the anti-*Plasmodium* defence, and it is unclear whether they operate together via the same mechanism or act independently. Future studies of the mechanistic attributes of pathogen recognition in insects will provide answers to this and other unanswered questions, and thereby elucidate the efficiency of the innate immune system in mounting broad but yet pathogen-specific responses.

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CHAPTER 6

Comparative genomics of insect immunity

Fotis Kafatos*, Robert Waterhouse, Evgeny Zdobnov, and George Christophides

6.1 Introduction

Insects are the most diverse and successful animals, encompassing the largest number of described metazoan species, many of them represented by huge numbers of individuals that often exhibit extensive within-species polymorphisms at the molecular and morphological levels. With the exception of the oceans, insects have successfully colonized nearly all habitats on the planet. Sharing these diverse environments with bacteria, fungi, viruses, and parasites, the insects provide fertile ground to study how innate immunity has evolved an assortment of strategies to recognize and combat multiple challenges. In the pioneering studies of innate immunity, the discovery of key features shared by the vertebrate and insect immune systems, such as the Toll-like receptors, led to the general concept that the innate immune system is highly conserved. However, increasingly available sequenced insect genomes have facilitated the application of novel comparative approaches to elucidating the biology and understanding the evolution of immunity. Indeed, comparative genomics have revealed a much finer and intriguing picture: conservation of core features of this system is accompanied by diversified inputs and outputs, possibly reflecting continuous readjustments between accommodation with and rejection of insect pathogens. Dissecting the evolutionary processes that have shaped their immune repertoires may illuminate how insects are able to face the wide variety of challenges in their diverse habitats. Conversely, experimental identification of novel immune components in insects may lead to a better understanding of the origins of immune functions in all organisms.

6.2 Insect comparative genomics

The unrivalled evolutionary diversity of the insect orders can be exploited to unravel the complexities of interactions between insects and their environments, including infectious micro-organisms. These ongoing interactions have allowed the Hexapoda to flourish in a myriad of ecological niches, by successfully defending against microbial challenges. Comparative analysis of insect genomes provides an exceptional opportunity to build an extensive knowledgebase combining genomic, molecular, and biological information; a powerful approach to elucidating trends and features that shape and distinguish this diverse animal group. These approaches can take advantage of multi-species comparisons, to explore the evolutionary processes acting on genes and genomes, and to understand how these processes translate into new functions and phenotypes. At the same time, however, insect comparative genomics faces a special challenge: to develop robust methodologies to handle high levels of sequence divergence.

*Fotis Kafatos dedicates this chapter to Tom Eisner, his mentor and friend who taught him to love all of biology.

Much of the progress in insect genomics has been fuelled by the positive and negative impacts of insects on the environment, agriculture, health, and the economy across the globe. Limiting the damaging effects of insects has traditionally involved their control through the use of pesticides, but with variable and seemingly declining success. Novel approaches to insect control require a detailed understanding of insect biology, to facilitate highly targeted interventions that address specific pests while limiting possible ecological knock-on effects. Elucidating the molecular mechanisms that underpin the key processes of insect innate immunity, and the metabolism of drugs and xenobiotics, is therefore of utmost importance. The recent rapid progress in genomics of innate immunity in disease vector insects reflects the great social relevance of these diseases.

As a prominent model organism subjected to decades of genetic and molecular research, the fruit fly *Drosophila melanogaster* was the obvious first target for insect genome sequencing. The release of its approximately 120 Mb euchromatic genome in 2000, validated the process of whole-genome shotgun sequencing for eukaryotes, and made the fruit fly the first insect, indeed the second multicellular organism, to have its genome sequenced completely (Adams *et al.*, 2000). Since then, the fruit fly genome has played a pioneering role in genomics research, including the development of analytical techniques for the interpretation of an ever-increasing volume and variety of data. This genome has served as the logical framework upon which to build a comprehensive biological knowledge base (Ashburner and Bergman, 2005). Next, as the prime vector of human malaria in Africa, the *Anopheles gambiae* mosquito was prioritized for genome sequencing. The availability of its complete genome sequence (Holt *et al.*, 2002), just 2 years after the fruit fly genome, provided the very first opportunity for extensive comparative genomics studies between two insect species (Christophides *et al.*, 2002; Zdobnov *et al.*, 2002).

In subsequent years, multiple species from several insect orders were chosen for genome sequencing because of their agricultural, economic, or health impacts. The genomes of the silk moth, *Bombyx mori* (Lepidoptera; Xia *et al.*, 2004), the honey bee, *Apis mellifera* (Hymenoptera; HGSC, 2006), the mosquito vector of arboviruses, *Aedes aegypti* (Diptera; Nene *et al.*, 2007), and a stored-food pest, the flour beetle, *Tribolium castaneum* (Coleoptera; TGSC, 2008), have provided an evolutionary perspective of holometabolous insects spanning over 300 million years of divergence. This wealth of data has presented new opportunities for expanding and even revising our understanding of insect biology and evolution, through multidimensional comparative analyses on a genomic scale. Indeed, phylogenomic approaches have placed Hymenoptera at the base of the holometabolous insect radiation (Savard *et al.*, 2006; Zdobnov and Bork, 2007) (Figure 6.1a), whereas morphological and molecular marker analyses had previously failed to provide a confident resolution. Newly sequenced genomes that are currently being analysed include a third mosquito species, *Culex pipiens quinquefasciatus*, the parasitoid *Nasonia* wasps, and outgroup species to the holometabolous insects: the human body louse, *Pediculus humanus*, and the pea aphid, *Acyrthosiphon pisum*. Genome projects that are under way include additional insects of relevance to global health, such as the tsetse, sand, and house flies, and the Hemipteran bug, *Rhodnius prolixus*, an important vector of Chagas' disease.

The increasing wealth of available genomic data has facilitated a quantitative approach to describing the incredible diversity of insects in the perspective of metazoan evolution as a whole. Assorted metrics of evolutionary diversification have been utilized, such as changing numbers of gene family members in different species, sequence divergence of orthologous proteins, and the extent of genome shuffling that disrupts ancestral gene arrangements. These metrics consistently reveal strikingly faster rates of genomic evolution among insects compared to vertebrates (Figure 6.1b) (Wyder *et al.*, 2007; Zdobnov and Bork, 2007). Insect genomics, therefore, represents a comprehensive resource that spans tremendous diversity and provides a broad framework to support and orient research in insect biology.

Even before the genome-sequencing era, pioneering work in insects had highlighted the exceptional power of multi-species comparative sequence analysis. Sequence comparisons of several chorion (eggshell) genes from diverse *Drosophila* species led to the recognition of conserved, short, putatively

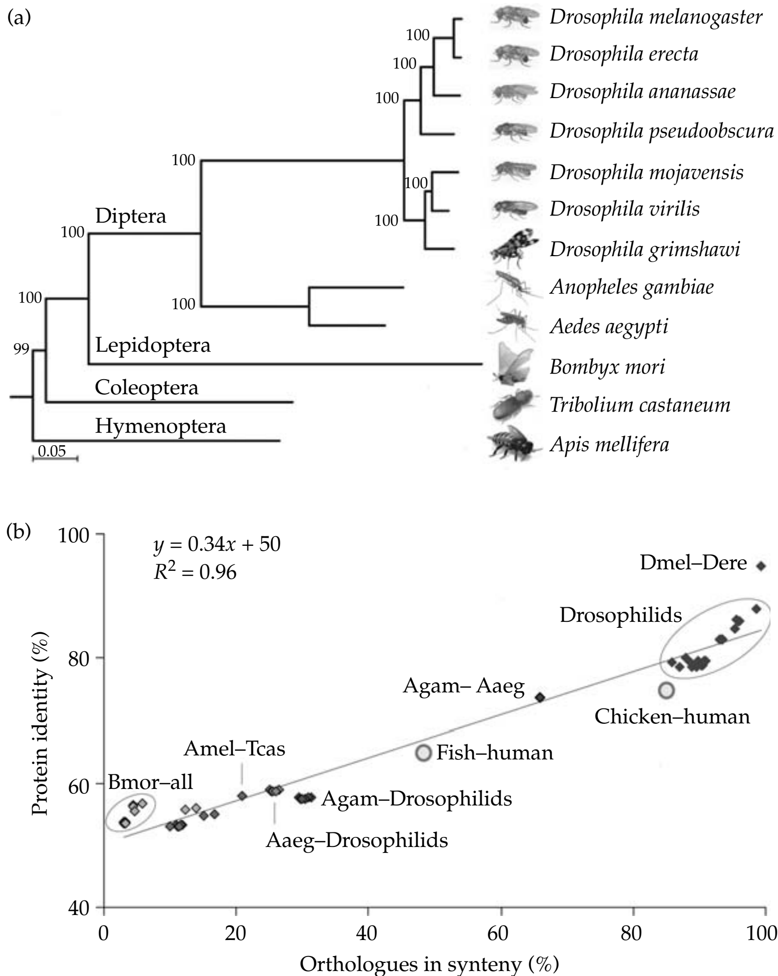

Figure 6.1 Insect phylogeny and insect genome divergence. (a) Phylogenetic tree based on the protein sequences of 2302 single-copy orthologues from the 12 insect genomes as described in Zdobnov and Bork (2007). (b) The genomic diversity across the insect orders is highlighted by two different measures of genomic evolution as described in Zdobnov and Bork (2007): the sequence divergence of orthologous proteins and the extent of genome shuffling, which disrupts ancestral gene arrangements. The pairwise average protein sequence identity of single-copy orthologues correlates well with the fraction of these genes remaining in synteny (maintaining ancestral gene arrangements). This diversity is markedly elevated compared with corresponding measurements of vertebrate data from human–chicken and human–fish genomic comparisons. Strikingly, measures of orthologue sequence identity and synteny show that despite diverging only about 250 million years ago, mosquitoes are more divergent from fruit flies than humans are to pufferfish, which are separated by some 450 million years. Aeag, *Aedes aegypti*; Agam, *Anopheles gambiae*; Amel, *Apis mellifera*; Bmor, *Bombyx mori*; Dere, *Drosophila erecta*; Dmel, *Drosophila melanogaster*; Tcas, *Tribolium castaneum*. Both panels are adapted from Zdobnov and Bork (2007).

functional regulatory sequence motifs (Martinez-Cruzado *et al*., 1988). Experimental testing using transgenic and nucleotide substitution technologies identified one of these motifs, TCACGT, as essential for chorion gene expression in both *Drosophila* and silk moths (Fenerjian and Kafatos, 1994). Despite major differences in promoter sequence and architecture, a short, bidirectionally active promoter region from a silk moth chorion gene pair was shown to direct proper gene expression in *Drosophila*, where chorion genes are totally different and unidirectionally oriented (Mitsialis *et al*., 1987).

Only 7 years after the release of the first fruit fly genome, continued advances in sequencing technologies have fuelled the growth of genome resources. The community is already able to take advantage of the fully sequenced genomes of 12 *Drosophila* species (Clark *et al.*, 2007) that span about 40 million years of divergence and inhabit a wide range of ecologies including rainforests, deserts, and islands, with generalist as well as specialist feeders. The combination of fast sequence diversification in insects and the plethora of closely related species opened new research territories. Comparative analysis of the 12 fruit fly genomes demonstrated how identification and characterization of evolutionary sequence signatures can accurately define encoded functional elements, improving protein-coding gene prediction, as well as discovering novel functional elements such as microRNA genes (Stark *et al.*, 2007; Lin *et al.*, 2008).

This analysis revealed the remarkable power of comparative genomic approaches to make revisions even to the 'gold standard' of *D. melanogaster* annotations, despite many years of intensive expert curation and experimental validation. Manual curation cannot possibly be scaled up to keep pace with the accelerating sequencing revolution. Thus, insect genomics has firmly established the comparative approach as immensely valuable for genome annotation and mining, despite the high rates of insect genome diversification. Annotation techniques have evolved along with the rapid increase in available sequence data: from initial single-genome *ab initio* methods, to dual- and then multi-species approaches, to full-genome alignments and the discovery of characteristic conservation patterns. Indeed, the *de novo* discovery of functional elements through analysis of evolutionary signatures across 12 *Drosophila* genomes represents a methodological milestone. Where high sequence divergence precludes reliable alignment at the DNA level, feature annotation must rely on: (1) single-genome *ab initio* methods that analyse sequence composition properties to recognize gene features and (2) knowledge-based approaches that utilize homology to the growing universe of known proteins, primary expressed sequence tags (ESTs) and cDNAs to recognize coding regions. These complementary approaches are integrated through strategies that evaluate all the available evidence, producing sets of consensus gene models that balance sensitivity and specificity to produce high-quality genome annotations: the basis for higher-level comparative analyses.

6.3 Concepts and methods in comparative genomics

The volume of data from genome sequencing presents a wealth of opportunities, but also immense challenges: to identify meaningful encoded elements, elucidate their functions, and interpret broad principles of genome evolution. Comparative methodologies have been instrumental for understanding important generators of diversity such as alternative splicing, and the extent and importance of non-protein-coding elements. It is now understood that recognizable biological functions are encoded by the interaction of a variety of elements: protein-coding genes, non-protein-coding RNA genes, and conserved non-coding functional elements. The insights gained from comparative genomics, in combination with functional data, can propel comparative analysis stepwise, to the systems level: from macromolecular complexes to regulatory networks, signalling pathways, and coordinated physiological reactions to environmental stimuli, such as responses and modulation of the immune system. To this end, large-scale comparative analyses employ an array of methodologies, often with a focus on characterizing evolutionary relationships among genes and genomes. Here we outline the key concepts and methods applied to the analysis of gene families, defining orthology and paralogy, and exploring dynamics of genome shuffling. Additional approaches, particularly those taking advantage of DNA evolutionary signatures to identify traces of selection, become more valuable with the increasing availability of the genomes of more closely-related species.

Comparative analyses of protein-coding genes often aim to trace the evolutionary histories of genes, and infer their putative functions, whether highly specific or widely shared. Pairwise sequence comparisons, such as the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997) are

complemented by profiles from multiple-sequence alignments like those employing Hidden Markov Model methods (Eddy, 1998). Together they provide a plethora of analysis tools for detection of homology; the shared ancestry of biological sequences. Curated or semi-curated alignments form the basis of many protein-domain recognition profiles, which capture patterns of defining amino acids exemplified by several resources integrated through the InterProScan application (Zdobnov and Apweiler, 2001). Protein families are based on recognizable domains, but may also be defined by particular domain combinations or indeed by sequence relationships that have not yet been described by current protein-domain resources. Therefore, clustering of scores from all-against-all full-length sequence comparisons provides complementary protein family definitions. Importantly, domain-based approaches classify a multi-domain protein into several groups, whereas clustering techniques usually produce mutually exclusive groups of proteins. Comparative analysis of gene family dynamics can identify major differences between families: it may reveal expansions and contractions that depart from a random gene birth/death model, or may even document family extinctions and the appearance of novelties reminiscent of the concept of punctuated equilibrium. For example, the *Anopheles–Drosophila* comparison identified several prominent genomic features, including a major mosquito expansion of fibrinogen-related proteins (FREPs), potentially implicated in antibacterial immune responses (Zdobnov *et al*., 2002). Honey bees exhibit an expansion of the major royal jelly proteins, important dietary components that function in caste differentiation (HGSC, 2006). In the flour beetle the odorant receptor family has expanded, with concomitant reduction in the number of opsin genes, probably reflecting adaptation to low light conditions and increased reliance on smell in the evolutionary line of this stored-food pest insect (TGSC, 2008).

The homology which defines protein families implies common ancestry, which can be further refined to distinguish between orthologous and paralogous genes (Koonin, 2005). Orthologues derive from a single gene in the last common ancestor and therefore, most likely retain the ancestral function, especially if they have remained as single-copy genes over a long evolutionary period. Paralogues arise from gene-duplication events in a given lineage; these copies may share the ancestral function, or may have acquired new, often related, functions. Accurate delineation of orthologues and paralogues is vital for confident functional inferences (Figure 6.2). The functional annotation of *D. melanogaster* genes, accumulated over many decades, is an invaluable resource for inferring putative gene functions in other species. As orthology is defined relative to the last common ancestor, classification is inherently hierarchical. Analysing distantly related species produces large gene groups, potentially all the descendants of an ancestral gene; analysis of closely related species identifies the one-to-one orthologous relations. All-against-all sequence comparisons are widely employed by genome-scale orthology analysis as a means to identify genes representing best reciprocal hits (Koonin, 2005). Phylogenetic methods that provide estimates of evolutionary distances calculated from refined models of amino acid substitutions generally produce more accurate orthology assignments, for example TreeFam (Ruan *et al*., 2008). However, these methods are computationally challenging and can be error-prone when scaled up to the level of whole-genome analysis. Better assignments are achievable by the development of hybrid methodologies, together with finely tuned distance measurements and clustering procedures, for example OrthoDB (Kriventseva *et al*., 2008), or by incorporation of additional evidence for orthology such as conserved gene neighbourhoods, for example SYNERGY (Wapinski *et al*., 2007).

High levels of sequence divergence common among insects may preclude detailed comparison based on whole-genome alignments. However, protein orthology assignments can help identify orthologous genomic regions, within which discrete genes are found to preserve their local gene neighbourhoods (synteny). The ancestral genomic state is eroded through evolutionary time by sequence rearrangements such as duplications, inversions, deletions, and accumulation of repetitive DNA arising from the activity of transposable elements. Nevertheless, regions exhibiting local conservation of orthologous gene arrangements can define

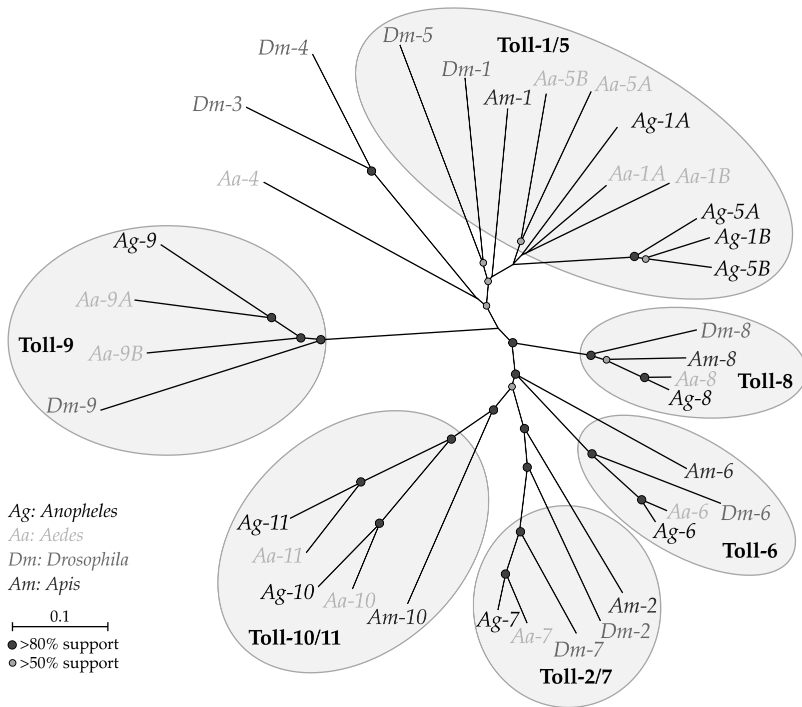

Figure 6.2 Phylogenetic tree of the Toll-like receptors from *Drosophila melanogaster* (*Dm*), *Anopheles gambiae* (*Ag*), *Aedes aegypti* (*Aa*), and *Apis mellifera* (*Am*), where orthologous groups can be clearly distinguished that together describe the evolutionary history of this gene family. The Toll-1/5 group shows expansions in both the mosquito species, creating groups of paralogous genes such as the *Anopheles* Toll-5A-5B-1B group. The Toll-8s and Toll-6s on the other hand remain as single-copy orthologues. Duplications appear to have occurred in *D. melanogaster* in the Toll-2/7 group and in *A. aegypti* in the Toll-9 group. The Toll-10/11 group might appear as a mosquito-specific duplicated group if only the dipterans were compared; however, the presence of *AmToll-10* rather points to the loss of this gene from *D. melanogaster*. The Toll-9s form a clearly distinct group which in fact shows more similarities with the mammalian Toll-like receptors than with other insect Toll family members.

synteny blocks despite such erosion (Zdobnov *et al.*, 2002; Zdobnov and Bork, 2007). Elevated transposable-element activity is likely to promote genomic instability and contribute to increased genome size. Almost half of the approximately 1.4 Gb *A. aegypti* genome is made up of recognizable transposable-element sequences, which result in increased shuffling as indicated by the approximately 2.5-fold higher level of estimated synteny breaks in *Aedes* compared to *Anopheles* (Nene *et al.*, 2007). Remnants of longer-range synteny at the level of their five major chromosome elements can also be identified between the mosquitoes and the fruit fly. However, few confident correspondences can be established with the 16 chromosomes of the more distant honey bee (HGSC, 2006). Although highly conserved gene arrangements such as the *Hox* gene cluster may reflect functional constraints, strict preservation of gene order appears to be under limited selection: only a few hundred genes maintain their local gene neighbourhoods across the insect orders (Zdobnov and Bork, 2007).

6.4 Comparative insect immunogenomics

6.4.1 *Drosophila*: establishing the framework of insect immunity

Genetic tractability established *D. melanogaster* as arguably the best-characterized model for dissecting the biology that underlies insect innate

immunity, although recognition of the capacity of insects to respond to invaders was initiated with the discovery of the antimicrobial peptide (AMP) cecropin in the giant cecropia moth (Steiner *et al.*, 1981). The microbicidal properties of the haemolymph and the identification of numerous inducible AMPs therein, pointed to an insect immune system able to recognize and respond to invading pathogens. Regulatory DNA sequence motifs, resembling those recognized by mammalian nuclear factor κB (NF-κB), were detected in AMP gene promoters, providing the first parallel with mammalian innate immunity (Sun *et al.*, 1991). These discoveries set the course for applying the power of *Drosophila* genetics to the elucidation of upstream components of two key immune-signalling pathways, Toll and Imd (immune deficiency). Together these pathways permit differential recognition and response to invading micro-organisms through signal transduction, leading to nuclear translocation of specific NF-κBs and transcriptional activation of AMPs and other effectors. These immune responses, along with phagocytosis and the production of reactive oxygen species (ROS), are processes reminiscent of vertebrate innate immune systems. Other insect responses, such as encapsulation and melanization are also ancient, but only prominent in invertebrates.

Fruit fly genetics and molecular biology shaped our understanding of the machinery and processes that define insect innate immunity, while studies of other insect systems provided important complementary, supporting data. Discovery greatly accelerated with the sequencing of the *D. melanogaster* genome (Adams *et al.*, 2000). This landmark placed the few immune proteins that were previously identified through intensive experimental investigations in the context of the full complement of related proteins. Genes that were genetically implicated in any of the characterized fruit fly immune responses led to screening of corresponding families of homologues identified in the genome, and thus facilitated generation of new hypotheses followed by targeted experimental testing. In a virtuous cycle, elucidation of innate immune components in *Drosophila* guided functional analysis of related factors in vertebrates, and *vice versa* (Leulier and Lemaitre, 2008).

Once the framework of innate immunity was established in both vertebrates and insects, it allowed classification of genes and families into broad functional categories: recognition, modulation, signal transduction, and effector components (Table 6.1). Recognition of foreign molecular features is the first step towards activating innate immune responses. Recognition is usually achieved through the binding of specialized pattern-recognition receptors (PRRs) of the immune system to cognate pathogen-associated molecular patterns (PAMPs), such as peptidoglycans, lipopolysaccharides, carbohydrates, and β-1,3-glucans (Lemaitre and Hoffmann, 2007). This recognition and binding to foreign bodies can result in direct and indirect outcomes, such as opsonization, phagocytosis, encapsulation, melanization, and lysis. These outcomes represent a coordinated immune system response to combat a recognized threat. The Toll, Imd, and Janus kinase/signal transduction and activators of transcription (JAK/STAT) signalling pathways are important for signal transduction events that link recognition of foreign bodies with initiation of effector responses. Modulation is important in controlling the balance between timely immune-signal amplification in response to a harmful pathogen, while preventing hyper-responsiveness to false or subcritical danger signals.

6.4.2 *Anopheles*: appreciating the diversity of insect immunity

Sequencing the *A. gambiae* genome (Holt *et al.*, 2002) in 2002 gave a great boost to research on disease vectors, and the availability of two insect genomes facilitated the introduction of new ideas, approaches, and recruits to the field of vector biology. Genome comparisons provided opportunities for comprehensive comparative studies of insect immunity and other physiological or developmental systems, with the *A. gambiae* genome being quickly adopted by the community of drosophilists for comparative bioinformatic and experimental studies. The acquisition of comparative information in both genomics and innate immunity created the new and dynamic field of immunogenomics.

Mosquitoes and fruit flies are both Diptera, but they have adopted very different life strategies.

Table 6.1 The established framework of insect innate immunity allows for the classification of genes and gene families into broad functional categories of recognition, modulation, signal transduction, and effector components as well as responses to oxidative stress and RNA. These categories consist of genes from pathways and protein families implicated in immune responses through experimental research, and together they form the basis of the currently recognized insect immune repertoire.

Functional category	Abbreviation	Full name
Recognition	GNBPs (BGBPs)	Gram-negative-bacteria-binding proteins (1,3-β-D-glucan-binding proteins)
	CTLs	C-type lectins (also important modulators)
	FREPs (FBNs)	Fibrinogen-related proteins (fibrinogen-domain immunolectins)
	GALEs	Galactoside-binding lectins
	LRIMs	Leucine-rich-repeat immune proteins
	MLs	MD2-like proteins (lipid recognition)
	PGRPs	Peptidoglycan-recognition proteins
	SCRs	Scavenger receptors (SRCR(A), CD36(B), and CCP-MAM(C) types)
	TEPs	Thioester containing proteins
Modulation	CLIPs	Clip-domain serine proteases (also serine protease homologues)
	IAPs	Inhibitors of apoptosis
	SRPNs	Serpins, serine protease inhibitors
Signal transduction	PATHWAYS	Toll and Imd pathway genes
	JAK/STATs	Janus kinases/signal transducers and activators of transcription
	RELs	Rel-like NF-κB proteins (transcription factors)
	SPZs	Spätzle-like proteins (cytokines)
	Tolls	Toll-like receptors
Effectors	AMPs	Antimicrobial peptides
	CASPs	Caspases
	LYSs	Lysozymes
	ProPOs	Prophenoloxidases (melanization)
Oxidative defence	CATs	Catalases
	PRDXs	Peroxidases (glutathione (GPX), haem (HPX), and thioredoxin (TPX))
	SODs	Superoxide dismutases (CuZn and MnFe types)
RNA defence	DCRs	Dicers
	AGOs	Argonautes

Haematophagy (blood feeding) provides the rich meal of proteins and lipids required by the female mosquito to develop a batch of eggs. However, blood feeding exposes her to pathogens in the vertebrate blood. The first genome-scale comparison between immune repertoires of two invertebrates identified 242 mosquito genes from 18 families with putative immune functions, based on experimental work in mosquitoes, or comparisons with genes of known immune-related functions in fruit flies and other insects (Table 6.1) (Christophides *et al.*, 2002). This study highlighted marked diversification between the two species, with prominent gene family expansions among immune recognition, modulation, and effector components.

Global comparative analysis of sequence identities in *Anopheles–Drosophila* orthologues highlighted enhanced divergence of proteins linked to defence and immunity through gene ontology (Zdobnov *et al.*, 2002). Additionally, functional-domain comparisons identified major immunity gene family expansions or reductions in these two species (Zdobnov *et al.*, 2002). Analysis of the immune repertoires against the backdrop of the entire proteomes revealed a clear deficit of orthologues, particularly in immune-related gene families implicated in recognition, signal modulation, and effector systems (Christophides *et al.*, 2002, 2004). In marked contrast, the key immune signal transduction components showed striking

orthology, in both the Toll and Imd pathways. These comparative studies laid down an initial conceptual framework of insect innate immunity that was further elaborated to accommodate major observed variations in sequence diversity, between distinct functional categories of immune gene families. Subsequent large-scale functional studies, including microarray analysis and reverse genetics via RNA interference (RNAi), revealed mechanisms underlying this framework. The *Anopheles* studies have suggested that mosquitoes are largely refractory to malaria parasites; this resistance is largely attributable to the mosquito's systemic and local epithelial immunity (Sinden *et al.*, 2004; Vlachou and Kafatos, 2005). The highly variable vectorial capacities of different mosquito species and strains reflect a dynamic relationship between pathogens and vectors: a balance between pathogen evasion or its effective detection and elimination by the vector immune system (see Chapter 7 in this volume).

6.4.3 Multi-species comparisons: exploring the evolution of insect immunity

The availability of multiple sequenced insect genomes has greatly facilitated the dissection of their immune repertoires into functional modules and sequential phases of an integrated innate immune system response. Concomitant analysis of orthology, sequence variation, and functional data from experimentation has provided important insights towards understanding the principles that govern the ongoing evolution of innate immunity. The emergent picture is one of a robust architecture, shared to some extent even with vertebrates, but with diversified inputs and outputs. The observations that different immune modules have distinct and even contrasting evolutionary dynamics have helped explain the overall flexibility of a system capable of adapting to a multitude of new challenges.

The sequencing of the *A. aegypti* genome (Nene *et al.*, 2007) permitted a comprehensive comparison of the immune repertoires of two very different mosquito species, separated by approximately 150 million years of evolution (Waterhouse *et al.*, 2007). *A. aegypti* is the major vector of dengue and yellow fever, as well as of several other important encephalitis-causing viruses, such as West Nile and Chikungunya. Thus, its successful adaptation to new, mostly urban environments presents a major challenge to global public health. In contrast, anopheline mosquitoes are infrequently associated with viral transmission, with O'nyong nyong being the only virus known to be transmitted primarily through *A. gambiae* and perhaps through *Anopheles funestus*. As *A. aegypti* can be infected with and transmit the avian parasite *Plasmodium gallinaceum*, as well as filarial nematodes that are also transmitted by *Anopheles*, comparative immunogenomics provides opportunities for studying a variety of mosquito–pathogen interactions, both common and unique.

Multi-species comparative immunogenomic studies of the two mosquitoes and the fruit fly that diverged from them approximately 250 million years ago, characterized and compared almost 1000 genes as components of immune-signalling pathways, or members of some 30 immune-related families or subfamilies (Waterhouse *et al.*, 2007). Rigorous phylogenetic analysis across this set of immune-related gene families enabled the identification of single-copy orthologous trios, mosquito-specific pairs, and genes showing characteristics of family expansions and gene losses, or high sequence divergence that precluded confident determination of phylogenetic relationships. The prevalence of orthologous trios, mosquito-specific orthologous pairs, and species-specific genes varied widely among immune gene families, clearly indicating that families vary in their degree of diversification (Figure 6.3a). At one extreme, families of enzymes involved in oxidative defence, the A- and B-type scavenger receptors, and inhibitors of apoptosis show a high proportion of orthologues. The C-type lectins, which can act as opsonins or modulators of melanization, display an intermediate level of conservation: they show large expansions (particularly in *Drosophila* and *Aedes*) while retaining a set of at least nine orthologous trios. The modulators, serine protease inhibitors, show a high level of conservation between the two mosquitoes, whereas few have confident orthologues in *Drosophila*. At the other end of the scale, only a few families of AMPs are shared among the three species: the effectors

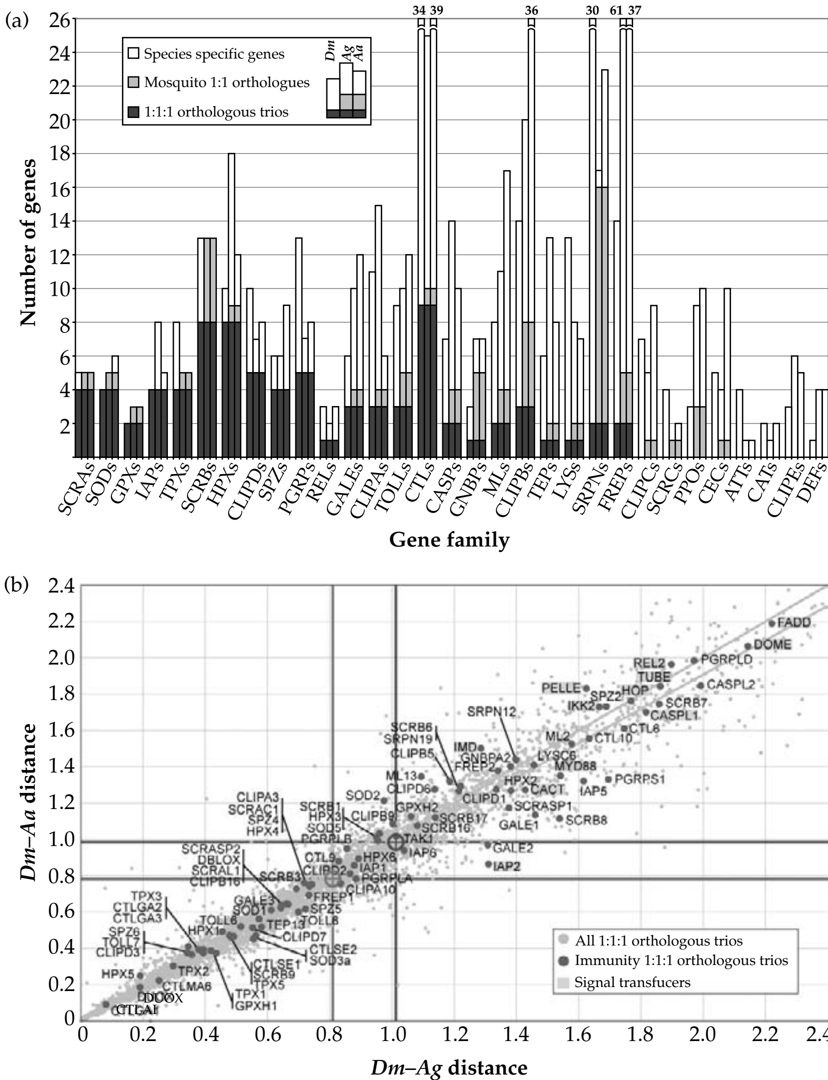

Figure 6.3 (a) The repertoire of putative immune-related gene families identified in *Drosophila melanogaster* (*Dm*), *Anopheles gambiae* (*Ag*), and *Aedes aegypti* (*Aa*) show highly variable levels of orthology conservation. The total number of genes identified in *D. melanogaster* (first bar), *A. gambiae* (second bar), and *A. aegypti* (third bar) are summed from the numbers of 1:1:1 orthologous trios (black), mosquito-specific 1:1 orthologues (grey), and species-specific genes (white) for each gene (sub)family. Families are arranged from left to right according to the decreasing proportion of 1:1:1 orthologous trios within the family. See Table 6.1 for the full list of gene family acronyms and definitions. (b) Sequence divergence among fruit fly–mosquito orthologous trios. All identifiable single-copy trios were compared with the subset of immune-related single-copy trios in terms of genetic distances of each mosquito (*A. gambiae* or *A. aegypti*) protein to the corresponding *D. melanogaster* orthologue. The distance means for immunity and all trios (dashed and solid lines, respectively) indicate that immunity trios (black dots) are significantly more divergent than all trios (grey dots). Signal transducers (boxed) are among the most highly divergent trios, despite high levels of conservation in terms of orthology. DEF, defensin. Both panels are adapted from Waterhouse *et al.* (2007).

show hardly any maintenance of single-copy orthologues. High diversity is also observed within the lysozyme family of peptidoglycan-hydrolysing enzymes. This family has expanded independently in each species, leaving only one gene that encodes a multiple-lysozyme domain protein with a clear three-way orthologous relationship. Across the recognized immune-related gene families shown in Figure 6.3a, some have undergone species- or lineage-specific expansions or have suffered independent losses resulting in fewer identifiable cases of single-copy orthology; other families are more conservative and retain many clear orthologous relationships.

In addition to differences in orthology conservation, the immune repertoires show interesting patterns of sequence diversification. Previous observations of elevated divergence in immune genes were based on sequence identities between orthologous *Drosophila*-*Anopheles* pairs (Zdobnov *et al.*, 2002). Multi-species analysis allowed more rigorous comparisons of immune and non-immune trios, in terms of the computed phylogenetic distances of each mosquito protein to their corresponding *Drosophila* orthologue. These distances revealed that immune trios display significantly higher levels of sequence divergence than the full set of identifiable trios in these genomes (Figure 6.3b). This three-way analysis detected several *Anopheles* immunity genes that appear considerably more divergent than their *Aedes* orthologues. This trend extends to the full set of trios and implies greater accumulation of amino acid substitutions in the conserved protein cores of *Anopheles* compared to *Aedes*. Remarkably, many components of signal transduction pathways show high conservation in terms of orthology (within Diptera and also other animal groups), but are in fact among the most highly divergent of the single-copy trios at the sequence level (Figure 6.3b). In contrast, multigene families with large species-specific expansions, for example C-type lectins (CTLs) and clip-domain serine proteases (CLIPs), retain orthologous trios that are highly conserved in sequence. Evidently, strict maintenance of copy numbers does not necessarily imply high conservation of protein sequence identities: these two measures represent distinct evolutionary processes.

6.4.4 Immune pathways: distinct evolutionary patterns in sequential phases of immune responses

Using the established framework, the immune repertoires can be dissected into several different phases. Commencing with molecular *recognition* of microbial patterns, the immune signals produced subsequently pass through a *modulation* phase, followed by *signal transduction* and activation of *effector* responses (Table 6.1). Signalling through the Toll or Imd pathways is known to be triggered by members of the recognition receptor families: Gram-negative-bacteria-binding proteins (GNBPs) or peptidoglycan-recognition proteins (PGRPs) (see Chapter 2). The repertoire of recognition receptors for universally encountered microbial groups such as bacteria and fungi appears to have evolved through species- and lineage-specific duplication events, of genes and domains, leading to expanded sets of related genes. These data, in conjunction with the sequence similarity between members of the recognition families, indicate that insects employ a conservative evolutionary strategy for the recognition of bacterial and fungal molecular patterns, complemented with species-specific fine-tuning via gene duplications and minimal sequence divergence.

Each of the genes encoding the Gram-negative peptidoglycan recognizing DmPGRP-LC and its *Anopheles* orthologue have three PGRP domains capable of alternative splicing. However, their domains apparently arose through phylogenetically independent duplications: they are more similar within each species than between species. In *Drosophila*, a separate duplication of two adjacent PGRP-LC domains has generated the *PGRP-LF* gene, which is not found in mosquitoes. PGRP-SD is fruit fly-specific and recognizes Gram-positive bacteria to activate the Toll pathway. The same activation is served by DmPGRP-SA, which has mosquito orthologues and functions together with GNBP1 to process polymeric peptidoglycan, making it accessible to PGRP-SA (Wang *et al.*, 2006). A large mosquito-specific expansion has generated a group of B-type GNBPs, distinct from the two A-type orthologous pairs that more closely resemble the three fruit fly GNBPs. DmGNBP3

recognizes fungi, possibly through binding β-1,3-glucans, suggesting that mosquito A-type GNBPs may serve a similar function; the B-type receptors may facilitate novel recognition interactions (Warr *et al.*, 2008).

Triggering of signalling through fungal and Gram-positive recognition in *Drosophila* activates an extracellular cascade of serine proteases and their serpin inhibitors, all of which lack mosquito orthologues. The cascade culminates in proteolytic cleavage of a cytokine precursor, Spätzle, releasing an active factor that binds to the Toll receptor (DmToll-1) and leads to intracellular Toll pathway signal transduction. No clear orthologues of this DmToll-1 have been identified in mosquitoes; instead, gene duplications have created a clade of mosquito genes related to both DmToll-1 and DmToll-5 (Figure 6.2). Toll pathway cytoplasmic signal transduction occurs through a chain of interacting partners—MyD88, Tube, Pelle, TRAF6, and CACT—which are strictly maintained as single-copy orthologues, but evolve extensively in sequence. The same is true for the components of the Imd pathway: Imd, FADD, Dredd (CASPL1), IAP2, TAK1, and IKKγ and β. This observed pattern of persistent orthology coupled with high sequence divergence also applies to the signal transducers Dome and Hop in the immune-signalling JAK/STAT pathway, which is activated in *Drosophila* by viral infections (Dostert *et al.*, 2005). These observations lead to the hypothesis that the requirement to interact productively with others in the same chain may drive escalating sequence divergence among these factors: mutations may be mutually acceptable between interacting partners, leading to coherent evolution rather than stasis. These characteristics highlight that the serially interacting signal transducers evolve in concert: strong selective pressure on pathway maintenance is combined with parallel diversification of their sequences.

The final stage of signal transduction leads to nuclear translocation of NF-κB transcription factors and activation of transcriptional responses; for example, the upregulation of immune effector genes. Several quite different effector mechanisms provide collective defence through the immune responses of lysis, melanization, encapsulation, or phagocytosis. Understanding the evolutionary patterns exhibited by these effector families requires an understanding of their modes of action. AMPs such as defensins and cecropins exhibit species- and lineage-specific duplications, while others such as gambicin appear as novelties, only in mosquitoes. Enzyme families implicated in oxidative defence, such as peroxidases show several expansions, but exhibit low sequence divergence, suggestive of constraints to preserve ubiquitous catalytic activities. Thus, effectors acting directly on microbes diversify rapidly or are species-specific, whereas enzymes that produce chemical cues to attack invaders remain conserved, but expand independently in each species.

6.4.5 Melanization: a conserved immune response based on species-specific regulatory modules

Melanization is an important, ancient defence reaction of arthropods that may be triggered by pathogens or wounding (see Chapter 3). Like immune signalling, melanization is structured into sequential phases: molecular pattern recognition produces signals that must be modulated before activating effector mechanisms. The melanization cascade is tightly regulated as it generates toxic byproducts, including ROS. It is positively and negatively regulated by a network of specific clip-domain serine proteases (CLIPBs), enzymically incompetent homologues (CLIPAs), CTLs, and serine protease inhibitors (SRPNs). Reverse genetics in *Anopheles* has identified a large set of regulators for melanization of the rodent malaria parasite *Plasmodium berghei* or Sephadex beads (Volz *et al.*, 2005, 2006; Paskewitz *et al.*, 2006; Barillas-Mury, 2007). Remarkably, all the regulators are members of mosquito-specific expansions, none has a definitive 1:1:1 orthologue, and only SRPN2 has a clear *Aedes* orthologue. Thus, while the melanization reaction is conserved among insects, its critical regulatory modules appear to be almost entirely species-specific.

On activation, proteolytic cleavage of prophenoloxidases (proPOs) into active POs initiates conversion of tyrosine to melanin with the assistance of additional enzymes, culminating with

cross-linking the wound or the invader in a melanotic capsule. The family of proPOs has expanded greatly in mosquitoes compared to *Drosophila* and larger model insects. Only one mosquito orthologous pair clusters with *Drosophila* proPOs; the remaining mosquito proPOs form a distinct extensive clade, created by reduplication events both before and since the *Anopheles/Aedes* divergence. The invariable catalytic activity of proPOs suggests that their observed expansions may accommodate differential regulation such as temporal, developmental, or topological activation. Indeed, several proPOs do show developmental or physiological specificity (Li *et al.*, 2005).

In *A. gambiae*, several genes have been implicated in the outcome of infections with the rodent malaria model parasite, *P. berghei*, and are classified as antagonist (negative) or agonist (positive) factors (Osta *et al.*, 2004) (see Chapter 7). The most important parasite antagonists are TEP1, member of a family of complement-like thioester-containing proteins (Blandin *et al.*, 2004), and members of a leucine-rich-repeat protein family (Osta *et al.*, 2004; Riehle *et al.*, 2006). The leucine-rich-repeat immune protein (LRIM) family appears as a mosquito evolutionary novelty and is discussed below. The TEP family is related to the vertebrate complement factors C3/C4/C5 and pan-protease inhibitors, the α2-macroglobulins (Blandin and Levashina, 2004). It exhibits only one orthologous trio, and otherwise shows two clades: one with both fruit fly and mosquito TEPs, and a mosquito-specific group that includes AgTEP1. Melanization or lysis are thought to be initiated when TEP1 kills parasites after binding to their surface (Blandin *et al.*, 2004). TEP1 also binds to bacteria, promoting their phagocytosis (Levashina *et al.*, 2001; Moita *et al.*, 2005). Melanization usually disposes of malaria parasites that have been killed by TEP1 binding (Blandin *et al.*, 2004). However, depending on the genetic background, melanization may itself cause parasite killing (Volz *et al.*, 2006).

Components of the critical regulatory modules appear to have been selected from large reservoirs of independently expanded gene families, in a so-called mix-and-match mode of evolution, giving rise to related but distinct sets of proteins that control the melanization response in each species. It would appear therefore, that the specificity of the otherwise ubiquitous process of melanization derives from its tight regulation by genetic modules that probably co-evolve with pathogens. The modular mix-and-match evolution hinders detailed knowledge transfer between species, but elegantly illustrates the flexibility of the immune system to correctly identify specific threats and then activate a potent immune response.

6.4.6 Honey bee: appreciating defence strategies other than immunity

The honey bee genome allowed the comparative analysis of an insect separated from Diptera by over 300 million years of evolution. Compared to the Dipteran genomes, the honey bee appears to have evolved slowly: the mean sequence identity of its single-copy orthologues with human genes is higher than that between fruit fly and human, or mosquito and human. Moreover, the honey bee retains more ancient introns, and its gene losses and gains appear to be lower than in Diptera (HGSC, 2006). The honey bee genome has allowed a unique comparison of the immune repertoires between social and solitary insects. Behavioural studies of honey bees and other social insects have highlighted strategies that may have evolved to protect them against disease: grooming and nest hygiene habits, prompt removal of infected larvae, and use of antimicrobial compounds in nest-building materials, result in a relatively sterile nest environment and a consequent dramatic reduction in exposure to pathogens. Indeed, the comparative analysis of the honey bee immune repertoire revealed maintenance of the overall architecture of the innate immune system. Genes with key roles in the immune signal transduction pathways are found across the insect orders. This is despite the overall reduction of the immune gene repertoire, which could indicate reduced reliance on immunity for pathogen recognition and elimination. Although members of most immune gene families were identified in the honey bee genome, including orthologues of the majority of pathway components, the total repertoire size in the honey bee is only one-third of the fruit fly's and the malaria mosquito's (Evans *et al.*, 2006). The lack of a

large arsenal of immune-related genes encoded in the honey bee genome may be directly compensated by sociality, which in turn may be reflected in enhanced brain function: this is suggested by the existence of four times as many neurons in the honey bee than in the fruit fly, and is consistent with the known complexity of honey bee foraging behaviours (von Frisch, 1974).

6.4.7 Exploiting the *Drosophila* phylogeny

The *Drosophila* 12 genomes project provided unprecedented opportunities for developing comparative genomic approaches, to examine features of sequence evolution across a moderately divergent insect phylogeny (Clark *et al.*, 2007; Stark *et al.*, 2007; Lin *et al.*, 2008). The high levels of sequence divergence that were observed previously, in more distant insect genome comparisons, prevented examination of molecular evolution for evidence of selective constraints. However, the limited divergence among six closely-related species of the *melanogaster* group facilitated robust estimation of rates of synonymous and non-synonymous substitutions in protein-coding genes. Substitution analysis of single-copy orthologues from this group with respect to gene ontology classifications found that most functional categories are strongly constrained, whereas the *defence response* class is a rare category that exhibits an elevated ratio of non-synonymous to synonymous divergence, suggesting positive selection, or reduction of selective constraints (Clark *et al.*, 2007). This result directly supports our previous observations that immune-related genes show elevated divergence in terms of pairwise *Drosophila–Anopheles* sequence identities (Zdobnov *et al.*, 2002) and three-way fruit fly–mosquito phylogenetic distances (Waterhouse *et al.*, 2007). Indeed, maximum likelihood analysis of gene family expansions and contractions across the 12 *Drosophila* species identified the defence response category as one of the most common annotations in gene families with elevated rates of gene gain or loss. These genomes represent a rich dataset for exploring the evolutionary processes (including selection and non-adaptive drift) which shape important phenotypes such as defence and immunity, metabolism and detoxification, sex and reproduction, or chemoreception.

Comprehensive annotation of the immune repertoire identified over 2500 candidate immune-related genes across the 12 *Drosophila* species, facilitating the investigation of evolutionary patterns in fruit fly immunity gene families and their sequence divergence (Sackton *et al.*, 2007). Orthology analysis identified single-copy orthologues, conserved paralogues, and lineage-restricted genes from various functional categories of such families across the 12 species. Maximum likelihood modelling of gene birth/death events provided an estimate of the rates of gene turnover (duplications and losses) over the entire phylogeny of each family. These estimates showed a deficit of single-copy orthologues among effector families, contrasting with high prevalence of orthologues among signalling proteins. The latter class also exhibited reduced levels of gene turnover compared to both effectors and recognition proteins. Lineage-restricted genes, identified through multi-species comparisons, represent evolutionary novelties potentially generated from rearrangements and/or truncated copies of existing genes. Examples of such novelties are examined in more detail below. The gene encoding the antifungal peptide drosomycin is used as an indicator of Toll-pathway activation in *D. melanogaster*, but is restricted to the *melanogaster* group. Thus, even within the *Drosophila* genus, there is considerable variation in the repertoires of recognition and effector families, while the core signalling components show much more stable prevalence.

Sequence analysis of all single-copy orthologues from the *melanogaster* group found that the immune repertoire exhibits a significantly high proportion of adaptively selected genes. The class of recognition proteins, particularly those implicated in phagocytosis, was largely responsible for this trend, whereas effectors showed little evidence of adaptive evolution. Codon-based selection analysis along the recognition proteins located significantly more sites of possible positive selection, within regions experimentally identified as putative pathogen-interaction domains. Assessment with lineage-specific codon models identified several *Imd* pathway genes, indicating accelerated evolution in *D. melanogaster* compared with *Drosophila yakuba* and *Drosophila erecta*. The positively selected sites among these proteins showed

significant clustering within regions thought to interact physically during signal transduction. These observations are in agreement with, and provide further evolutionary insight into, findings from the earlier fruit fly–mosquito comparative immunogenomic analyses (Waterhouse *et al.*, 2007). Uncovering patterns of sequence variation which point towards adaptive evolutionary processes, can identify key components of the innate immune repertoire which may shape host–pathogen co-evolutionary dynamics.

6.4.8 Immune novelties revealed through comparative genomics: the LRIM and NIMROD examples

Leucine-rich repeat (LRR) sequence motifs are present in many proteins implicated in immune responses, and have emerged as powerful immune-recognition domains in multicellular organisms. The discovery that the Toll receptor is important in *Drosophila* innate immunity was the catalyst for elucidating the roles of Toll-like receptors (TLRs) in mammalian immunity. The LRR ectodomain of *Drosophila* Toll recognizes and binds to a proteolytically activated cytokine, Spätzle, resulting in intracellular NF-κB signalling and immune-effector production. Activation of the homologous mammalian TLRs also leads to immune transcriptional responses through NF-κBs. However, TLR ectodomains bind directly to components of infectious agents. Similarly, LRRs encoded by plant R genes can confer resistance either by interacting directly with pathogen virulence factors, or indirectly by binding to perturbed host proteins (Bent and Mackey, 2007). Many R proteins combine a variable number of LRRs with a nucleotide-binding domain (NBD) and an N-terminal Toll-interleukin-1 receptor (TIR) homology region or a coiled-coil domain. These R proteins show striking structural and functional similarities to animal nucleotide-binding leucine-rich repeat (NLR) proteins, which combine LRRs and NBDs with a variable N-terminal domain associated with apoptosis and/or signalling: they sense PAMPs such as peptidoglycan or flagellin, initiating immune responses through activation of NF-κBs and mitogen-activated protein kinases (Shaw *et al.*, 2008). The power of forming recognition domains from a variably repeating, structure such as the LRR, is also the basis of the adaptive immune system of the primitive jawless vertebrates, the lamprey and hagfish. Instead of using immunoglobulin gene segments, these organisms create a repertoire of variable lymphocyte receptors built from highly diverse combinatorial assemblies of gene segments from a library of LRR cassettes (Pancer *et al.*, 2004).

Novel discoveries and insights from extensive functional analyses continue to reveal genes and families that make up the insect immune repertoire. Microarray studies in *A. gambiae* identified numerous novel genes with putative roles in defence and immunity (Dimopoulos *et al.*, 2002). Two LRR-containing genes were differentially activated in cell cultures by septic, but not sterile injury and massively upregulated by heat-killed bacteria and microbial components. One of these genes, later named *LRIM1*, was also highly upregulated during mosquito infection by *P. berghei* (Dimopoulos *et al.*, 2002; Vlachou and Kafatos, 2005). RNAi-mediated silencing of *LRIM1* revealed a striking increase in *P. berghei* oocyst numbers, identifying LRIM1 as the prototype antagonist of the development of this rodent malaria parasite (Osta *et al.*, 2004). The same study identified two CTLs that act as inhibitors of parasite melanization, and revealed that *LRIM1* acts upstream of the CTLs in initiating the melanization reaction. However, the *LRIM1/CTL4* genetic module appeared to not have an effect against the human parasite *Plasmodium falciparum* (Cohuet *et al.*, 2006). The second LRR gene was later found in a population survey of West African *A. gambiae* mosquitoes and mapped to the *APL1* genetic locus, with major effects on *P. falciparum* development and melanization (Riehle *et al.*, 2006). RNAi silencing of *APL1* in laboratory mosquitoes produced a similar phenotype to that observed for *LRIM1*, with dramatically increased numbers of *P. berghei* oocysts. Furthermore, a recent study showed that orthologues of *LRIM1* and *APL1* in the malaria non-vector mosquito, *Anopheles quadriannulatus* species A, are involved in the melanization response that these mosquitoes mount naturally against *P. berghei* (Habtewold *et al.*, 2008).

The important functions of LRIM1 and APL1 in *Anopheles* innate immunity, and specifically the

wide-ranging recognition roles of LRR domains, provide a basis for bioinformatic characterization of a novel immune-related gene family (Povelones *et al.*, 2009). *LRIM1* and *APL1* both have signal peptide sequences followed by a stretch of LRRs that create an alternating α-helix/β-strand pattern, with some irregularities that likely translate into subtle structural variations of their characteristic horseshoe fold. The C-terminal sequences of both these genes exhibit characteristic seven-residue (heptad) repeats that define the primary structure of coiled-coil domains; a distinctive cysteine-rich pattern can be identified in the hinge region between the LRR and the coiled-coils. Comprehensive scans employing these LRIM features identified over 20 regions encoding potential LRIM-like genes in each of the three available mosquito genomes. They encompass short LRIMs with six to seven LRRs and long LRIMs with 10–13 LRRs, such as LRIM1 and APL1 (Povelones *et al.*, 2009).

The structural integrity of the LRR domain rests with a conserved pattern of leucine residues that tolerates only limited substitutions with similar amino acids: the intervening positions are far less constrained. The coiled-coil heptads must maintain a pattern of hydrophobic and polar residues within the repeat, but the identity of individual amino acids is not critical. Thus, sequence conservation within the LRIM family varies considerably, hindering robust phylogenetic analysis for resolving orthologous relationships. Nevertheless, a comparative approach employing synteny among the three mosquitoes does resolve and confirm relationships suggested by initial protein sequence analyses. Indeed, orthologous genomic clusters of both short and long LRIMs can be identified in all three mosquito species. *APL1* is found within a cluster of mostly long LRIMs, located between conserved BRACA2-like and zinc-finger genes that delineate the synteny (Figure 6.4a). In *A. gambiae*, three of these genes, *APL1A*, *APL1B*, and *APL1C*, are very similar in sequence and likely originated from recent gene duplications; only *APL1C* has an effect against *P. berghei* (Riehle *et al.*, 2008). A second cluster contains only short LRIMs and the synteny is supported by a gene encoding a guanine nucleotide exchange factor in all three species. Both clusters exhibit striking examples of local gene shuffling, duplication, and even a clear case of pseudogenesis after duplication. The identity of LRIM1 orthologues in the culicine mosquitoes is somewhat obscured by high sequence divergence. However, detailed inspection reveals convincing evidence of orthology: the same number of LRRs, matching profiles of coiled-coil heptad repeats, and preservation of synteny (Figure 6.4b).

The variable repeating units that characterize the LRIM family of putative recognition receptors are thought to provide both the structural and evolutionary flexibility required to facilitate recognition of diverse immune-stimulating pathogen structures. Such repetitive features are also found in a recently identified family of another class of recognition receptors in *Drosophila*, characterized by additional complex protein-domain architectures. The distinct epidermal growth factor (EGF)-like repeats of the *Drosophila* phagocytosis receptors Eater (Kocks *et al.*, 2005) and Nimrod C1 (NimC1) allowed the characterization of a novel family of putative receptors (Kurucz *et al.*, 2007). The EGF-like repeats of Eater and NimC1 exhibit a conserved six-cysteine consensus (NIM repeat) separated by loops of variable length. *NimC1* neighbours the haemocyte-specific *Hemese* gene on chromosome 2, where a cluster of genes containing NIM repeats is found. These Nimrod-like genes make up three distinct classes based on their additional domain features. NimA exhibits domain organization similar to *Draper*, another *Drosophila* phagocytosis receptor (Manaka *et al.*, 2004). It consists of a large intracellular domain, an extracellular EMI domain (with possible protein–protein interaction properties), and eight-cysteine EGF-like repeats. NimBs are probably secreted proteins, but apart from a weakly conserved N-terminal region, they do not have additional identifiable features. Finally, the NimC class encodes transmembrane proteins with a partially conserved N-terminal region upstream of the NIM repeats.

Evolutionary analysis of these NIM-repeat-containing proteins suggests how the Nimrod superfamily may have evolved from an EGF-repeat-containing ancestor via a *Draper*-like gene, containing both EGF repeats and a single NIM repeat (Somogyi *et al.*, 2008). The genomic cluster of Nimrod genes remains broadly conserved across the 12 sequenced *Drosophila* genomes, but

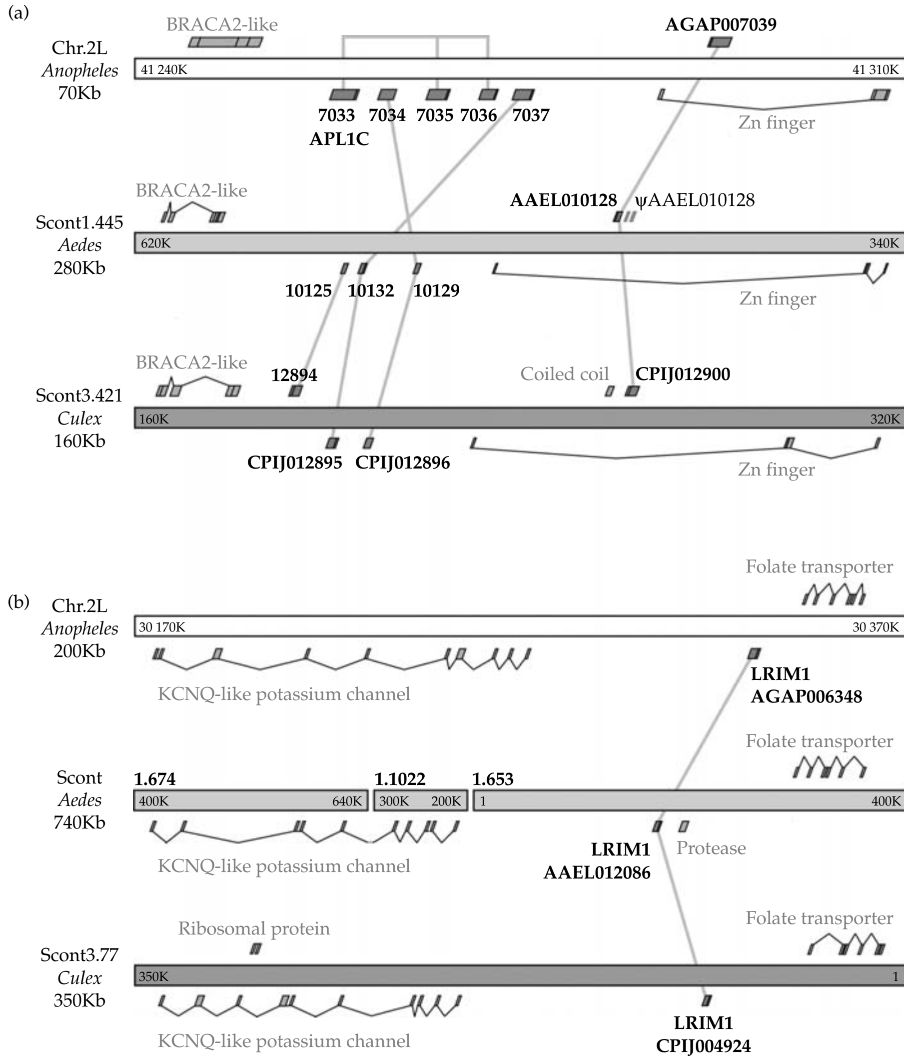

Figure 6.4 Two genomic loci encoding multiple leucine-rich repeat immune protein (LRIM) genes in three mosquito species. (a) *Anopheles gambiae* APL1C (AGAP007033) is found in a genomic cluster of LRIM genes where the orthologous genomic regions in *Aedes aegypti* and *Culex pipiens*, delineated by conserved neighbouring BRACA2-like and zinc-finger genes, also encode LRIMs. Two *Anopheles* LRIMs in this cluster (AGAP007035 and AGAP007036) are closely related to APL1C; however, putative orthologues are not found within these synteny regions in *Aedes* and *Culex*. The syntenic cluster nevertheless exhibits three LRIM orthologous trios, whose relative orientations indicate the occurrence of genomic shuffling events since the anopheline/culicine divergence. One of the *Aedes* LRIM genes appears to have undergone a duplication event followed by pseudogenesis leaving a dysfunctional copy, ΨAAEL010128. (b) The preservation of synteny at the LRIM1 gene locus delineated by the neighbouring KCQN-like potassium channel and folate transporter genes helps to confirm the putative LRIM1 orthologues in *Aedes* and *Culex* which, at the protein-sequence level, are somewhat obscured by high levels of divergence. Identification of the orthologous genomic region in *Aedes* was hampered by incomplete assembly and sequence gaps as indicated on the figure, but a better assembly in *Culex* serves to confirm the synteny among these regions. Chromosomes (Chr) and supercontigs (Scont) are labelled in *Anopheles gambiae* (white), *Aedes aegypti* (light grey), and *Culex pipiens* (dark grey) with start and end positions of the displayed genomic regions. LRIM genes are indicated in bold typeface and neighbouring genes are in grey typeface. For clarity, the species code and leading zeros have been removed from some gene identifiers.

expansions and lineage novelties are also identified (Sackton *et al*., 2007). *Hemese* is only found in the *melanogaster* group, prompting the hypothesis that this gene has originated from a truncated duplication of an ancestral *NimC1*. Novel *NimD* genes are found in the *Sophophora* subgenus and *NimE* in the *Drosophila virilis*/*Drosophila mojavensis* clade. Nimrod-like genes exhibiting most of the family-defining features are identifiable in other insect genomes, including *A. gambiae* (which also has an *Eater* homologue) and *A. mellifera*. *NimB*-like genes have been described in *B. mori* and *Holotricia diomphalia*. Proteins exhibiting Nimrod-like features have also been identified beyond insects; several of them have been implicated in phagocytosis and/or binding to microbes. The NimA-like *Caenorhabditis elegans* CED-1 protein is a receptor for phagocytosis of apoptotic cells, and mammalian Ced-1-like genes may perform similar roles (Mangahas and Zhou, 2005).

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CHAPTER 7

Physiological integration of innate immunity

David Schneider

7.1 Introduction

The field of insect immunity has made enormous progress in describing the basic molecular mechanisms of the immune response to microbes. This work not only told us how insects work but also how the human innate immune system works, because many of the basic mechanisms are conserved between these two organisms. Three serious problems remain in our description of innate immunity. The first stems from our basic definition of the process; innate immune systems are defined as responding to threats using germ-line-encoded receptors. This is in contrast to a so-called adaptive immune system, which can increase the specificity of its detectors and effectors through somatic recombination and mutation. Often one is given the impression that an innate immune response is static and stereotypical, producing the exact same molecular output every time it encounters an elicitor. Nothing could be further from the truth; innate immune responses are supple and responsive. Innate immunity fluctuates with changes in the native microbiota, energy availability, feeding, circadian rhythm, age, and even past exposure to microbes. Sometimes it is surprising that we can even get experiments to repeat, given the responsiveness of the insect.

A second problem with our description of innate immunity is that this subject has become a victim of its own success. Past work focused on the molecular mechanisms behind elicitor recognition and the signalling pathways regulating initial transcription events. As this generated excitement in the larger world of vertebrate immunology it further focused our attention on these molecular studies. Unfortunately, however, we still don't know why most insects die when they are infected with a pathogen, yet this type of knowledge is critical if insects are to be used as a model for disease or even if we are to simply understand the physiological regulation of immunity.

Third, we tend to describe the immune response of insects very simply. However, ecological studies of plant–herbivore interactions predict that hosts evolve two methods of retaining fitness when faced with a predator; they can increase either their resistance or their tolerance of the threat. Resistance is defined as the inverse of the herbivore intensity. Tolerance is the reaction norm found when the fitness of the plant is plotted at different herbivore levels. In tolerant plant strains the slope of this tolerance curve is shallow, indicating that the plants do not suffer a large loss in fitness as herbivore levels increase. Together these two properties comprise the defensive capabilities of a host. In insect studies, we have focused most of our attention on resistance and have largely ignored tolerance. However, tolerance has been shown to play a role in insect immunity and measurements of tolerance need to be taken in future experiments (Corby-Harris *et al*., 2007; Ayres *et al*., 2008).

To understand how insect immunity is regulated we need to study the interactions of all those aspects of physiology that impact immunity. This includes both resistance and tolerance aspects of defence as well as all of the other assorted physiological systems of the insect that alter the immune response.

Our hypothesis is that an insect's innate immune response sits in the centre of a physiological net and the immune response is sensitive to changes throughout this net. The goal of this chapter is to try to tie all of these physiological strands together and demonstrate how innate immunity alters the gross physiology of an insect and how the gross physiology, in turn, alters the immune response. An emergent property that falls out of this analysis is the prediction of several types of physiological collapse; these collapses result from positive-feedback loops that lead to amplified and damage-inducing immune/physiological responses.

7.2 Insect immune defence mechanisms

I refer the reader to detailed descriptions of the individual components of insect immune systems provided in other chapters of this volume and just summarize them here. I count seven layers to the immune response, moving from the outside of an insect to the inside. First, is the native microbiota, which occupies niches on the surfaces of insects (both on the outside of the body and within the gut, for example) and can prevent colonization by other microbes. Second is the barrier epithelial immune response, which is induced to produce antimicrobial peptides (AMPs) when it recognizes a pathological event. Third is the clotting response, which can entrap microbes in a fibrous net. Fourth is the haemocyte-driven immune response, which can lead to phagocytosis, encapsulation, or nodulation of invading parasites. Fifth is the melanization response, which can produce reactive oxygen that presumably kills microbes. Sixth is the AMP response, in which the fat body releases large quantities of AMPs into the circulation. Finally there is the RNA interference (RNAi) response that can limit viral growth. Alongside all of this lies tolerance and we do not yet have a good picture of the physiological mechanisms that underlie tolerance.

I concentrate on realized immune responses rather than potential immune responses in this chapter. By that is meant that most attention will be devoted to experiments that challenged insects with microbes that cause pathology and measure the effects of the immune response on this pathology. This is in contrast to experiments that treat the insect with an elicitor and measure a transcriptional output. We took this approach because this is where we find the greatest number of examples of interactions between immunity and gross physiology. Not enough studies have been performed linking molecular mechanisms to gross changes in physiology to tell a meaningful story.

I draw my examples from the world of insects but focus on work done in *Drosophila melanogaster* using injected microbes. This is done for the sake of consistency because different insects may have come up with different evolutionary solutions for a problem. One should avoid the trap of oversimplification by saying 'insects work like this...' and try to limit this problem by focusing on one insect. Injected microbes are the focus because this type of experiment makes up the bulk of the literature. So-called natural infection models in the fly require that larvae or adults be fed a paste of the infecting bacteria and, as it will become clear below, this is predicted to have spectacular effects of the physiology of the insects that are difficult to control.

7.3 Energy management and immunity

Energy flow in an organism is strictly regulated. One of the most common arguments in ecology seems to be that physiological programming decisions evolve in part based on the internal competition for resources. Many experiments imply that immune activation utilizes energy and takes that energy from other physiological activities but such changes can be difficult to assess directly because of compensatory changes in these other systems. Moret and Schmid-Hempel (2000) have provided a particularly clean example of this resource-allocation issue because the design deliberately limited the effects of compensation by starving the bumble bees that were the subject of the experiments. Unfed bumble bees die at a reproducible rate and obviously cannot compensate for energy use by eating more. If these bees are forced to raise an immune response then they die faster than un-manipulated bees, suggesting that the immune response is using energy that would otherwise be

used to keep the bees alive. The authors conclude that the cost of an immune response can be hidden by compensatory physiological changes.

Starvation provides an interesting and drastic example of the cost of immune response but doesn't answer the question of how energy use changes in an infected insect that is given food *ad libitum*. Dionne and colleagues (2006) provide some answers to this question through their studies of *Mycobacterium marinum*-infected flies. This microbe initially causes intracellular infections in haemocytes following injection into the haemocoel. Ultimately the microbes invade adjacent tissues and grow free in the haemolymph. Microarray analysis of progressively sicker flies infected with *M. marinum* suggested an answer to the energy question; the authors noted that catabolic metabolism was turned down as the flies grew moribund. Analysis of glycogen and fat content confirmed that these sick flies were suffering from a wasting disease but a peculiar aspect of this was that the glucose levels in the flies were rising while the fat and glycogen levels fell. This doesn't resemble starvation so much as diabetes. Further analysis of insulin signalling pathways showed that these sick flies did not appear to activate the these pathways appropriately. Forced activation of insulin signalling, induced by a mutation in the transcription factor FOXO, partially rescued the flies and allowed them to survive infections significantly longer. This FOXO mutation did not affect the resistance of the sick flies as they were found to have the same number of *M. marinum* as the wild-type flies. This suggests that the insulin signalling pathway was affecting the tolerance of the flies to this infection and highlights the importance of measuring both tolerance and resistance when performing immunity experiments.

This insulin story is complicated because insulin signalling appears to have different effects depending upon on the infectious agent tested. Work from Libert and colleagues (2008) tested the effects of inhibition of the insulin pathway on *Enterococcus faecalis* and *Staphylococcus aureus* infections, two microbes that cause extracellular infections when injected into the fly haemocoel. Part of the drive behind these experiments was to understand the link between ageing, diet restriction, reduced insulin signalling, and immunity. The authors found that decreased insulin signalling, induced by the mutation of the insulin signalling pathway component chico, could improve the survival of *E. faecalis*- or *S. aureus*-infected flies. The effects this mutation has on bacterial growth were not tested and thus it is not possible to determine whether this increase in survival was due to changes in resistance or tolerance.

These two sets of experiments produced opposite results; increases in insulin signalling reduce lethality during an *M. marinum* infection while decreases in insulin signalling reduce lethality during *E. faecalis* and *S. aureus* infections. There have not been enough experiments analysing insulin signalling to determine mechanistically how insulin signalling alters immunity and we cannot yet derive simple rules regarding its effects on infections. One thing that is clear is that insulin signalling has important effects on immunity. It will become clearer below that insulin is a prime candidate for a master regulatory protein that affects most of the physiological systems interacting with insect immunity.

Important outstanding questions remain: how does the innate immune system alter insulin signalling? Does this occur indirectly because the immune system drains energy from the system or are there more direct links between an immune response and insulin production/recognition? Answers to these questions will help us tie immunity to other physiologies in the fly.

7.4 Feeding behaviour and immunity

The greatest regulation of insulin levels will probably be food related and therefore it is important to look at how immunity and feeding behaviour interact. As flies eat less, circulating sugar levels will fall and insulin should also fall. Having seen that insulin signalling is altered by an infection and that insulin signalling can alter the outcome of infections it is easy to predict that feeding regulation will have large effects on immunity.

Unsurprisingly, outright starvation alters the immune response in bad ways. For example, starvation of *Rhodnius prolixus* reduces its ability to fight *Enterobacter cloacae* infections (Azambuja *et al*.,

1997). Likewise, the restriction of dietary yeast reduces a larval *Drosophila*'s chances of killing a parasitoid wasp egg (Vass and Nappi, 1998).

In nature, feeding levels presumably don't have just two states: unlimited food and starvation. Unfortunately there are not a lot of papers describing the effects of more subtle nutritional changes on the innate immune response of insects. One place to start is the field of dietary restriction; diet restriction, the physiological state during which animals fed a small diet can increase their lifespan, was shown to have no effect on the realized innate immune response for *Drosophila* against *E. faecalis* and *S. aureus* (Libert *et al.*, 2008). This is surprising because diet restriction is anticipated to reduce insulin signalling and a reduction in insulin signalling alone was shown to increase the ability of these flies to defend against these two microbes. As an explanation, Libert and colleagues proposed that diet restriction suppressed the activation of defences that were induced by a loss of insulin signalling.

It appears that some sick insect larvae engage in a type of self-induced chemotherapy; for example, larvae of *Estigmene acrea* sequester antiparasitic plant-derived pyrrolizidin alkaloids (Bernays and Singer, 2005). These authors demonstrated that taste sensitivity of parasitized caterpillars increases towards these antiparasitic compounds while at the same time they show decreased responsiveness to normally deterrent chemicals. This suggests that infected caterpillars might switch to food that would be unpalatable to an uninfected caterpillar with the purpose of eating more antiparasitic compounds.

Certainly, drug-containing foods can alter immunity but the general quality of the food has effects as well; Lee and coworkers (2006) found that *Spodoptera literalis* larvae fed protein-rich diets were better able to survive nuclear polyhedrosis virus infections. When caterpillars were allowed to choose their own diets, those choosing protein-rich diets were also better able to survive the infections. Infected larvae tended to choose a higher level of dietary protein late during infections. This suggests that these insects can deliberately change the structure of their diet in a way that helps them fight infections.

Infections can produce more prosaic changes in appetite than an attempt to go out and consume drugs or switch to a more healthy diet; simple infection-induced anorexia has been observed in a number of situations. In some cases the reasons for this anorexia are structural, such as when *Pseudomonas entomophila* causes gut-blocking lesions when fed to *Drosophila* larvae (Liehl *et al.*, 2006). In other cases, the activation of the immune response appears to trigger a decrease in appetite (Adamo, 2005; Adamo *et al.*, 2007). Although infection can reduce food intake, in no case has it been shown yet that this decrease in consumption has an effect on immunity.

Given that in the anorexia experiments the insects were not offered a choice of food, we cannot distinguish between the following two responses: 'yuck, this food is not what I want' and 'I don't feel like eating anything.'

In flies, one of the pathways regulating appetite is the neuropeptide F family, which includes NPF and short NPF (sNPF) along with their two receptors. Appetite is positively regulated by sNPF and its receptor (Lee *et al.*, 2004). Inhibition of sNPF signalling by RNAi treatment reduces the appetites of affected flies. sNPF itself is an inducer of insulin signalling (Lee *et al.*, 2008). When flies are hungry, as defined by sNPF expression, they induce insulin. Dilp 1 and 2, two of the seven *Drosophila* insulin family members, are regulated by sNPF. The regulation of sNPF by insulin has not been tested experimentally but we predict that its activity will be inhibited by high insulin levels.

NPF regulates the fly's response to noxious substances (Wu *et al.*, 2005). High levels of NPF reduce the negative effects of repulsive-tasting chemicals like quinine. NPF signalling is negatively regulated by insulin apparently through an inhibition of signalling through the NPF receptor. When insulin levels are low and NPF levels are high the flies are more likely to eat bad-tasting food; hungry flies will eat anything.

The peptide NPF has other interesting effects on fly behaviour and physiology that might be predicted to change during an infection. NPF is a suppressor of aggression; flies lacking this signalling pathway are more aggressive, as assayed by male flies battling for food and females (Dierick and

Greenspan, 2007). If NPF drops during an infection, flies might be expected to become feistier.

In an uninfected animal we anticipate that low levels of nutrients would lead to low levels of insulin. This would increase NPF induction and we predict would also increase sNPF production. These molecules would raise the appetite of the fly and reduce its avoidance of noxious food. Something different appears to be happening in sick flies, however. Insulin signalling is reduced, but appetite is also reduced. This suggests that the connection between appetite and insulin signalling is altered in these infected animals. The flies should be eating like crazy but are not; why?

There aren't any descriptions in the literature yet where the entire cycle of the immune regulation of appetite and the nutrient regulation of immunity have been completed and both sides were found to be important. However, putting together what we know about immunity and insulin signalling from other models, we can imagine that this apparent conflict between immunity and appetite-regulating systems could lead to physiological collapse. Normally energy depletion increases hunger; in infected flies, energy is being depleted and yet the flies reduce their eating. In the case of an *M. marinum* infections, this regulatory circuit is expected to lead to a physiological collapse where the fly dies because it wastes away, and this wasting does not help fight the infection. In contrast, we know that reduced insulin signalling is expected to help fight infections caused by *E. faecalis* and *S. aureus*. This isn't a perfect counter example, however, because here diet restriction does not affect survival. We anticipate that the fly has evolved in this way because this particular energy-regulation circuit increases fitness when the flies encounter real fly pathogens in the wild. Our laboratory experiments can direct us to those physiological systems that are of interest to the insect immunologist, but they don't tell us how the reactions of these systems help the insects in the field.

There are a several important lessons to be learned from this section that we should apply to our experiments. The first is that food is an important consideration in immunity experiments and thus the exact food composition should be reported in all experiments. The above results suggest a difficulty in performing well-controlled experiments using orally delivered pathogens. If the immune response alters appetite, then the dose of an orally delivered pathogen will vary when immunity mutants are tested. Some immune signalling mutants might appear more susceptible to oral infections but this could be due to an increased uptake of the pathogen, if the anorexia response were blocked. During infections with microbes like *S. entomophila*, which appear to cause a complete blockage of the gut, other problems can be anticipated; these infected flies are anticipated to be starving and that has been shown to cause enormous changes in the immune responses in all creatures.

7.5 The immune response varies with the time of day

The immune response and circadian clock regulate each other, potentially causing feedback loops that are predicted to cause a physiological collapse in some situations but in others might increase an insect's defences. Flies exhibit a circadian rhythm in which they are active during the day and less active at night. The molecular pacemaker that controls these rhythms is defined by the genes *period*, *timeless*, *clock*, and *cycle* (*per*, *tim*, *clk*, and *cyc*) (Nitabach and Taghert, 2008). The genes *per* and *tim* are transcribed and the Tim and Per proteins together form a negative transcriptional regulators of the clock and cycle genes. The Clock and Cycle proteins are positive transcriptional activators of the *per* and *tim* genes. Thus as Per and Tim levels rise, this represses the production of Clock and Cycle which ultimately causes Per and Tim levels to fall. Mutants in *per* or *tim* show an activity pattern where active and inactive periods are dispersed throughout the day rather than being clustered during the day or night, respectively. Environmental stimuli, like the ambient photoperiod, are called zeitgeibers and set the phase of this oscillator. These oscillator genes function both in a collection of clock neurons in the brain as well as in peripheral tissues, which can have their own rhythms. The mechanisms behind the synchronization of these clocks is a field of active research.

The first hint that circadian rhythms affected immunity came from a paper monitoring the role of genetic variation in the ability to resist *Serratia*

marcesans infection (Lazzaro *et al.*, 2004). Lazzaro and colleagues injected genetically different fly lines with *Serratia* and then plated out crushed flies at 7, 15, 26, and 37 h post-infection to determine bacterial levels. For experimental simplicity, the flies that would be homogenized at 7 and 26 h were injected in the morning while the 15 and 35 h timepoint flies were injected in the evening. To their surprise, the authors found a strong correlation in resistance depending on the time of day that the flies were infected; flies injected in the evening had higher bacterial loads, suggesting that flies differ in their capacity to fight microbes depending upon the time of day they are challenged.

Microarray analysis demonstrates that immune-related transcripts are among the many the levels of which rise and fall during the course of the day and this could provide a simple explanation for how resistance is regulated in a circadian manner. Williams and colleagues (2007) probed this subject by looking at the bidirectional regulation of circadian rhythm and immunity. In particular, the transcription factor Relish, the major transcription factor regulating Imd signalling, was found to cycle in a diurnal fashion. They found that knockdown of the Imd pathway with a *relish* mutation could alter sleep patterns. This suggests that Imd signalling plays a previously unrecognized role in controlling circadian rhythm. This phenotype could be rescued by expressing Rel only in the fat body, suggesting that it really was the immune response that was causing this phenotype and not some role for Relish in an unknown tissue. This is a rather surprising result as there was no *a priori* reason to anticipate that immune-signalling mutations in uninfected flies would affect rhythm. One possibility is that daily encounters with microbes act as a zeitgeiber; perhaps if a fly does not have its immune system activated during the day, it doesn't feel ready for bed. Williams also demonstrated that physically depriving flies of sleep by tapping their vials would increase their resistance to *Escherichia coli* as measured by the clearance of *E. coli* colony-forming units.

Shirasu-Hiza and colleagues (2007) demonstrated a bidirectional regulation of immunity and circadian rhythm. Flies infected with *Streptococccus pneumoniae* or *Listeria monocytogenes* were found to have altered circadian rhythms, starting approximately 3 days before the flies died. The total activity of sick flies over the full day did not change but their sleep distribution was altered; instead of sleeping for long periods at night, the sick flies slept for short periods through the entire 24 h day. This resembles the effect seen by Williams *et al.* (2007). To determine whether circadian disruption could affect the immune response, mutants in *per* and *tim* were tested and found to be sensitive to these two bacteria and died more rapidly during infections. This is the opposite of what was observed for *E. coli* infections by Williams *et al.*

Experiments from Lee and Edery (2008) provided a more detailed picture of how immunity varies through the circadian cycle. They infected flies with *Pseudomonas aeruginosa* around the clock and measured survival. Their results showed that survival varied depending upon the time of injection. If the circadian oscillator was broken by mutating *tim*, *clk*, or *cyc* (but surprisingly not *per*) they found that this eliminated the difference seen in survival at different injection times, supporting the hypothesis that the immune response is regulated in a circadian manner. They also demonstrated that the flies injected during the night are better able to clear the bacteria, suggesting that circadian rhythm alters the resistance to these microbes. A small but statistically significant increase in a limited number of immune-regulated genes was found for injections performed at night rather than in the day. The authors suggest that this enhancement of the early response to an infection is critical for deciding the outcome of the infection. This is an interesting idea and it hasn't been well addressed by the current literature. Experiments to date have tended to measure peak expression of AMPs and don't follow differences in expression over time or the impact that such differences might have on a realized immune response.

The study of the circadian–immune connection is still in its infancy and it is too soon to define precise rules yet. From what we have seen from the work by Shirasu-Hiza *et al.* it seems possible that during some infections there will be a positive-feedback loop that will lead to physiological collapse; infection with *L. monocytogenes* or *S. pneumoniae* will disrupt sleep. This sleep disruption in turn could reduce the defences of the fly, which might then be expected to disrupt sleep still further. This seems

like a maladaptive signalling loop. The work of Williams *et al.* suggests that sleep disruption might be beneficial for fighting some microbes and thus we can imagine that in those cases infection leads to sleep disruption in such a way as to increase immune defences. We predict that this kind of interaction between the immune and circadian systems will be found to be more common where flies have co-evolved with the pathogens that infect them in the wild.

The mechanistic links between immunity and circadian rhythm are unknown. It isn't clear yet whether the central clock is important for regulating immunity or whether this regulation occurs at a tissue or cellular level. However this works, insulin again appears to be a possible linking signal. The fly clock is sensitive to oxidative stress and this sensitivity increases in *foxO* mutants (Zheng *et al.*, 2007). This sensitivity shows up as reduced cycling of both central and peripheral clocks as measured by period protein levels, determined by fluorescence microscopy. This *foxO* phenotype can be rescued non-autonomously by expressing FOXO in the fat body, suggesting that cellular circadian clocks throughout the body are sensitive to the metabolic status of the fly. When FOXO activity is low, which should resemble the status of flies when insulin levels are high and the flies are well fed, the clocks are sensitive to oxidative stresses. From what we have seen above, at least in *M. marinum* infections, insulin signalling appears to be decreased in sick flies, which would be expected to harden the clock against potential perturbations. This could be useful to protect the clock against reactive oxygen producing immune responses like melanization. The reactive oxygen produced during an immune response could also provide a signal from the immune system to the clock, by resetting the clock or adversely affecting its timekeeping ability and thus altering the balance of rest/activity cycles in the fly.

Not much has been described about the circadian regulation of eating in flies. If feeding is regulated over the course of a day, flies may find themselves with a circadian rhythm of rising and falling insulin levels. Sleep-disrupted flies might eat throughout the day rather than at the normal times and this could have drastic effects on their immune responsiveness by damping the circadian rhythm of insulin.

There are some important experimental lessons to be learned from this section: it is clearly important to include the time of day that a challenge is given as a variable when performing infection experiments. Nighthawk graduate students might be expected to get different results from day-walkers. As it is important to let your flies have a good night's rest, those same nighthawk graduate students going into and out of the incubators throughout the night might disturb the sleep and results of ongoing infection experiments.

7.6 Mating and reproduction

The large percentage of the fly's body devoted to reproduction makes clear its importance; female flies are essentially ovaries with wings. It is therefore unsurprising that an immune response that might compete for energy would have an effect on reproduction. Even a simple immune response to a non-pathogenic elicitor like *E. coli* will reduce egg-laying (Zerofsky *et al.*, 2005). Experiments with *Salmonella typhimurium* strains of various levels of virulence suggest that as pathogenicity increases so egg-laying decreases. In infected females, eggs do not appear to progress beyond stage 8 of oogenesis, when yolk deposition begins (Brandt and Schneider, 2007). It should be unsurprising to the reader at this point in the chapter that this is a checkpoint in egg development that is known to be responsive to insulin signalling (Drummond-Barbosa and Spradling, 2001).

Ovaries themselves can become infected with intracellular pathogens like *L. monocytogenes* and *S. typhimurium* as these microbes infect macrophage-like cells in the oviduct (Brandt and Schneider, 2007). It isn't clear whether this direct infection is responsible for changes in fertility or whether fecundity changes are linked to effects occurring elsewhere in the body. These two models are not mutually exclusive.

A curious phenotype has been observed in male flies linking mating and immunity; McKean and Nunney (2001) demonstrated that mating males have a reduced capacity to clear injected *E. coli*. Carney (2007) showed that within minutes of mating male flies downregulate AMP expression, which could account for the observed difference in the realized immune response in these flies.

Alterations in circadian rhythm provide another explanation for this phenomenon (Fujii *et al.*, 2007). When male flies are placed in a circadian activity meter they show the standard circadian rhythm of activity during the day and rest at night; however, when they are housed with females they stay active almost continuously, resting only at dusk. Perhaps the sleep disruption resulting from this 24-h activity is enough to alter the fly's immune response.

The act of mating itself induces an immune response in females. This is due to the activity of a sex peptide injected along with the sperm (Peng *et al.*, 2005). This peptide can activate both the Imd and Toll signalling pathways and induces the transcription of AMPs. The purpose of this response is unknown but it is easy to speculate that it could be a prophylactic attempt to block sexual transmission of diseases. The cost of mating on the female in terms of lifespan reduction does not appear to involve changes in diet and is suspected to involve the activity of this sex peptide, perhaps by forcing the activation of the immune response. What is really puzzling is that female flies show an immediate activation of their immune response and this is driven by the male, whereas males flies turn down their own immune system following mating. What evolved for the goose did not evolve for the gander here and the logic behind this difference is unknown.

7.7 Ageing and immunity

The fly's innate immune response clearly has an effect on ageing rates; the constitutive induction of the Imd signalling pathway reduces the lifespan of male flies. Induction was initiated through the drug-induced over-expression of the receptor peptidoglycan-recognition protein long chain C (PGRP-LC) (Peng *et al.*, 2005). The effects of both the immune induction and ageing could be blocked by mutating genes downstream in the pathway. Ageing rates can be increased by reactive oxygen damage and thus it is possible that it is the reactive oxygen produced during the immune response that alters the ageing rate.

Ageing has several effects on the immune response. As microarrays became available, several groups rushed to determine the difference between young and old flies. Old flies tend to express more AMPs. One explanation for this increased level of AMPs comes from the work of Zerofsky and colleagues (2005). They found that young and old flies expressed similar levels of AMPs when they were initially induced; however, the old flies were slower at turning the AMPs off. Immune senesence in ageing may involve the alteration of pathways that can downregulate an immune response.

There are a variety of molecular signalling events required for the negative regulation of immune responses and these might be altered during ageing (Schneider, 2007). Alternatively, the flies may be less able to clear the infecting bacteria, which persist and continue to stimulate the host. There is some evidence that this might be the cause of immune senescence in these experiments as the injection of dead bacteria, which should act merely as immune elicitors, produces a smaller induction of AMPs in old flies than it does in young flies, the opposite result of what is seen with live bacteria.

Most of the connections between ageing and immunity have focused on this induction of AMPs in old flies and thus pursue the changes in AMP-dependent resistance that might result in ageing flies. One study, using a unique injection model, implicates the senescence of tolerance as an important change in ageing flies. Ramsden and colleagues (2008) injected flies with a rather large dose of *E. coli*, on the order of 500 000 bacteria per fly. This is approximately 100–500 times more than is typically used in *Drosophila* experiments. High doses like this are lethal, whereas when low doses of *E. coli* (1000 colony-forming units) are injected into the fly it is non-pathogenic in wild-type flies. The authors found that old flies were as effective as young flies at clearing these high doses of bacteria, which rules out resistance as something that is affected by ageing in this model; instead, these results suggest that tolerance suffers from ageing senescence.

It may be possible to link ageing to immunity and some of the other physiological systems discussed above by examining dietary restriction. Dietary restriction in flies, as in many animals, will reduce ageing rates. Evolutionary arguments about diet restriction suggest that it represents a method for animals to find a broader optimum for diet and fitness. Given large amounts of food animals

will produce a lot of offspring and die rapidly but when given lower amounts they will live longer and space out their offspring production. Dietary restriction functions in part through a reduction in insulin signalling although the effector mechanisms involved in this process remain a mystery.

Given that sick insects can become anorexic, we propose that this provides another regulatory feedback loop, this time between immune-induced changes in feeding and ageing. Anorexia induced by infections might be expected to shift flies into a diet-restriction state that may help broaden the optimum for survival and fecundity. Sick flies show reduced egg-laying but the life extension derived from reduced eating could possiblyrestore some fitness to these insects.

7.8 The contribution of native microbiota to immunity

Immunologists can give the impression that microbes are a continuous and terrible threat and we would be better off living germ-free. Perhaps this comes from work *in vitro* where microbes indeed make it difficult to maintain cultured cells. Living animals are different from cultured cells and exist in close association with their microbes. We engage in simply commensal relationships with some microbes and in other cases the relationship is mutually beneficial. In this section we discuss how the native microbiota of a fly might contribute to its immune response.

A simple way in which the native microbiota support the immune response of an insect is by supporting the normal physiology of the host. In cases where there are mutalistic microbes living within an insect the disruption of these microbes can be anticipated to cause physiological changes that would alter the immune response of the animal. For example, in a termite that depends upon its native microbiota for the digestion of cellulose, it might be expected that a gut infection that altered the number and diversity of bacteria could adversely affect both the amount and quality of available nutrients, and thus alter the immune response. Changes in nutrient availability and insulin signalling could be described as a way of monitoring the health of a host's native microbiota.

Native microbes also help protect a host against invading microbes. Early work done by Bakula (1969) in *Drosophila* showed that flies raised under axenic conditions could be forced to support the growth of *E. coli*. If the *E. coli* gnotobiotic flies were exposed to a normal gut flora in a food vial, the *E. coli* would be quickly replaced with the native flora. This suggests that the native flora can prevent the invasion of foreign bacteria, at least by occupying a niche, but perhaps by more active means. Perhaps this is why oral 'natural infection' models in the fly require that larvae be challenged with bacteria at an optical density of 50 for the rest of the larva's life to generate a phenotype. The insect gut is a resilient reactor that resists colonization by foreign microbes in part because it maintains a natural microbiota that excludes non-adapted competitors (Dillon and Dillon, 2004).

A mechanistic description of this sort of gut microbe effect was published recently by Ryu *et al.* (2008); they showed that flies carefully regulate the expression of AMPs in their gut and this in turn regulates the indigenous microbes. A simplistic explanation for the role of AMPs in the gut would be that they are present to sterilize the gut and to limit the possibility of infections. Ryu and colleagues found that when AMPs were misregulated and over-expressed it led to an alteration in the gut microbiota and that one particular *Gluconobacter* strain became numerically dominant and caused pathology. They proposed that the normal gut flora prevents the growth of this particular pathogen and the disruption of the native microbiota through AMP over-expression is the cause of pathogen overgrowth. It appears as though the flies were 'farming' their gut microbes, trying to create an optimal balance of bacteria to maintain health.

The native microbiota can be critical in defining the sensitivity to infections by blocking pathogens but native gut microbes can also be a cause of pathology. Broderick and colleagues (2006) showed that sensitivity of caterpillars to orally administered *Bacillus thuringiensis* toxin depended upon the presence of indigenous microbiota. Elimination of endogenous bacteria by antibiotic treatment eliminated sensitivity to the toxin. The reason appears to be that the toxin damages the gut, allowing the gut

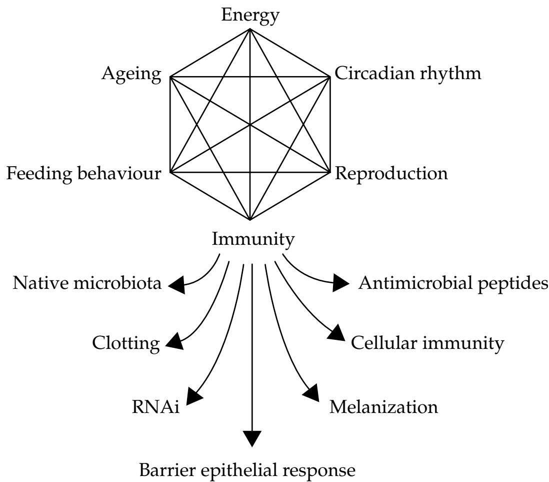

Figure 7.1 Summary of potential interactions between immunity, feeding behaviour, ageing, energy use, circadian rhythm, and reproduction in *D. melanogaster*. All potential interactions are listed. Below are listed the seven potential immune responses. These are all shown springing from immunity. Presumably, once the entire signalling matrix has been studied, it will be possible to distinguish the individual effects of, say, circadian rhythm on cellular immunity. There are not enough data yet to begin filling in this side of the figure.

bacteria to cause a fatal septicaemia. In this case, the native microbiota which are normally harmless become pathogenic when the insect is stressed by the toxin.

Ageing can be introduced into this story because Ren and colleagues (2007) demonstrated that as flies age they accumulate bacteria; they recorded as much as a 100 000-fold increase in the number of microbes. Surely this affects immunity; if these microbes act as a barrier to the introduction of pathogens then this aspect of the immune response will increase drastically as flies age. The immune response of aged flies suffering from a natural infection will reflect the sum of all of its defences: senescence of antibacterial responses may be offset by increases in protection from the native microbiota.

Native microbiota are something that simply hasn't been controlled in immunity experiments but we should start; first by learning what the native microbiota of our favorite insects are and then be figuring out how these alter infections.

7.9 Conclusions

The strength and quality of innate immune responses varies in response to the environment. In many cases, clear stories predict how these environment/physiology interactions will lead to adaptive changes in an insect's immune response. There are other situations where conditions appear to conspire against the insect and infections induce positive-feedback loops that crash the system. Figure 7.1 illustrates the potential for interactions between immunity and physiology.

To fully understand the immune response of insects we must record and control these environmental variables in our experiments; more than that, we need to understand how these environmental changes alter immunity because some of these environmental effects alter immunity to the same extent as knock-down of the central Toll and Imd signalling pathways.

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SECTION B

Immune interactions and evolution

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CHAPTER 8

The inherited microbiota of arthropods, and their importance in understanding resistance and immunity

Gregory D.D. Hurst and Alistair C. Darby

8.1 Introduction

In much of this volume, the focus has been on interactions between antagonists—viruses, bacteria, and parasitoids—whose presence is not welcome within the host, and where death of the host is often required for the propagation of the virus, bacterium, or parasitoid. However, the central importance of pathology in the life history of microparasites that interact with insects is by no means universal. For microbes that benefit from vertical transmission—that is, through the gamete to the next generation—the maintenance of a healthy female host is a pre-requisite for the survival of the microbe. In these cases, the microbes are termed symbionts to reflect that their whole existence is within the host. Symbiosis, in this context, does not judge whether the interaction is net beneficial or deleterious to the parties. Rather, it is a value-free term, in the sense of de Bary (1879), that reflects 'living together'.

Why should a book on insect infection and immunity carry a chapter on symbionts? There are two main reasons. First, recent work has indicated that some of these symbionts are themselves involved in defence of the host against pathogens and parasites. Whereas these bacteria are not part of the classical immune armoury as discussed elsewhere in this volume, recent studies have led to their emergence as important components of resistance to pathogens in aphids and elsewhere. As such, they will have important effects on the ecological and evolutionary dynamics of host–pathogen interactions. Second, because bacterial symbionts live within an insect with a 'fully loaded' immune system, they represent an interesting area of interaction that may be important in the design and evolution of insect immune systems. For inherited symbionts that are parasitic, they represent antagonists that the immune system is selected to detect and remove. However, for symbiotic bacteria that are beneficial (indeed, necessary) to the host, selection must act on the host to maintain a fully functional immune system while not killing off its partner. We will discuss how this occurs, and how possession of a necessary symbiont might affect the evolution of the immune system which runs alongside it. We will raise the hypothesis that immune-system evolution may be best understood not solely as a product of antagonistic coevolution with parasites, but is additionally subject by the need to accommodate a changing array of beneficial partners. In this chapter, we first review briefly the diversity of insect–symbiont interactions, before examining these two topics in turn.

8.2 The natural history of the interaction between bacterial symbionts and their hosts

Bacteria were first recognized as being commonly found as symbionts of insects from morphological

and microscopy studies. A seminal synthesis was made by Anton Buchner, whose treatise on *Endosymbioses of Animals with Plant Microorganisms* detailed the presence of organs of host origin within the bodies of many insects that carry bacteria in large numbers, reviewed the means by which these bacteria were transmitted to the next generation, and made some comments on their function (Buchner, 1965). The examples given in Buchner's text are mainly of bacteria that are integrated into the anatomy and function of insects. Two different modes of transmission were observed. In transovariol transmission, the bacteria were generally internalized within the insect, and passed from a female into her egg at the point of fertilization. In transovum transmission, material containing the bacteria were commonly present in gut diverticulae, and were physically placed onto the egg or in the vicinity of larvae, and then ingested by the larvae on hatching.

Aside from Buchner's work, the discovery of symbionts followed from two different lines of research. First, microscopy work found evidence of bacteria inside particular cells, rather than organs carrying bacteria. The inherited bacterium *Wolbachia* was first described in this way (Hertig and Wolbach, 1924; Hertig, 1936). This strand of discovery became particularly fruitful with the advent of electron microscopy, which revealed the presence of intracellular bacteria in many species. *Rickettsia* bacteria were likewise first described in this manner.

Aside from microscopy, inherited bacteria were also discovered from observation of their phenotype, in particular maternally inherited sex-ratio biases and incompatibilities. Sporadic records of insect lines producing all-female broods were recorded in butterflies, woodlice, ladybirds, and fruit flies over the period from 1920 to 1950, where the trait was inherited down the female line (Simmonds, 1928; Vandel, 1941; Lus, 1947; Magni, 1952). Following the discovery of male-killing in the *Drosophila willistoni* group of flies (Malogolowkin, 1958), experimental work then established that the agents were infectious through microinjection rather than an established component of the fly genome (Malogolowkin *et al.*, 1961), and the combination of antibiotic sensitivity of the trait with the inability of the agent to pass through filters of 450 nm gauge indicated that the causal agents were bacterial.

Aside from sex-ratio distortion, incompatibility between lines of particular insects from different populations was also ascribed to maternally inherited elements. In the work of Laven (1951), different mosquito lines were incompatible, but this incompatibility was associated with maternal line and independent of nuclear background. Microscopy and sensitivity of the trait to antibiotics demonstrated associations with maternally inherited bacteria (Yen and Barr, 1971). Inherited bacteria causing incompatibility have since been observed in many species.

8.2.1 Which microbes are symbionts?

The discovery of symbionts through microscopy and phenotype rather than through culture, reflects the fastidious nature of the symbionts. The co-adaptation of symbiont to host environment has led to these bacteria commonly being hard to establish in culture (although there have been several notable successes, and cell culture has also proven useful). The first robust taxonomic insights into the diversity of bacteria that have evolved into insect symbionts followed the advent of PCR amplification of 16S rDNA, and its sequencing. This development also allowed the creation of PCR assays that permitted survey for the presence of candidate bacteria in a wide range of insects. Bacteria from many clades have been demonstrated to have evolved symbiosis with insects, and in many cases are inherited symbionts.

Within the proteobacteria, many members of the Enterobacteriacae (gamma proteobacteria) have independently evolved into symbiotic interactions with their host, presumably evolving from a gut association into one that existed within the body of the insect. One particular gamma proteobacterium, *Arsenophonus*, is known to be widespread, being found in about 5% of all insect species (Duron *et al.*, 2008a), and there are implications (from repeated individual records) that others such as *Sodalis* may also be widespread (Fukatsu *et al.*, 2007; Novakova and Hypsa, 2007). Within the alpha proteobacteria, members of the genera *Rickettsia* and *Wolbachia* are

obligate symbionts of arthropods. *Rickettsia* often combine horizontal transmission via an arthropod vector with maternal transmission to host progeny, but many are now thought to be dedicated arthropod symbionts (Perlman *et al.*, 2006). The population biology of *Wolbachia*, in contrast, is strongly based on maternal transmission, with occasional horizontal transfer events creating new infected lineages or species. Both *Wolbachia* and *Rickettsia* exhibit a number of so-called reproductive parasite phenotypes, manipulating sex ratio, and in the case of *Wolbachia* also induce incompatibility. Other alpha proteobacteria members have also entered into symbiosis with insects, albeit a more narrow subset. Ants, for instance, have recently been observed to carry vertically transmitted *Bartonella* relatives (Stoll *et al.*, 2007).

Outside of the proteobacteria, there are two clades commonly found in a range of arthropods: the spiroplasmas and members of the genus *Cardinium*. *Cardinium hertigii*, a member of the Bacteroidetes-Flavobacteria-Cytophaga, was found in 5–7% of arthropod species sampled (Weeks *et al.*, 2003; Zchori-Fein and Perlman, 2004), and has a population biology akin to *Wolbachia*, dominated by maternal transmission with occasional horizontal transmission between species. Like *Wolbachia*, it exhibits a variety of parasitic manipulation phenotypes (Weeks *et al.*, 2001; Zchori-Fein *et al.*, 2001; Hunter *et al.*, 2003).

Members of the genus *Spiroplasma* represent common associates of arthropods. Derived from the mollicutes (mycoplasma relatives), these are arthropod specialists similar to *Rickettsia* in population biology. Some strains are propagated only by vertical transmission; others combine vertical and horizontal transfer, with some potentially being horizontally transmitted only (and causing insect disease) (Ammar and Hougenhout, 2006). Where *Spiroplasma* differ from *Rickettsia* is their locale in the host: whereas *Rickettsia* are almost completely confined to the intracellular milieu, spiroplasmas commonly additionally live outside of cells, free in the haemolymph.

A clade of bacteria that are emerging as partners of insects are the actinomycetes. These bacteria are often found on the exterior of insects in specialized glands. They are commonly placed into the local environment of the insect, where they act in a defence role against fungi that degrade either scavenged food provided for the insects' young (Kaltenpoth *et al.*, 2005) or mutualistic fungal gardens provided likewise (Currie *et al.*, 1999, 2003; Scott *et al.*, 2008). They are commonly observed to show maternal inheritance. Although this is often contact-based, without internalization of the bacteria, transmission through feeding in the larval stage is suggested in one case (Kaltenpoth *et al.*, 2005).

There are a range of other bacteria that are important in particular host groups. *Chlamydia* relatives have been found in symbiosis with the plant-sucking bug *Bemisia tabaci* and scale insects (Thao *et al.*, 2003). Cockroaches, termites, and many scale insects require the presence of a Flavobacterium, specifically *Blattabacterium* in the case of cockroaches and termites (Bandi *et al.*, 1995). Mealybugs carry a member of the beta division of proteobacteria, *Tremblaya* (Baumann and Baumann, 2005).

Aside from bacteria, eukaryotes of the phylum Microspora are commonly associated within insects. Whereas many microsporidia are oral pathogens that invade through the gut wall, propagate intracellularly, and kill their host, others are obligately vertically transmitted and have very weak (if any) pathology, or combine vertical and horizontal transmission with the timing of pathogenesis and horizontal transmission strictly controlled (Terry *et al.*, 2004). All members of the Microspora appear able to co-exist over significant periods with their host individual. Yeasts are also found in obligate interactions with insects, again with vertical transmission (Noda and Kodama, 1996).

8.2.2 Many insect species exist in symbioses with bacteria and microsporidia

Where there are discrete clades of bacteria and protists that live symbiotically with insects, screens of a wide range of species have been conducted to investigate how many species carry these bacteria. *Wolbachia* has been estimated as infecting between 16 and 70% of arthropods, depending on assay method, intensity of sampling, and method of numerical analysis (Werren and Windsor, 2000; Duron *et al.*, 2008a; Hilgenboecker *et al.*, 2008).

Cardinium, *Arsenophonus*, and *Spiroplasma ixodetis* relatives are each found in between 5 and 10% of all arthropod species (Duron *et al.*, 2008a). All except *Arsenophonus* appear to be more common in spiders and mites than in insects, with over half of spider species carrying inherited symbionts (Goodacre *et al.*, 2006; Duron *et al.*, 2008a).

8.2.3 What maintains inherited symbionts within their host?

Inherited symbionts have been traditionally classified into those required for host function (primary symbionts), and those where the host is competent to survive and reproduce without them (secondary symbionts). Primary symbionts are generally defined as being present in all individuals, combined with a strongly deleterious impact following treatment of the host with antibiotics.

Primary symbionts exist in a wide variety of taxa, but are particularly important in host species that live on nutritionally depauperate diets: phloem or xylem feeders; those feeding on wood; and those feeding throughout their life on vertebrate blood (Moran and Baumann, 2000). Within these taxa, the symbionts variously synthesize essential amino acids where the host diet lacks particular elements, and they are thought to be involved with the supply of B vitamins, and also in nitrogen metabolism. They are often co-adapted into host physiology, being present in large numbers in a special organ within the host (bacteriome for bacteria; mycetome for yeasts). They also commonly have very long associations with particular groups. Concordance of the phylogeny of aphid host and *Buchnera* symbiont (co-cladogenesis) indicates that *Buchnera* has passed vertically through aphid lineages for 200 million years (Moran *et al.*, 1993).

Secondary symbionts, defined by their non-essential nature, are of more recent ancestry: they are rarely shared by pairs of related species. In contrast to primary symbionts, they all show at least occasional movement between host species. They vary in the quantity of intraspecific horizontal transmission. An appreciable number show significant levels of intraspecific horizontal transmission in addition to their maternal inheritance. Horizontal transmission may occur, either directly—for example, following sexual contact (Moran and Dunbar, 2006) or through honeydew (Darby and Douglas, 2003)—or indirectly through a secondary host such as a plant or vertebrate. Secondary symbionts may be found in a bacteriome if one is present for housing primary symbionts, but more commonly are found diffusely among tissues (Cheng and Aksoy, 1999; Moran *et al.*, 2005b).

These symbionts may be maintained by one or more of four factors. First, they may have sufficient horizontal (infectious) transmission to balance any inefficiency in vertical transmission. This transmission may be either direct (such as sexual transmission), or through an intermediary host such as a mammal (through blood feeding) or plant (through phloem feeding). Second, they may provide a direct fitness benefit to the host. This is covered extensively below. Third, they may manipulate host sex ratio towards the production of infected daughters: sex-ratio distorters. Finally, they reduce the fitness of uninfected females through making them incompatible with infected males (cytoplasmic incompatibility). These latter two classes are termed reproductive parasitisms, as they involve the manipulation of host reproduction by a maternally inherited symbiont that has no interest in the production or fitness of male hosts. A brief summary of the distribution of reproductive parasitic phenotypes for various inherited bacteria is given in Table 8.1.

8.3 Symbionts that increase insect resistance to invading pathogens and parasites

As reflected elsewhere in this volume, insects and other arthropods possess a formidable immune system, comprising both cellular and humoral responses, that responds to a wide array of pathogens and parasites. This response, which can cope with an array of opportunistic infections, is combined with specific resistance to particular pathogens, that may be encoded within this system or in other nuclear genes that interact with the particular parasite or pathogen.

Recently, it has been observed that variation in resistance to pathogens is sometimes not associated with genetic variation in nuclear genes. Rather,

Table 8.1 The taxonomy of bacteria known to show reproductive parasitism, with notes on phenotypes observed, and the host range within which these are observed. For sources, see main text.

Species	Male-killing	Feminization	Parthenogenesis induction	Cytoplasmic incompatibility	Notes
Wolbachia	Many arthropods	Isopoda, Lepidoptera, Hemiptera	Many haplodiploid arthropods	Many arthropods	One strains shows antiviral resistance.
Rickettsia	Coleoptera		Hymenoptera		Some strains show horizontal transmission through plant/animal.
Spiroplasma	Many arthropods				Many strains show horizontal transmission through plant/animal.
Cardinium		Acari	Acari, Hymenoptera	Acari, Hymenoptera	Some strains are likely not to be reproductive parasites.
Bacteroidetes (unnamed)	Coleoptera				Apparently uncommon
Arsenophonus nasoniae	Hymenoptera				Also has horizontal transmission; relatives in the genus Arsenophonusare likely to be secondary symbionts.

variation in resistance was maternally inherited, and associated with the presence/absence of secondary symbionts. Detailed study of the effects of secondary symbionts has been largely confined to the resistance of pea aphids, *Acrythosiphum pisum*, to a variety of pathogens. This has been studied both experimentally through comparison of the susceptibility to parasitism of an aphid clone differing in infection status, and through testing whether parasitism is necessary and sufficient to maintain infection in population cages. Five different secondary symbionts are known to occur in this species, alongside the primary symbiont *Buchnera*, but two of these—a *Rickettsia* and a member of the *S. ixodetis* clade—have not been subject to functional study with respect to resistance to natural enemies (Table 8.2).

Secondary symbionts were first established as a major component of aphid resistance to parasitoid wasps (Oliver *et al.*, 2003, 2005; Ferrari *et al.*, 2004). *Hamiltonella defensa* and *Serratia symbiotica* infection reduced the success of *Aphidius ervi* parasitism. The defence was not a deterrant to *A. ervi* oviposition, but defence against the wasp within a challenged aphid. In both cases, laboratory populations of aphids maintained these symbionts in the presence of the host's natural enemy, but the symbiont reduced in frequency in their absence. Following this work, a third secondary symbiont, *Regiella insecticola*, was observed to be associated with resistance to infection by the fungus *Pandora neoaphidis* (Scarborough *et al.*, 2005). The ability to resist fungi has also been observed in actinomycete symbionts of insects, albeit in this case outside of the insect in the environment the insect occupies (Currie *et al.*, 1999, 2003; Kaltenpoth *et al.*, 2005; Scott *et al.*, 2008).

The intense level of research on *Wolbachia* has also provided evidence that this bacterium can impact on resistance to pathogens. A strong positive effect of *Wolbachia* on resistance to RNA virus infection has been revealed recently. Presence of *Wolbachia* in *Drosophila melanogaster* protected against three RNA viruses—C virus, Nora virus, and Flock House virus—but not a DNA virus, insect iridescent virus 6 (Hedges *et al.*, 2008; Teixeira *et al.*, 2009). This result has several ramifications. First, it indicates that symbiont-mediated protection extends to viruses. Second, the protecting symbiont can be an existing reproductive parasite. In this case, the bacterium is the *w*Mel strain of *Wolbachia*, which can also induce weak cytoplasmic incompatibility. Third, resistance can be delivered to a variety of pathogens with similar biology.

Table 8.2 The symbiotic microflora of the pea aphid, *Acrythosiphum pisum*. All bacteria show maternal transmission. Synonyms given are those used in papers before taxonomic description. References to the data may be found in the corresponding text section. Note, whereas *Buchnera* is present in all populations and all individuals, secondary symbionts vary in frequency both geographically and temporally.

Symbiosis type	Bacterium: division	Species	Fitness effect	Horizontal transmission	Synonym
Primary	Gamma proteobacteria	*Buchnera aphidicola*	Provision of essential amino acids; essential for normal reproduction	No	
Secondary	Gamma proteobacteria	Candidatus *Hamiltonella defensa*	Increased parasitoid resistance	Common but variable; sex and oral/faecal	PABS, T type
	Gamma proteobacteria	Candidatus *Serratia symbiotica*	Increased parasitoid resistance	Common but variable; sex and oral/faecal	PASS, R type
	Gamma proteobacteria	Candidatus *Regiella insecticola*	Increased fungal resistance	Common but variable; sex and oral/faecal	PAUS, U type
	Alpha proteobacteria	*Rickettsia* sp.	Elevated temperature tolerance	Yes, rarely, mechanism unknown	PAR, S type
	Mollicutes	*Spiroplasma* sp.		Yes, rarely, mechanism unknown	

Perhaps most surprising is that defence against natural enemies goes beyond immunity. An inherited *Pseudomonas* symbiont encodes protection of the rove beetle *Pederea* against predation by spiders. The bacterium is responsible for the synthesis of the small molecule pederin, which is a potent toxin of spiders (though apparently does not harm *Pederea*) (Kellner and Dettner, 1996; Kellner, 2001, 2002; Piel *et al*., 2004).

Although records of increased resistance to natural enemies dominate, secondary symbionts may also sometimes negatively affect the chance of parasitization. *Sodalis*, a secondary symbiont residing in the gut epithelia, increases vector competence of its tsetse fly host, probably by altering the ability of trypanosome to establish in the midgut (Baker *et al*., 1990; Geiger *et al*., 2007). In *Drosophila simulans, Wolbachia* presence was associated with increased susceptibility to parasitoid infection (Fytrou *et al*., 2006). Infection with *Wolbachia* strain wVulC in the woodlouse *Armadillidium vulgare* is associated with lowered haemocyte density, and also increased titre of culturable bacteria (i.e. not *Wolbachia*) in the haemolymph, implying that infection was associated with immunosuppression (Braquart-Varnier *et al*., 2008). Reduced longevity associated with wVulC infection was also observed.

Notwithstanding these data, positive effects on resistance to natural enemies are probably of greatest importance. It is unlikely that secondary-symbiont-encoded resistance is limited to fungi, viruses, parasitoids, and predators. The resistance of secondary-symbiont-infected individuals to other natural enemies will be of interest. Resistance to entomopathogenic nematodes seems likely, given the ability of *Photorhabdus* to infect both nematodes and insects (and in effect be a secondary symbiont of nematodes), and interactions with other common natural enemies (such as microsporidia, and nucleopolyhedrosis viral infection) should also be investigated.

Perhaps the most fertile ground will be interaction with other bacterial infections. This is most likely to occur as a defence against infectiously transmitted pathogens rather than vertically transmitted symbionts, as in general vertically transmitted symbionts share a common 'desiderata' of mutual transmission. There are two reasons to believe it is likely that secondary symbionts will provide resistance to other bacteria. First, bacteria are well known for their ability to secrete a number of small antimicrobial molecules, such as colicins, to which they themselves are resistant (Cascales *et al*., 2007), and phage may also be more active in other bacterial hosts. The only requirement for these systems to play a role in secondary symbiosis is that these are induced in response to bacterial challenge of the host. Second, *Photorhabdus*

(which can be considered a secondary symbiont of nematodes) possesses an array of genes encoding defence against other bacteria, in this case to defend the corpse of its insect host against incursion from other microbes after it has killed it (Sharma *et al.*, 2002; Duchaud *et al.*, 2003).

Symbiont-mediated protection is a relatively recent discovery in host–parasite interactions. There are three clear lines of research for the future. First, how commonly is resistance to natural enemies mediated by symbionts? Second, how are the effects produced mechanistically, and are these exploitable? Third, what is the population and evolutionary ecology of these interactions?

8.3.1 How commonly is resistance to natural enemies symbiont-mediated?

As argued above, secondary symbionts are very common in arthropods. What is not known is what proportion of secondary symbionts are reproductive parasites, how many propagate through horizontal transmission combined with vertical transmission, how many produce a direct benefit, and, of these, what proportion produce resistance to pathogens and parasites.

In this context, it is particularly interesting to examine the 'common bacteria': *Wolbachia*, *Rickettsia*, *Spiroplasma*, *Cardinium*, and *Arsenophonus*. For *Wolbachia*, there are a number of cases where reproductive parasitism phenotypes have been sought, but have proved either weak or absent (e.g. Hoffmann *et al.*, 1996). Study of the frequency of these infections in natural populations, and comparison of infection frequency in male and female hosts, has indicated that reproductive parasitism is a poor explanation for infection presence in many cases (Duron *et al.*, 2008b).

Without reproductive parasitism, maintenance of the symbiont requires either horizontal transmission or a direct benefit to infection. Whereas secondary symbionts can produce direct benefits outside of immunity (for instance, increasing the spectrum of host plants that can be utilized or improving thermal tolerance; Montllor *et al.*, 2002; Tsuchida *et al.*, 2004; Dunbar *et al.*, 2007), it is quite likely that many secondary symbionts have unrecognized effects on insect resistance to pathogens and parasitoids. In some cases, there is circumstantial evidence of this from geographical association between infection frequency and frequency of parasitization, for instance in the case of *Arsenophonus* in psyllids (Hansen *et al.*, 2007).

8.3.2 How do symbionts produce the effects observed?

With the phenotypic influence of symbionts having been only recently discovered, it is inevitably the case that we can only speculate on the mechanistic basis of symbiont-induced protection. There are two basic possibilities. The first is that the effect is mediated through existing host systems. The second is that the effect is direct, and has no element of interaction with the host. The former hypothesis has been suggested for *Wolbachia*-induced resistance to viruses. Teixera *et al.* (2008) suggested that resistance might be mediated through *Wolbachia* actively interfering with pro-apoptotic pathways of the host, in order to produce their maintenance. This interference prevents viruses accessing these pathways during their life cycle, slowing their transmission within the host. One can imagine also that bacterial symbionts may affect host innate immunity: they may prime it, giving prophylaxis, or secrete molecules that downregulate it, which would be associated with increased susceptibility.

The alternative mechanism by which symbiont effects on resistance may function is a direct effect. Direct effects could come from secreted molecules that affect the invading species alone. A useful model may be the biology of the gamma proteobacterium *Photorhabdus luminescens*, which is a nematode-transmitted pathogen of insects. It inhabits nematode guts without pathology. When the nematode host invades an insect, *Photorhabdus* moves from nematode gut to insect haemocoel. In this latter context, the bacterium is a virulent pathogen, with a formidable array of secreted compounds that protect against the host innate immune system, and which cause active pathology to the host (for instance, through damage to the gut epithelia). *Photorhabdus* thus demonstrates context-dependent virulence. Change in host species (from nematode to insect) leads to radical change in bacterial behaviour (from commensal to pathogenic).

For a secondary symbiont bacterium, ingestion by a parasitoid may produce a similar change in bacterial behaviour towards virulence. For *Hamiltonella* and *Serratia*, *RTX* genes are notably present, as well as a variety of toxin genes associated with phage, such as a homologue of *Stx* (Shiga toxin), *cdtB* (cytolethal distending toxin), and YD-repeat containing open reading frames (allied to the *Toxin Complex* genes commonly found in entomopathogenic bacteria such as *Photorhabdus*), each of which are known to harm eukaryotic cells (Moran *et al.*, 2005a; Degnan and Moran, 2008). Whereas a role for these genes in symbiosis is possible, a role in pathology on exposure to parasitoids or other natural enemies is a very tempting hypothesis.

Aside from toxicity, the other feature required for this hypothesis is context-dependent behaviour. Cases where bacteria, including pathogens, alter behaviour in response to environmental cues are well known, and commonly encoded through two component systems (Hentschel *et al.*, 2000). These are sensory-response circuits operating through kinase genes whose activity varies with environmental conditions. Under appropriate conditions, they are activated and alter the phosphorylation state of their cognate protein. In many cases the cognate protein is a transcription factor, and the alteration of phosphorlyation effects a change in the genes that are expressed, appropriate to the environment. In other cases, the cognate protein is a protease or demethylase, whose activity is then altered, producing a change in bacterial behaviour. The involvement of the PhoP/PhoQ two-component systems in the switch to insect symbiosis is established for *Photorhabdus*, and represents a promising avenue of research for regulation of symbiont behaviour in general, especially in the gamma proteobacteria (Derzelle *et al.*, 2004).

8.3.3 Will the population ecology of secondary-symbiont-encoded resistance differ from that of nuclear-encoded resistance?

Although not well studied, there are good theoretical reasons to believe that resistance mediated by secondary symbionts may have a rather different dynamic from resistance genes that are encoded by nuclear loci. In terms of theory, both represent traits whose positive effect on fitness is dependent on the presence of natural enemies. However, two factors may make secondary-symbiont-encoded resistance more likely to decrease in frequency in the absence of selection: transmission inefficiency and a difference in the cost of resistance.

First, we can compare cost-free nuclear and cytoplasmically encoded resistance. Selection from pathogen threat will drive these genes up in frequency. For nuclear genes, a reduction in pathogen threat would make the resistance genes subject to random drift processes. In a large population, these will cause only small changes in frequency each generation, and an increase and decrease in frequency are equally probable. For a maternally inherited element, in contrast, there is likely to be progressive loss of infection over time through inefficient transmission. In order to be maintained in the absence of selection for its presence, each daughter of an infected female must inherit the infection. Whereas vertical transmission efficiency can be near perfect (e.g. Jiggins *et al.*, 2002), imperfect vertical transmission would mean progressive loss of the symbiont over time. Thus, when pathogen threat recedes, the expectation is that the infection will decline in frequency.

Aside from this, a cost of possessing the factor would render it deleterious in the absence of natural enemy threat, or when the threat was low. A cost can, of course, occur for both nuclear genes and for maternally inherited bacteria. However, there are perhaps reasons to believe that costs will be greater and more certain for secondary symbiont infections. First, these infections represent organisms that have a metabolic cost associated with their activity. This cost cannot be avoided by not expressing them: they must use ATP and nutrients to survive and to replicate. Second, as argued in the section below, they may also carry a cost associated with their interaction with the host innate immune system, or indeed other symbionts. Whereas all maternally inherited agents have common interest in the fitness of the female host, there may be unexpected interactions between bacteria. Secondary symbionts often cohabit with primary symbionts in the bacteriome, and co-infections with different symbionts can produce changes in density of the parties (Oliver *et al.*, 2006).

These ideas suggest that resistance encoded through maternally inherited agents will generally decline in frequency in the absence of the natural enemy more rapidly than nuclear-encoded elements. Rapid reduction in frequency in the absence of natural enemies is observed when infected and uninfected clones compete in experimental population cages (Oliver *et al.*, 2008), although fitness costs are not always evident in the absence of competition (Darby *et al.*, 2003). In the field, this flux is perhaps reflected in the profound geographical variation in secondary symbiont prevalence (Tsuchida *et al.*, 2002). The hypothesis is that, when unused, symbiont-encoded resistance is a trait that is lost.

This hypothesis awaits mathematical modelling, as well as empirical work to verify or refute the basic tenets (that resistance is more costly if symbiont-encoded), and to investigate its reality in the field. The view also needs to be extended to incorporate effects on the dynamics of the natural enemies against which the symbionts protect. The degree to which natural-enemy dynamics are driven by resistance, and by other factors external to resistance, will of course be important in this.

This field is in its infancy, and the above is a very simplified treatment of the population biology of the system. Secondary symbionts may provide resistance against a variety of enemies (e.g. *Wolbachia* in *D. melanogaster* provides resistance to more than one RNA virus). Further, they may evolve towards mutualism, as found for *Wolbachia* in *D. simulans* (Weeks *et al.*, 2007), or be counteracted by adaptation on the part of the natural enemy, which would alter the dynamics of the element.

8.3.4 The evolutionary ecology of secondary-symbiont-encoded resistance

Pathogens, parasites, and their hosts are involved in evolutionary arms races. The evolution of resistance on the part of the host is often followed by counter-adaptation on the part of the pathogen. To date, the hypothesis that natural enemies can counter-adapt to the resistance encoded by secondary symbionts has not been tested, although it is likely that they do. First experimental avenues would be to examine natural enemy populations for genetic variation in the ability to remain unaffected by secondary symbiont mediated resistance, and to conduct experimental evolution studies where the capacity of natural enemies to evolve when faced with symbiont mediated resistance is examined.

The above experiments would determine whether counter-adaptation in the natural enemy is possible. If it is, it is not clear whether or not the trajectory of coevolution will differ in interactions between natural enemies and nuclear- and symbiont-encoded resistance factors. The difference in population biology of secondary symbiont and nuclear resistance alleles outlined above suggest they may do; symbiont-encoded resistance is more likely to be lost in the short term, leaving less scope for counter-adaptation.

8.4 Symbiosis and immunity

When injected with *Escherichia coli*, the insect system of cellular and humoral immunity is upregulated, and the invading bacteria are killed through phagocytosis, nodulation, and the expression of antimicrobial peptides (AMPs). Removal of elements of this system (e.g. mutations that disrupt the cascade leading to AMPs) is accompanied by sepsis and death in response to challenge. As argued in Chapters 2 and 6 in this volume, the insect innate immune system is a formidable system for protection against natural enemies. Notwithstanding this, insects possess a complex flora of bacterial symbionts. Parasitic symbionts must either not induce cellular and humoral responses, or, if they do induce it, survive this induction. As close antagonists, they may co-evolve with host systems in arms races. Primary symbionts, and secondary symbionts that induce resistance to natural enemies or confer ecological adaptation to host plants, differ from this in that both host and bacteria have an interest in the persistence of the bacteria, and their transmission to the next generation. The systems of destruction that are normally induced by bacterial presence must either not be invoked by the bacteria, or not affect them significantly.

The explanation for symbiont survival in a hostile host has largely relied on the intracellular location of many of these agents. We first discuss why this may matter, and whether the view of the intracellular location is a fair one. We then discuss

whether the absence of response to intracellular bacteria is sufficient to make ignorable for the purpose of interaction with the immune system.

8.4.1 Is the intracellular location of inherited bacteria key in their ability to live in insects?

A pervasive (but possibly unwritten, and indeed potentially misleading) view in the field was that inherited symbionts do not interact with the insect immune system because of their intracellular location. Humoral immunity involves secreted peptides, which are unlikely to be active intracellularly. The cascade leading to their production likewise is induced by free bacteria, not bacteria inside cells. Cellular immunity likewise involves recognition of bacteria that are extracellular, followed by their subsequent ingestion and lysis. Infected host cells are not targeted in this process. This makes the intracellular milieu a potential safe haven for bacteria, and it is tempting to suggest that the intracellular environment being a place of safety has in fact the driven the evolution of maternal inheritance for many bacteria. Under this thesis, entry into cells and adopting the intracellular habitat evolved as a way of escaping immune system activity. This entry into cells led to maternal inheritance, as bacteria were then found inside egg cells, and this to the evolution of both reproductive parasitism phenotypes and also evolution towards active contribution to host function.

This caricature is plausible, but is it reflected in evidence? With regard to innate immunity, it has been noted that *Wolbachia*-infected *Drosophila* do not upregulate genes encoding AMPs. However, *Wolbachia* infection does not prevent induction in response to septic shock, implying that the bacteria are likely to be avoiding activation of the system, rather than actively downregulating it (Bourtzis *et al.*, 2000). In contrast to this, a study by Xi *et al.* (2008) in *Drosophila* S2 cell lines did establish genes associated with innate immunity as being upregulated in infected cells compared with uninfected ones. However, it was noted that the upregulated elements were not AMP genes (concordant with the whole-organism study), but those involved in the nuclear factor κB (NF-κB) signalling cascade leading to AMP induction. The inference from this data is not obvious, however, as these genes also have roles in non-immune contexts.

The humoral response is only one of the ways in which hosts disable or kill invading pathogens. Cellular responses including digestion of microbes in a phagolysosome, and also exposure to high levels of reactive oxygen species. Although humoral immunity may be ineffective, both of these mechanisms are potentially available to combat intracellular infections. With respect to the former, studies on *Legionella* and *Coxiella* have recently demonstrated the importance of secreted proteins carrying ankyrin-repeat motifs in manipulating the cellular environment, preventing fusion of endosomes with the vacuole in which the bacteria reside, and inhibiting endocytic maturation and the formation of a phagolysosome that would destroy the bacterium (Pan *et al.*, 2008). It is notable that *Wolbachia*, the most common intracellular symbiont of insects, has an expanded range of genes containing ankyrin-repeat domains (Wu *et al.*, 2004), and these represent an excellent area of focus with respect to how *Wolbachia* maintains itself inside cells.

The potential involvement of reactive oxygen species in defence against intracellular bacteria has been examined in *Wolbachia* infections in *Drosophila* S2 lines. Elevated levels of reactive oxygen species were observed, although it is unclear whether this was a host response to *Wolbachia*, or a result of *Wolbachia* respiration (Brennan *et al.*, 2008). Proteomic analysis demonstrated increased levels of the superoxide dismutase and peroxidase enzymes of host origin, and also dismutase enzymes of *Wolbachia* origin, that may represent 'coping mechanisms' on the parts of host and bacterium. The proteins of host origin were broadly reflected in upregulated genes in the transcriptomic study of Xi *et al.* (2008).

8.4.2 Many inherited bacteria can be found outside of cells in a hostile immune environment

The conjecture that intracellular lifestyle reduces the interaction with host innate immunity seems broadly fair. However, does this mean that inherited symbionts simply do not interact with the

immune system? It is, in fact, commonly observed that many symbionts have both intra- and extracellular phases (Moran *et al.*, 2005b), in some cases moving outside of cells during certain life-history phases. Other microbes that are maternally inherited symbionts, such as *Arsenophonus*, have an extracellular location (Huger *et al.*, 1985).

Movement of intracellular symbionts into the haemolymph during certain host life-history stages is observed commonly. Primary symbionts move from the bacteriome during various phases of the host life history. For instance, SZPE, a gamma proteobacterium and the required symbiont of weevils, is extensively found free in the haemocoel during nymphal maturation, alongside expression of *inv/spa* genes associated with entry into cells (Dale *et al.*, 2002). In lice, *Riesia* bacteria usually found intracellularly within bacteriomes are observed to undergo two extracellular migrations during their host's life history (Perotti *et al.*, 2007). Reproductive parasites such as *Wolbachia* can be observed on the exterior of ovarioles, migrating into forming oocytes through the germ-line stem cells following microinjection (Frydman *et al.*, 2006), and the male-killing Flavobacteria in ladybirds can be observed adjacent to the sheath surrounding the host ovary (Hurst *et al.*, 1999).

Other bacteria live both inside and outside cells. A dark field micrograph of *Drosophila* haemolymph can reveal thousands of *Spiroplasma poulsonii* free in the haemolymph. While spiroplasmas can be found in cells (notably, in the embryo), the haemolymph is likely the usual habitat for spiroplasmas outside the *S. ixodetis* clade. Secondary symbionts like *S. symbiotica* and *H. defensa* are found in the cells surrounding the bacteriome, but are also found widely in other host tissues, and are found free in the haemolymph (Moran *et al.*, 2005b). Indeed, this location may be a requirement for them to display their antiparasite defence capability. *Sodalis* is also found in diverse tissues, including the haemolymph, and has both extracellular and intracellular phases (Cheng and Aksoy, 1999).

Finally, some inherited bacteria are primarily extracellular. *Arsenophonus nasoniae*, which is largely transovarially transmitted, invades its host by its mouth each generation, enters through the gut wall, and then is transmitted again at oviposition alongside the egg (although not in it). It can be transmitted horizontally on superparasitism of a host fly pupa by more than one wasp individual (Skinner, 1985), and this represents an intermediate between infectious transmission and vertically transmitted symbiosis. This transmission process is also mirrored in some *Wolbachia* strains, which can cross between host individuals following co-infection of a host by two parasitoid individuals.

These observations indicate that many symbionts are found outside cells at some point in their life history, and that this will lead to them being exposed to interaction with the host immune system. For parasites, avoidance of immune system activity must be a property of the bacterium. For beneficial symbionts, which the host has an interest in maintaining, it may be a property of the bacterium, the host, or both.

8.4.3 Bacterial mechanism of immune avoidance

There are two types of solution to the 'problem' of the host immune system. The first is to limit the time spent outside of cells. The second is to evolve mechanisms that either stop induction of host immunity, or be insensitive to the host systems.

The former—limited time in the haemolymph—appears to be the strategy adopted by *Riesia*, the primary symbiont of lice. Although it would not be advantageous for a host to attack a beneficial symbiont, it does appear to occur in this case. Observations by Perotti and coworkers of the migration of *Riesia* out of the bacteriome into the ovary record the bacterium being 'chased by hemocytes' (Perotti *et al.*, 2007). As the bacteria are released from the cells of the stomach disk, and migrate to the ovariole, they are pursued by haemocytes which attempt to engulf them. The bacteria form pores in the tunica surrounding the ovariole, each pore being then covered by a haemocyte. Perotti *et al.* note that the host is morphologically adapted to the bacterium—including surface properties of the tunica that aid adhesion and entry—but very poorly adapted in terms of immunological co-operation. *Riesia* survives by possessing a surprising turn of speed.

SZPE, the primary symbiont of *Sitophilus* weevils, also appear to survive through limited time spent outside cells. As previously noted, SZPE moves

from the bacteriome to the haemocoel during the nymphal phase, from where it invades the ovary. Experiments have demonstrated that SZPE injected into the haemocoel is recognized as an invader that does upregulate the host immune cascades (Anselme *et al.*, 2008). Thus, despite SZPE being a required symbiont, the haemocoel is a hostile environment to it during its passage through it.

Other bacteria—notably secondary symbionts such as *Arsenophonus*, *Spiroplasma*, *Serratia*, *Sodalis*, and *Hamiltonella*—spend considerable periods of time outside cells, and thus must either not induce host responses or be insensitive to the systems. This has been examined in the case of *S. poulsonii* infecting *Drosophila in vivo*, and for the case of *Sodalis* in tsetse flies, *in vivo* and *in vitro*.

For the *S. poulsonii*–Drosophila interaction, no induction of AMPs was observed in this system (Hurst *et al.*, 2003), and a lack of generalized immune activation was corroborated by microarray studies (G.D.D. Hurst, unpublished results). Ectopic immune activation did appear to reduce *Spiroplasma* titre in this system, implying that the organism did not induce host defences, but was susceptible. It is notable that spiroplasmas are like all mollicutes in possessing virtually no polysaccharide cell coat, and this may be a reason why they do not elicit a response.

For *Sodalis*, in contrast, titre is unaffected by ectopic immune activation. When the tsetse system of humoral immunity was upregulated through feeding with pathogens, there was no observable reduction in the titre of *Sodalis* (Rio *et al.*, 2006). *Sodalis* exists intracellularly (in the gut epithelia) and also free in the haemolymph. Experiments indicate that *Sodalis in vitro* is not strongly affected by tsetse AMPs (Hao *et al.*, 2001; Hu and Aksoy, 2005). Indeed, tsetse flies constitutively express a homologue of *diptericin*, which has been suggested to be a result of exposure to symbionts throughout the host life history (Hao *et al.*, 2001).

The 'lack of coat' explanation for failure of spiroplasmas to elicit an immune response is a conjecture. It is unlikely to be generally true of haemolymph-associated bacteria, as *Arsenophonus*, *Serratia*, *Hamiltonella*, and *Sodalis* are all gamma proteobacteria likely to carry significant cell walls, just as *Photorhabdus* does. Whereas alteration of their coat as a means of reducing the host response is possible, perhaps most likely is that they, like *Photorhabdus*, also have means of surviving when phagocytosed and of inhibiting phagocytosis, combined with a means of either downregulating AMP production or resisting the effects of AMPs. *Photorhabdus*, for instance, can induce apoptosis in haemocytes through the gene *mcf* (Daborn *et al.*, 2002), and secretes unidentified diffusible molecules that reduce phagocytosis (Au *et al.*, 2004), protecting the bacterium against cellular immunity.

The genomes of these bacteria do suggest some candidate molecules for interaction with the immune system. The genome of *A. nasoniae*, for instance, possesses a homologue of ecotin, within the operon of a type-three secretion system (making it highly likely to be a secreted peptide) (T. Wilkes, A.C. Darby, and G.D.D. Hurst, unpublished results). Ecotin encodes a protein belonging to the serine protease inhibitor class. Serine protease inhibitors (serpins) operate in many host systems as inhibitors of response cascades initialized by serine proteases. Ecotin has been demonstrated to be able to inhibit host processes that are initiated by protease activation, such as blood clotting (Castro *et al.*, 2006). Perhaps most interestingly, ecotin has been observed to affect the ability of neutrophils to neutralize ingested *E. coli*. *In vitro* studies indicate that elastase, a serine protease secreted into the phagolysosome by neutrophils, is inhibited by ecotin (Eggers *et al.*, 2004). Whereas a role for *Arsenophonus* ecotin as a general mechanism of resisting protease activity cannot be discounted (the bacterium does need to resist proteases encountered in gut transit to enter the haemocoel, and probably also needs to be able to inhibit its own arsenal of secreted proteases), a role for this gene in inhibiting elicited defence cascades is tempting, and worthy of investigation. The genome of *A. nasoniae* contains other genes whose homologues are known to function in the inhibition of phagocytosis, such as cytotoxic necrotizing factor 1 (T. Wilkes, A.C. Darby, and G.D.D. Hurst, unpublished results).

8.4.4 Host mechanisms of immune avoidance

The above describes work, and speculation, about bacterial mechanisms of surviving host

anti-microbial defences. For symbionts that are beneficial, the host also carries an interest in the maintenance of the symbiont. While not seen in some cases (e.g. *Riesia* is not ignored by the host systems when extracellular), there is some evidence of it in others, notably the SZPE–weevil interaction. As discussed above, this bacterium moves from bacteriome into the haemolymph during nymphal maturation. In this system, bacteriome tissue expresses a peptidoglycan-recognition protein long chain B (PGRP-LB) homologue. PGRP-LB homologues in *Drosophila* have the ability to cleave peptidoglycan, reducing exposure to the bacterial elicitors of immune cascades that would potentially cause harm to primary symbionts. Interestingly, this gene is particularly strongly expressed at the time when bacterial release occurs (Anselme *et al.*, 2006). An interpretation of this is that the host is actively upregulating an enzyme that removes elicitors of humoral immunity, and acting prophylatically to prevent immune response from prior infections from killing off the beneficial symbiont. A prediction of this hypothesis is that the weevil will be more prone to opportunistic pathogens in this phase.

8.4.5 Antimicrobial activity and culturing of symbionts

Recent work on the SZPE–*Sitophilus* symbiosis has demonstrated that bacteriome tissue, aside from expressing PGRP-LB homologues that may help in downregulating AMP production in the haemolymph, also constitutively expresses an AMP of the coleoptericin family. The function of this is not clear: it may be to avoid 'contamination' of the bacteriome with other bacteria or to keep SZPE number in check (Anselme *et al.*, 2008).

8.5 Conclusion: inherited symbionts and the evolution of host immune systems

The previous two sections argue first that symbionts can actively contribute to resistance to natural enemies, and second that many of them will also interact with the immune system of the host. What are the consequences of these for immune system evolution? With respect to the former, the resistance functions provided by symbionts may alter selection pressures on the components of host immunity that they complement. If a dominant parasitoid natural enemy is resisted by symbiont infection, this may alter the pattern of selection on the host's own surveillance and response mechanisms. The requirement to maintain standing prophenoloxidase- and haemocyte-based defences may decline. Thus, the conjecture is that symbiont-induced natural enemy resistance is likely to be accompanied by changes to the host's input to standing defences. This may have ramifications for the success of parasites and pathogens of the host to which the symbiont does not provide protection. The extent to which this occurs will depend on the extent to which symbiont-mediated protection is independent of hosts, or driven through them. If it is a case of the symbionts potentiating existing systems, then selection for the removal of the host standing components will be weak or non-existent. If the symbiont effect is direct, however, host standing systems become redundant, and potentially subject to weakened selection for their maintenance.

The second issue raised in this chapter was the interaction between symbionts and the immune system of the host. The observation that symbionts can interact with host immune systems indicates we should ask whether symbionts have produced selection for immune systems that accommodate them? It is notable that the insect immune system, despite being classically considered 'generalist' in its action, still shows some evidence of being fast-evolving, both in terms of the strength of positive selection and the turnover of elements within the systems. The signature of positive selection is particularly strong in the signalling components of the humoral immune cascade (Begun and Whitley, 2000; Jiggins and Kim, 2007), although interestingly not in the receptors or the effectors (Jiggins and Hurst, 2003; Lazzaro and Clark, 2003), and is strong also in receptors associated with phagocytosis (Lazzaro, 2005).

Turnover of the genes comprising immunity occurs in subtly different compartments. It is particularly pronounced in the complement of AMPs, which, despite showing little evidence of positive selection, vary in constitution from species

group to species group (Sackton *et al*., 2007). This observation implies that new AMP molecules are being recruited to kill bacteria and fungal enemies over evolutionary time, and, as importantly, other AMP molecules are being lost. The complement of receptors for phagocytosis similarly shows variation between species.

This signature of rapid evolution is not typical of a generalist system. Rather, it implies that the immune system faces common parties with which it interacts, as well as providing a generalist system of defence. The most common view of immunity has been one of a system driven by antagonistic co-evolution. Lazzaro (2008) reflects this, stating 'Natural selection may act strongly on immune systems as hosts adapt to novel, diverse, and coevolving pathogens'. Although this view is of course fair, and indeed this chapter would argue that parasitic symbionts with extracellular phases present some strong interacting partners that may comprise part of this process, a complementary view is that the evolution of these systems may be driven in part by the need to accommodate partners as much as from antagonistic interactions with parasites.

This thesis is made from the knowledge that these bacteria are common and strong interactors with insects, and that the bacteria that are present in these roles alter over time and between species. This creates the evolutionary scenario in which they can drive the evolution of these systems on a continuous basis. One can imagine the loss of an AMP being selected for by presence of a beneficial symbiont which is sensitive to it. Any subsequent loss of the symbiont (as natural enemy pressure changes) would produce selection for a replacement. Likewise, receptors that promote phagocytosis of beneficial bacteria may be lost, and replaced with other elements with a different spectrum of sensitivity.

One prediction of the thesis that immune systems evolve to accommodate the presence of beneficial symbionts is that a history of interaction with beneficial symbionts will alter sensitivity to pathogens. It is notable that many secondary symbionts occur within the gamma proteobacteria, and are closely allied to pathogens. How does any host accommodation or bacterial action that accompanies possession of a *Pseudomonas* strain by *Pederea* beetles affect susceptibility to related pathogens, and, likewise, *S. symbiotica* for aphids?

8.6 Acknowledgements

We would like to thank Tim Wilkes for reading the manuscript, and the NERC for funding our work on host–symbiont interactions.

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CHAPTER 9

Polydnaviruses as tools to deliver wasp virulence factors to impair lepidopteran host immunity

Sebastien Moreau, Elisabeth Huguet, and Jean-Michel Drezen

9.1 Introduction

The associations between several thousands of parasitic wasps and viruses (polydnaviruses or PDVs) represent a unique example where a complex virus machinery has been domesticated by one organism (the parasitic wasp) to manipulate the physiology of another (the parasitized lepidopteran larval host). The viral machinery produces particles used to transfer and express virulence genes encoding factors that impair host immunity. PDV particles, injected along with parasite eggs into the host body, manipulate host immune defences and development, thus enabling wasp larvae to survive and develop in a potentially harmful environment. Viral particle production occurs exclusively in specialized cells of the wasp's ovaries and PDVs are vertically transmitted. Two PDV genera have been described, Ichnovirus and Bracovirus, which are associated with thousands of wasp species from the subfamilies Campopleginae and Braconidae respectively.

In the first part of this chapter we will present the characteristics of PDVs and the origin of the genes they encode. We will then describe the general effect of PDVs on the insect immune system, and finally we will review functional analyses of PDV products.

9.2 General characteristics of PDVs

9.2.1 The unique nature of PDVs

Unlike classical pathogenic viruses, PDV particles do not replicate in the infected host tissues (Wyder *et al.*, 2003) where they act as potent regulators of lepidopteran host immunity. Experimental evidence for this role came from the initial observations by Vinson (1972) that fluids from the calyx, a tissue located between the ovarioles and the lateral oviducts of some parasitoid females, protected the parasitoid eggs from being encapsulated by host lepidopteran circulating immune cells (haemocytes). PDVs were later found in these fluids, and their life cycle investigated from their production by calyx cells to their uptake by host cells (Rotheram, 1973; Krell and Stoltz, 1980) (Figure 9.1 and Figure 9.2). In 1981, Edson *et al.* demonstrated that virus-free eggs of the ichneumonid parasitoid *Campoletis sonorensis* artificially injected into the haemocoel of their permissive host, *Heliothis virescens*, were always encapsulated (Edson *et al.*, 1981). In contrast, addition of calyx-fluid extracts or purified *C. sonorensis* ichnovirus (CsIV) to the injected eggs had a protective effect, reducing encapsulation rate by approximately 75%. The protection afforded to the injected eggs was lost if calyx fluid or purified viruses were previously irradiated with ultraviolet light, a treatment which alters DNA, indicating that PDVs were responsible for the impairment of the encapsulation process via the expression of encapsidated genes, that would thus act as virulence genes.

9.2.2 PDV genomics reveal signatures of mutualist and parasitic lifestyles

Since these early observations several potential virulence genes have been characterized through

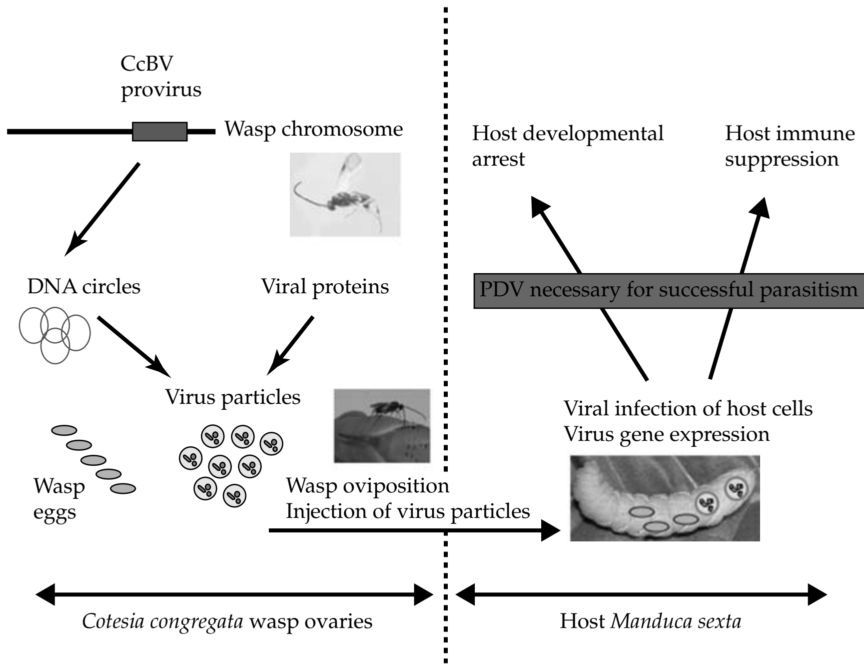

Figure 9.1 PDV life cycle. Life cycle of parasitoid wasps as examplified by *Cotesia congregata* and its interactions with the tobacco hornworm, *Manduca sexta*. Mature female wasps inject eggs (oviposition) bathed in calyx fluid containing PDV virions. The wasp eggs hatch and the larvae develop within the host, feeding on haemolymph components or host tissues. The wasp larvae emerge (emergence) from the parasitized insect, spin a cocoon, pupate, and emerge as adults to mate and search for new host insects. CcBV, *C. congregata* bracovirus.

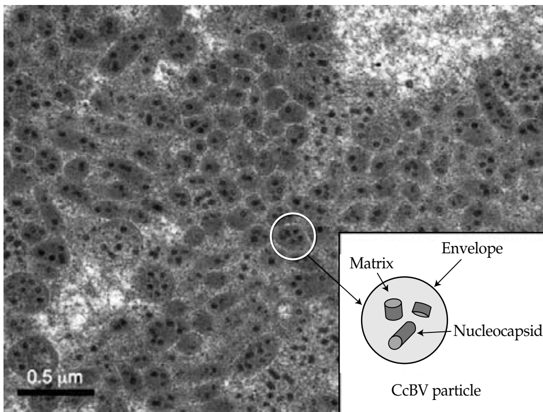

Figure 9.2 PDV particles observed under transmission electron microscopy in the nucleus of cells from the wasp *Cotesia congregata* replicating the bracovirus CcBV. Several rod-shaped nucleocapsids are present per particle, each probably containing a unique DNA circle as shown for *Chelonus inanitus* bracovirus (CiBV) (Albrecht *et al.*, 1994) and *Microplitis demolitor* bracovirus (MdBV) (Beck *et al.*, 2007). The nucleocapsids are embedded in a matrix and surrounded by an envelope.

sequencing projects, many of them in the past few years, allowing the acquisition of a considerable amount of data on the DNA contained in PDV particles injected into caterpillars by parasitoid wasps. The sequences of six PDV genomes have been published: *Cotesia congregata* bracovirus (CcBV) (Espagne *et al.*, 2004), *Microplitis demolitor* bracovirus (MdBV), CsIV (Webb *et al.*, 2006), *Glypta fumiferanae* ichnovirus (GfV) (Lapointe *et al.*, 2007), *Hyposoter fugitivus* ichnovirus IV (HfIV), and *Tranosema rostrale* ichnovirus (TnIV) (Tanaka *et al.*, 2007). Moreover, sequencing of the *Glyptapanteles indiensis* bracovirus (GiBV) genome is currently nearing completion (Desjardins *et al.*, 2007) and partial data have been obtained for several other viruses such as *Cotesia vestalis* bracovirus (CvBV) (ex- *Cotesia plutellae* bracovirus; Choi *et al.*, 2005) and *Chelonus inanitus* bracovirus (CiBV) (Annaheim and Lanzrein, 2007).

A characteristic feature of PDV genomes is the organization of their genes into gene families most

likely produced by duplications of segments of the viral genome and tandem duplications of an initial copy (Friedman and Hughes, 2006; C. Serbielle *et al.*, unpublished results). In CcBV, for example, almost half of the genes are organized in gene families (Dupuy *et al.*, 2006), including the protein tyrosine phosphatase (PTPs; 27 genes), cactus/inhibitory κB (IκB)-like (six genes), cystatin (three genes), and cysteine-motif (cysteine-rich protein (CRP); four genes) gene families. In addition, several bracovirus gene families encode conserved proteins that show no similarities to entries in the sequence databases. Few of these factors have been studied due to the difficulty of predicting and thus assessing their possible roles, but they are likely to represent new potential functions.

Strikingly, few PDV genes share significant similarities with genes from other viruses. The paucity of 'virus-like' genes may be explained by the fact that the virus does not replicate in the host tissues (Wyder *et al.*, 2003). Therefore the genes involved in the production of calyx-fluid virus particles are not required to be present on the DNA circles themselves, and might reside permanently in the wasp genome, as shown for a gene coding a structural protein of CsIV particles (Deng *et al.*, 2000). Recently, we have identified the viral machinery producing bracovirus particles and confirmed that none of the genes involved are encoded in the DNA of the particles (Bézier *et al.*, 2009). Thus most of the genes delivered by the particles appear to be exclusively devoted to the production of factors involved in the manipulation of lepidopteran physiology (Dupuy *et al.*, 2006).

9.2.3 Origin of PDVs

Important progress has been made recently towards the understanding of the origin of PDVs and their relationship with other viruses. Detailed phylogenetic studies have shown that bracovirus-associated wasps form a monophyletic group: the microgastroid complex (Whitfield, 2002). This finding led to the hypothesis that all current associations involving bracoviruses originate from a unique integration event of a viral genome as a provirus in a chromosome of the ancestral braconid wasp. The vertically transmitted viral DNA would then have been maintained because of its contribution to successful parasitism. Subsequently, viral DNA encapsidated in the PDV particles has presumably evolved differently depending on the wasp lineage, thus contributing to the diversification of the microgastroid complex, which now comprises at least 17 500 species (Whitfield, 2002). It has been estimated from fossils preserved in amber that the ancestral wasp lived during the Cretaceous period approximately 100 million years ago (Murphy *et al.*, 2008). Recently, the use of transcriptomic and proteomic approaches to identify viral structural genes expressed in wasp ovaries offered us the opportunity to reveal that the virus ancestor was most probably a nudivirus (nudiviruses constitute a sister group of baculoviruses) (Bézier *et al.*, 2009). Indeed, using these approaches we characterized the machinery producing CcBV and CiBV particles and showed that the genes involved are related to those of nudiviruses. Different subunits of the nudiviral RNA polymerase are expressed in wasp ovaries and about one-third of CiBV particle components have retained similarities with nudiviral proteins (B. Lanzrein, personal communication). The same approach did not allow the characterization of virus-related genes producing ichnovirus particles from *Hyposoter dydimator* ichnovirus (HdIV), indicating that the ancestor of ichnoviruses, if it ever existed, was not a nudivirus nor any other virus characterized to date. The picture has become even more complex with the recent characterization of viruses associated with wasps from the subfamily of Banchinae, which are proposed to form a third group. These viruses associated with ichneumonid wasps differ in morphology from ichnoviruses and contain PTPs, which so far have only been identified in bracoviruses (Lapointe *et al.*, 2007). These viruses are therefore likely to originate from a third ancestral event of wasp–virus association indicating that the domestication of viruses was selected several times during the evolution of parasitoid wasps. The association with viruses allowing gene transfer might have been selected repeatedly because it allows the production of a larger set of factors at a lower physiological cost for the wasp compared to the synthesis of proteins directly by the ovaries or the venom gland. This larger arsenal could also limit the selection of host resistance.

9.2.4 Gene content of the injected PDVs

Despite their independent origin all PDVs have common structural features, most probably because they have to evolve within the constraints imposed to maintain an effective association with the wasp, leading to successful parasitism. For example, all PDV genomes comprise large gene families: the existence of multiple variants of the same gene in PDVs may provide the means to interact with related signalling pathways in different tissues of the parasitized host. Moreover, the gene content of the particles appears to reflect the physiology of the host–parasitoid interaction rather than the type of virus captured originally. Indeed, no common genes are found within CiBV and other bracovirus genomes (Weber *et al.*, 2007), although they are produced by the same viral machinery and originate from the same ancestral wasp–virus association (Bézier *et al.*, 2008). The particular gene content of CiBV might be explained by the Cheloninae wasp life cycle. *C. inanitus* females oviposit into the eggs of the lepidopteran host (i.e. they are ovo-larval parasitoids). Although lepidopteran embryos have been shown to respond to parasitism by expressing a number of immune-related proteins (Abdel-Latief and Hilker, 2008), it is possible that *Chelonus* eggs and embryos are subject to only a limited response by the host cellular immune system. Therefore the genes maintained in CiBV particles are likely to be involved mainly in the control of host development. In contrast, bracoviruses from other wasps that oviposit directly into larval hosts are required to defend themselves against host cellular defences immediately. For this reason they have IκB and cysteine-motif genes in common with ichnoviruses (Dupuy *et al.*, 2006; Falabella *et al.*, 2007), as well as PTP genes in common with GfV (Lapointe *et al.*, 2007), suggesting that these factors have been selected by convergent evolution and may thus play a key role in the control of host immunity.

An interesting question concerns the origin of virulence genes and the way in which they have been acquired by PDVs. It was originally proposed that PDV virulence genes might originate from genes encoding venom products involved in parasitism success (Webb and Summers, 1990). Accordingly many potential virulence products are made up of a truncated form of a conserved eukaryotic protein (such as cactus/ IκB proteins) or a single protein domain (such as PTPs). However, surprisingly, they are not particularly close to insect proteins, most of them sharing less than 60% similarity with proteins from insects, birds, or mammals (Bézier *et al.*, 2008). The lack of a clear phylogenetic link between bracovirus and insect proteins may reflect the fact that bracovirus factors are evolving at a very fast rate due to their involvement in host–parasite interactions. In support of this interpretation, bracovirus PTPs and IκB-like proteins are less closely related among themselves than are the corresponding homologous proteins from different insect orders while *C. congregata* housekeeping genes are closely related to those of *Apis mellifera* and *Nasonia vitripennis*, indicating that the high divergence observed for IκB-like and PTPs sequences is not a general trend of the parasitoid wasp genes but a specific feature of bracovirus genes (Bézier *et al.*, 2008).

It is possible that some bracovirus virulence genes might have been present in the genome of the ancestral virus since viruses are known to pick up cellular genes that are beneficial for their life cycle in infected hosts (Herniou *et al.*, 2003). This might explain the high divergence rate of bracovirus genes, which, as genes from pathogenic viruses, are likely to have evolved rapidly and over a long period of time. An alternative hypothesis is that bracovirus virulence genes were acquired after the integration of the ancestor virus; originally residing in a non-viral region of the wasp genome, these genes were transferred to the proviral form at different times during the radiation of the microgastroid complex, leading to their incorporation into virus particles. In the case of bracovirus cystatins (Espagne *et al.*, 2005) and ichnovirus vinnexins (Turnbull and Webb, 2002) PDV genes lack the introns present in the cellular copies, suggesting these genes were acquired via integration of cDNA into the proviruses (Espagne *et al.*, 2005). Human long interspersed element retrotransposons have been shown to integrate transcribed DNA sequences in the genome. This results in genes which may fulfil new physiological functions (Esnault *et al.*, 2000). In the case of the aspartyl protease present in the *Toxoneuron nigriceps* bracovirus

(TnBV) genome, this gene has a clear retroviral origin: it was most probably acquired following the integration of a retroviral element in the bracovirus chromosomal form (Falabella *et al*., 2003). The fact that this gene is highly expressed in parasitized host haemocytes, fat body, and prothoracic glands suggests it has a physiological function.

Some data suggest the possibility that virulence genes may also have been acquired within lepidopteran hosts during the development of wasp larvae. Co-infection of hosts by wasps from different species does not result experimentally in detectable genetic exchange between their associated viruses (Stoltz *et al*., 1986). However, over large periods of time rare events of this type may occur, and additionally genes could also be acquired from other viruses not associated with parasitoids (Drezen *et al*., 2006; Bigot *et al*., 2008). In what appears to be an example of the latter type, bracoviruses from the *Cotesia* genus contain a copy of the baculovirus GP94 gene (Drezen *et al*., 2006) which is probably functional in CvBV. Surprisingly the bracovirus gene is phylogenetically most closely related to a particular lineage of lepidopteran baculoviruses (*Xestia-c nigrum* granulovirus) and not to hymenopteran baculoviruses, suggesting that the gene was acquired from a baculovirus of this lineage before the radiation of the *Cotesia* genus (Drezen *et al*., 2006). The bracovirus genomes of *Cotesia* species also contain a gene conserved in ascoviruses, a group of lepidopteran viruses (Drezen *et al*., 2006). These potential horizontal gene transfers could be explained by the intimate relationship between the parasitoid and the lepidopteran host, combined with the high concentration of virus particles during pathogenic virus infection. The penetration of virus particles in the wasp tissues might result fortuitously in the integration of a gene at the proviral locus in the wasp germline. The new gene could then be maintained if it provided a selective advantage to the parasite. A hypothetical gene transfer may also explain the intriguing similarities between IκB-like genes from different PDVs. Indeed, bracovirus and ichnovirus IκB-like proteins share a molecular signature, indicating that they have a common history (Falabella *et al*., 2007). Hyperparasitism involving an ichneumonid and a braconid wasp may have resulted in transfer of IκB genes from one PDV to another, which might explain their common features despite the different viral origins of the PDVs. The alternative hypothesis that they were obtained independently from an endogenous wasp gene having this signature is less likely since the molecular signature of PDV IκB-like proteins is not found in available sequences of cactus/IκBs from Hymenoptera (*A. mellifera*, *N. vitripennis*).

Thus PDV virulence factors most probably originated from multiple, different sources: viruses, mobile elements, wasps, and Lepidoptera. To enquire further into how these factors control host immunity it is necessary to describe first what is known of the immune responses of Lepidoptera and the overall effect of PDVs.

9.3 Effect of PDVs on host immunity

Unlike other species that act as predators, eating the host from the inside, wasps associated with PDVs are all koinobiont parasitoids: they develop together with the host and preserve host tissues. Thus they have to exert subtle effects without damaging the caterpillar's essential functions. Some factors target host immunity while others redirect resources to the haemolymph to feed the parasite or control the developmental programme of the host. We shall now focus on the PDV effects on host immunity.

9.3.1 Effect of PDVs on host haemocytes

Three main immune responses implicating haemocytes have been described in Lepidoptera. Phagocytosis corresponds to the engulfment of bacteria or yeast by individual cells, whereas nodulation and encapsulation involve haemocytes that will respectively, surround masses of bacteria or necrotic tissues, or form capsules around larger targets such as parasitoid eggs. Four circulating haemocyte types have been described in Lepidoptera and have been shown to be implicated in these cellular immune responses (Ribeiro and Brehelin, 2006). Plasmatocytes and granular cells are numerically the two main classes of haemocytes, accounting for 85–95% of all the haemocytes in a caterpillar (Loret

and Strand, 1998). In *Pseudoplusia includens* it has been shown that capsule formation requires that both granular cells and plasmatocytes are modified from being non-adhesive to highly adhesive cells that can adhere to the foreign target (Pech and Strand, 1996). In this process release of granulocyte inclusions allows the recruitment of plasmatocytes to form a multilayer of cells before a final layer of granulocytes completes the capsule (Pech and Strand, 1996; Gillespie *et al*., 1997). Granulocytes of this species also have strong phagocytic abilities, which enable clearance of microbial pathogens and dying cells from the haemolymph (Akai and Sato, 1973). Oenocytoids are large fragile cells that synthesize prophenoloxidase (proPO), a precursor of phenoloxidase (PO) (Essawy *et al*., 1985). The proenzyme is locally released in the plasma when these cells lyse (Ashida *et al*., 1988) and directly participates in the darkening of melanin and hardening of capsules and nodules (Marmaras *et al*., 1996). Finally, all lepidopteran species studied so far possess spherule cells, which are filled with crystal-like inclusions (Ribeiro *et al*., 1996). It has been suggested that these cells transport cuticular components (Locke *et al*., 1994) but their exact functions remain unknown.

PDVs can severely alter the function of host haemocytes (Figure 9.3). Depending on the virus and the hosts studied, the adhesion properties of plasmatocytes and granulocytes can be inhibited and their spreading at the surface of non-self material strongly reduced (Guzo and Stoltz, 1987; Strand and Noda, 1991; Luckhart and Webb, 1996; Beck and Strand, 2003). These effects were notably observed in the following host–parasitoid–PDV associations: *Malacosoma disstria*/*H. fugitivus*/HfIV (Stoltz and Guzo, 1986), *Pseudaletia separata*/*Microplitis mediator*/*M. mediator* bracovirus (MmBV) (Tanaka, 1987), *H. virescens*/*C. sonorensis*/CsIV (Davies and Vinson, 1988), *P. includens*/*M. demolitor*/MdBV (Strand *et al*., 1997; Strand and Clark, 1999), and *Spodoptera littoralis*/*C. inanitus*/CiBV (Stettler *et al*., 1998). Some PDVs can also trigger the apoptosis of granulocytes (Strand and Pech, 1995a), or target potential haematopoietic tissues (Strand and Pech, 1995b), thus quantitatively limiting the ability of the host's immune cells to encapsulate the parasitoid eggs (Figure 9.4). In *H. virescens*, CsIV induces a reduction in the number of circulating plasmatocytes, and the remaining haemocytes are altered in morphology and in spreading ability (Davies and Vinson, 1988; Webb and Luckhart, 1996). The phagocytic abilities of granular cells can also be altered upon injection of PDVs as in the case of the pierid *Pieris rapae* infected by *Cotesia rubecula* bracovirus (CrBV)

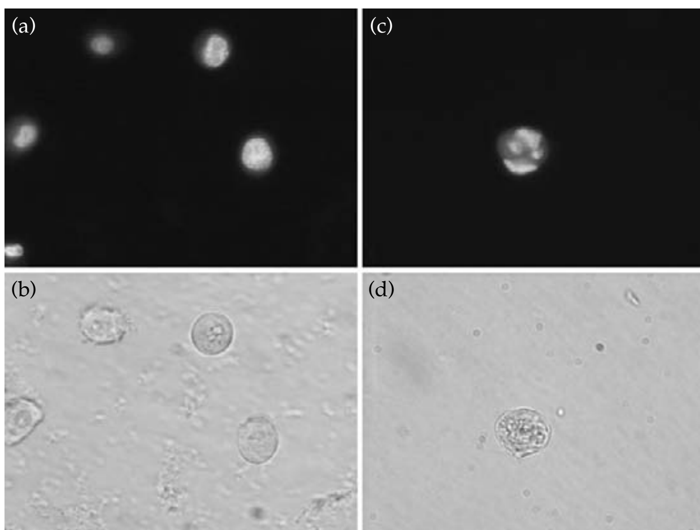

Figure 9.3 Comparison of haemocytes from *Manduca sexta* larvae parasitized by the wasp *Cotesia congregata* (24 h post-oviposition) and from unparasitized larvae. Cells were stained with neutral red for staining of the cytoplasm and Hoechst 33258 for nuclei staining. (a, b) Haemocytes from unparasitized larvae. (c, d) Haemocytes from a parasitized larvae showing typical nucleus fragmentation and condensation evocative of parasitism-induced apoptosis. This phenotype was also reported by Amaya *et al.* (2005).

(Asgari *et al.*, 1997). Recently, it has been suggested (Schmidt *et al.*, 2005) that these various virus-induced alterations in host haemocyte function could have in common actin-cytoskeleton breakdown and rearrangements (Tanaka, 1987; Li and Webb, 1994; Strand, 1994; Asgari *et al.*, 1997). Thus certain PDVs, probably in combination with other maternal factors, converge in their respective functions to disrupt one subcellular component of the haemocytes, the cytoskeleton.

The pathologies induced by MdBV on the haemocytes of two lepidopteran hosts, *P. includens* and *H. virescens*, were compared to those observed upon injection of bracovirus particles from *M. mediator* (MmBV) and *Microplitis croceipes* (McBV) (Kadash *et al.*, 2003). Interestingly the effects induced by the three PDVs differed. For instance, while MdBV induced in both hosts the apoptosis of granulocytes and prevented plasmatocyte adherence to foreign surfaces, MmBV caused a loss of adhesion in less than 50% of plasmatocytes and McBV had little impact on the haemocytes. However McBV was able to cross protect approximately 50% of *M. demolitor* or *M. mediator* eggs from encapsulation in *H. virescens*. This observation indicates that protection of parasitoid eggs from encapsulation does not necessary rely on the induction of detectable haemocyte pathologies, as has been observed in other models involving parasitoids devoid of PDVs (Asgari *et al.*, 2002; Moreau *et al.*, 2003). However, the protected eggs were not able to develop fully, indicating that host suitability for the development of a parasitoid not only results from the ability of PDVs to manipulate the host immune system but also depends on other aspects of host physiology.

9.3.2 Impact of PDVs on host humoral immunity

Activation of the PO cascade is necessary for the melanization of the capsule formed by host immune cells around a foreign body such as a parasite egg (for review see Marmaras *et al.*, 1996). This enzymic cascade constitutes one of the PDV targets (Stoltz and Cook, 1983; Lavine and Beckage, 1995; Strand and Pech, 1995b; Doucet and Cusson, 1996). For example, injection of CsIV in *H. virescens* larvae induces a significant reduction of the activities of several enzymes of the PO system (phenoloxidase, dopachrome tautomerase, DOPA decarboxylase) and a reduction in the concentrations of several important immune-related molecules: dihydroxyphenylalanine, *N*-acetyl dopamine, and precursors of reactive quinines (Shelby and Webb, 1999; Shelby *et al.*, 2000).

In addition, some PDVs have been shown to interfere with the synthesis of some antimicrobial compounds, such as cecropin and lysozyme (Shelby *et al.*, 1998), haemolymphatic phospholipase C (Shelby and Webb, 1999), attacin, lectins, or serine proteases (Gillespie *et al.*, 1997; Faye and Kanost, 1998). Whether the alteration of the expression of these compounds is directly advantageous to the parasitoid or constitutes a side effect of suppression of the host's immune response is not clearly established. It might be considered disadvantageous for the parasitoid to suppress the natural protections of its hosts against microbial agents, thus exposing the parasitized insect to infections that would kill the host before parasitoid development is completed. However, some well-characterized antimicrobial peptides, such as attacin, can also display antiparasitic properties against metazoan parasites of insects (Hu and Aksoy, 2005). The possibility that attacin or other antimicrobial peptides could have a toxic effect against parasitoid eggs and larvae would explain that the pathways controlling their production are targeted by PDVs. Parasitoids could counterbalance the suppression of their host's antimicrobial defences by producing their own antimicrobial substances. Such antibacterial and antifungal agents have been identified from adult and larval stages of parasitoids, associated or not with PDVs (Willers *et al.*, 1982; Führer and Willers, 1986; Dani *et al.*, 2003). In addition the temporal pattern of PDV gene expression could limit the duration of suppression of host immune responses.

9.3.3 Cumulative effects of PDVs and other virulence factors on host immunity

PDVs constitute a component of the parasitoid arsenal to overcome the host immune response

but other factors are also involved. The eggs of *C. rubecula* are deposited in caterpillars of *P. rapae* and are protected from encapsulation during their early embryogenesis by calyx-fluid glycoproteins (Asgari *et al.*, 1996). Among them, the ovarian protein Crp32 coats the eggs and the viral particles and provides protection during the short temporal window necessary to the expression of viral genes (Asgari *et al.*, 1998). Some venom proteins of *C. rubecula* were shown to inhibit melanin formation when added to host haemolymph (Asgari *et al.*, 2003a, 2003b) during the early phase of parasitization. A small venom peptide of 1.5 kDa is required for the expression of CrBV genes in host haemocytes (Zhang *et al.*, 2004). This peptide could facilitate virus chromatin restructuring, uncoating of genomic DNA, or expression of CrBV genes at the transcriptional level. This finding confirmed earlier reports which established that venom of *C. rubecula* was indirectly necessary for the protection of the parasitoid eggs from encapsulation (Kitano, 1982). Indeed, once expressed in host haemocytes, the PDV of *C. rubecula* induces profound modifications of cell-surface properties, actin cytoskeleton structure, and adhesion capacities of the immune cells within 6 h of their injection into hosts (Asgari *et al.*, 1996). In this system, a combination of factors thus provides a synergistic effect and ensures short-term and long-term protection to the developing parasitoid. In the closely related species *Cotesia kariyai*, venom is also required for successful parasitism (Wago and Tanaka, 1989) and combined injections of *C. kariyai* bracovirus (CkBV) and venom induced apoptosis of circulating haemocytes in the host *P. separata* (Teramoto and Tanaka, 2004). In the late stages of parasitism, the parasitoid larvae are protected by teratocytes, cells that derive from their extraembryonic serosa, and which do not divide inside the host's haemolymph but undergo a considerable increase in size. These cells release a yet-to-be-identified inhibitor of PO activity (Tanaka and Wago, 1990). In *Cotesia melanoscela*, the venom is required for the uncoating and persistence of viral DNA but not for viral entry into host cells (Stoltz *et al.*, 1988). In other braconid models, the venom is not strictly necessary for the function of bracoviruses but can increase their impact on host physiology. This is notably the case for the venom of *M. demolitor*, which amplifies the effects of MdBV on the immune system and the development of the host *P. includens* (Strand, 1994). In most ichneumonid wasps, PDVs and venoms appear to act independently (Stoltz and Guzo, 1986; Asgari, 2006).

In the ichneumonid wasp *Tranosema rostrale* an uncommon association between the PDVs and the egg chorion has been reported (Cusson *et al.*, 1998). The authors suggested that the delivery of the virus to specific host tissues could be enhanced by the fact that the virus lodges in the fine hair-like projections of the egg chorion. This example is evocative of some non-PDV-carrying parasitoids, in which structural properties of the egg chorion avoid encapsulation (Prevost *et al.*, 2005).

In the ichneumonid wasp *C. sonorensis*, venom, ovarian proteins, and PDVs co-operatively protect parasitoid eggs and larvae by disrupting the host encapsulation response and other aspects of innate immunity (Edson *et al.*, 1981; Li and Webb, 1994; Webb and Luckhart, 1994; Luckhart and Webb, 1996; Webb, 1998). During parasitization by *C. sonorensis* encapsulation is transiently inhibited by ovarian proteins, and in the longer term by PDVs. The ability of PDVs to establish a persistent infection of parasitized hosts determines the host range of this wasp (Cui *et al.*, 2000). Interestingly, Webb and Summers (1990) have revealed the existence of antigenic and sequence homologies between some proteins of the viral envelope of CsIV and venom proteins of *C. sonorensis*. According to the authors, expression of venom-related viral genes would enhance the survival of parasite eggs.

9.4 Functional analysis of PDV virulence genes

The characterization through genome sequencing of molecules encoded by PDVs gives insights into how they contribute to the parasite's success. The goal of current studies is to define more precisely the role of each gene product in this complex manipulation of host physiology. The fact that reverse genetics is not possible in these host–parasite systems has, however, made functional analysis of PDV genes difficult. Most functional approaches have therefore consisted of a combination of solid expression data (when applicable),

in vitro biochemical activity assays, *in vitro* bioassays, and finally transient expression assays in cell culture and more rarely *in vivo*. The use of RNA interference (RNAi) technology is still limited to cell-culture assays (albeit with one exception: Bonvin *et al.*, 2005) but is likely to develop and help us to characterize PDV gene function or function of PDV host targets *in vivo* in the future.

Another approach for testing the involvement of PDV-encoded factors in parasitism consists of studying their evolution by comparing viral genes in different species of a wasp genus to identify whether selection pressures promoting divergence of the sequences are operating on these genes, thus indicating their involvement in dynamic molecular interactions between hosts and parasites. Using this method we have been able to show that genes belonging to two families present in the CcBV genome (cystatin and PTP) have been under diversifying selection pressures, indicating they most probably play an important role in parasitism success (Serbielle *et al.*, 2008; C. Serbielle *et al.*, unpublished results). Similar results were obtained for cysteine-motif proteins in ichnoviruses (Dupas *et al.*, 2003). Certain PDV gene families encode proteins with characterized domains, which enable prediction of their biochemical activity and possible involvement in host physiology. In the cases of PDV cystatins, cysteine-motif proteins, and PTPs, for example, these groups of proteins are likely to target immune functions and/or development of the lepidopteran host.

We now present an overview of the PDV genes for which the functional evidence of involvement in host immune disruption is the most advanced (see Fig. 9.4. and Table 9.1).

9.4.1 PTPs

Gene families encoding PTPs are widespread in PDVs, having been identified in two bracovirus subfamilies (Microgastrinae and Cardiochilinae) and recently in a PDV associated with Banchinae wasps (Provost *et al.*, 2004; Webb *et al.*, 2006; Lapointe *et al.*, 2007). Furthermore, these genes have been subject to intensive expansion, leading to the largest gene families of PDV genomes (27 PTP genes in CcBV, 13 PTP genes in MdBV and TnBV). PTPs, together with protein tyrosine kinases, are known to play key roles in the control of signal transduction by controlling the levels of cellular protein phosphorylation (Andersen *et al.*, 2001). Each PTP dephosphorylates phosphotyrosine residues on a specific substrate. Bracovirus PTPs show considerable diversity in their amino acid sequences suggesting that each PTP has the potential to interact with a different specific substrate (Provost *et al.*, 2004). PDV PTPs are therefore likely to target signal transduction involved in multiple processes such as host immunity and development.

Mammalian immune-cell actin dynamics depends on the phosphorylation of proteins localized in focal adhesions. Certain mammalian bacterial pathogens have been shown to inhibit phagocytosis by injecting PTPs, which disrupt these actin rearrangements (DeVinney *et al.*, 2000). PDV PTPs were therefore also proposed to be able to disrupt signalling pathways controlling haemocyte cytoskeleton dynamics, thereby inhibiting encapsulation. In accordance with this prediction, certain bracovirus PTPs were found to be expressed in haemocytes and in certain cases bracovirus-infected host haemocytes showed more PTP activity than mock-infected controls (Provost *et al.*, 2004; Gundersen-Rindal and Pedroni, 2006; Ibrahim *et al.*, 2007; Pruijssers and Strand, 2007). Furthermore, transient expression of biochemically active MdBV PTP-H2 or PTP-H3 in *Drosophila* S2 cells led to a reduction of phagocytosis of *Escherichia coli* by these cells. This reduction was even more drastic if cells were co-transfected with PTP contructs and Glc1.8 (see below). Both PTP-H2 and PTP-H3 were shown by immunofluorescence to localize to focal adhesions in *Drosophila* S2 cells (Pruijssers and Strand, 2007). Taken together these results indicate that PTP-H2 and -H3 have antiphagocytic activity. Bracoviruses have been shown by *in vitro* biochemical assays to encode both catalytically active and inactive PTPs (Provost *et al.*, 2004; Pruijssers and Strand, 2007; Ibrahim and Kim, 2008). The inactive forms may have a different biochemical activity such as trapping phosphorylated tyrosine proteins. Transient expression in host (*Spodoptera exigua*) haemocytes of active CvBV PTP1 and inactive PTP5 resulted in reduced cell spreading and encapsulation of beads, suggesting that both PTPs are

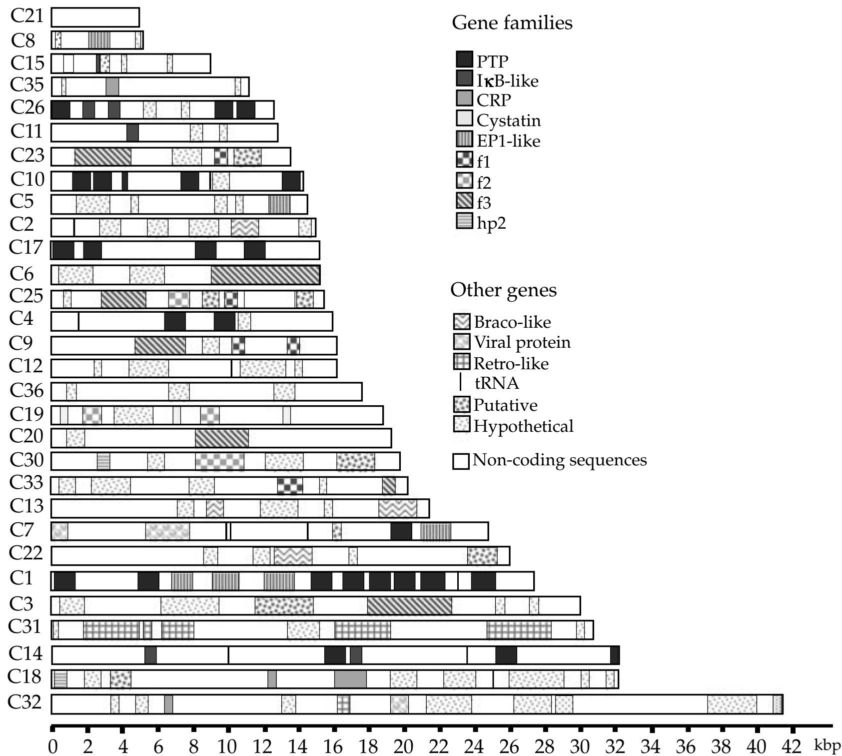

Figure 9.4 Gene content of the double-stranded DNA circles included in bracovirus particles: *C. congregata* bracovirus (CcBV) contains nine gene families, four of which encode proteins with well-known conserved domains (IκB-like, cysteine-motif (CRP), protein tyrosine phosphatase (PTP), cystatin), another four of which encode proteins of unknown function conserved in bracoviruses associated with wasps of the *Cotesia* genus (EP1-like, f1, f2, f3), and one of which is not present in the available sequences of other bracoviruses (hp2). Approximately 40% of the genes encode proteins showing no similarity to proteins in the databases (hypothetical) or having similarities with predicted proteins of different vertebrate or invertebrate species (putative). Some encoded proteins have similarities with products of mobile elements (retro-like), different viruses (viral protein), and unique proteins conserved in bracoviruses associated with wasps of the *Cotesia* genus (braco-like). Several lines of evidence indicate that at least some IκB-like and PTP gene products contribute to impairing host immunity.

involved in altering haemocyte behaviour, either by direct dephosphorylation of tyrosine residues or through competition with host PTPs (Ibrahim and Kim, 2008).

In certain host–parasitoid interactions haemocyte cell death and even apoptosis have been described (Schmidt *et al.*, 2001; Lavine and Strand, 2002). For instance, MdBV infection of *P. includens* and *Spodoptera frugiperda* leads to a large proportion of granulocytes (but not plasmatocytes) dying by apoptosis (Strand and Pech, 1995a; Suderman *et al.*, 2008). It has been recently shown that transient expression of MdBV PTP-H2 in the Sf21 cell line induces caspase-dependent apoptosis of these cells, in contrast to seven other MdBV genes and a PTP-H2 phosphatase-inactive mutant, all of which lacked apoptosis-inducing activity (Suderman *et al.*, 2008). If Sf21 cells were cultured under conditions in which apoptosis was inhibited, PTP-H2 was found both to inhibit the ability of the cells to engulf bacteria, and also to reduce proliferation. PTP-H2 has therefore been suggested to induce apoptosis by directly or indirectly perturbing the cell cycle. The MdBV PTP-H2 protein therefore appears to have different effects depending on target cells.

9.4.2 Mucin-like glycoprotein, Glc1.8

PTPs are not the only PDV proteins targeting haemocyte function. MdBV Glc1.8, a PDV gene that encodes a cell-surface mucin-like glycoprotein, has

Table 9.1 PDV genes for which the functional evidence of involvement in host immune disruption is the most advanced.

Protein	PDV and host	Predicted function	Observed physiological disruption	Functional data	Methodology	References
PTP	CcBV, *Manduca sexta*	Disruption of signalling pathways involved in hormone biosynthesis or haemocyte cytoskeleton dynamics		PTPA is a functional tyrosine phosphatase. PTPM is non-functional.	Activity of Sf21 cell lysates infected with recombinant baculovirus	Provost *et al.* (2004)
	CvBV, *Plutella xylostella*	As above	Loss of haemocyte adhesion Loss of phagocytosis	PTP1 expression in *Spodoptera exigua* haemocytes leads to increased PTP activity, reduction of cell spreading, and reduction of encapsulation.	*In vivo* transient expression in *S. exigua* haemocytes	Ibrahim and Kim (2008)
	MdBV, Noctuid moths	As above	As above MdBV infection of *S. frugiperda* induces apoptosis of granulocytes	MdBV-infected haemocytes have higher PTP activity. PTP-H2, -H3 are functional tyrosine phosphatases. PTP-H2 in combination with Glc1.8 reduces S2 cell phagocytosis. PTP-H2 expression in Sf21 cells induces apoptosis by caspase activation.	Parasitism of *Pseudoplusia includens* Bioassays in *Drosophila* S2 cell lines expressing PTP Protein expression in Sf21 cell lines	Pruijssers and Strand (2007) Suderman *et al.* (2008)
Glc1.8	MdBV Noctuid moths	Disruption of capsule-forming haemocytes	Loss of haemocyte adhesion Loss of phagocytosis	Loss of adhesion of Hi5 cell lines infected with MdBV RNAi using Glc1.8 restores adhesion. Expression of Glc1.8 causes loss of adhesion and reduced phagocytosis in Hi5 and S2 cell lines.	RNAi in MdBV-infected cell cultures Recombinant expression in cell cultures	Beck and Strand (2003) Beck and Strand (2005)
CrV1	CrBV, *Pieris rapae*		Inactivation of haemocytes Actin-filament disorganization	Injection of recombinant CrV1 in *P. rapae* modifies haemocyte spreading and disrupts actin filaments	Recombinant protein injection in natural host	Asgari *et al.* (1997)
CcV1	CcBV, *M. sexta*	Disruption of haemocyte cytoskeleton		CcV1 interacts with hemolin. CcBV1 inhibits hemolin binding to lipopolysaccharide. Hemolin-induced bacterial agglutination is abolished in the presence of CcBV1. *B. mori* haemocytes, S2 cells, and Sl2b cells show reduced phagocytosis ability in the presence of rec CcBV1. CcV1 and hemolin interact at the cell surface.	Yeast two-hybrid and co-immunoprecipitation *In vitro* assays using purified recombinant protein Immunofluorescence co-localization experiments	Labropoulou *et al.* (2008)

Table 9.1 *Cont.*

Protein	PDV and host	Predicted function	Observed physiological disruption	Functional data	Methodology	References
VHv1.1 Cys-motif	CsIV, *Heliothis virescens*	Disruption of immunity/ development	Translation inhibition during parasitism	*H. virescens* infection with VHv1.1-expressing baculovirus reduces encapsulation response to washed wasp eggs.	*In vivo* expression in the natural host using baculovirus	Li and Webb (1994)
				Injection of recombinant VHv1.1 increases susceptibility to baculovirus infection.	Recombinant protein injection in natural host	Fath-Goodin *et al.* (2006)
				Recombinant VHv1.1 and VHv1.4 inhibit translation of host RNA.	*In vitro* translation assays	Kim (2005)
Vinnexins	CsIV, *H. virescens*	Disruption of cellular immunity by altering gap junctions	Disruption of cellular immunity	cs-Vnxd and cs-Vnxg expression in *Xenopus laevis* oocytes form functional gap junctions.	Protein expression in *X. laevis*	Turnbull *et al.* (2005)
Lectin	CvBV, *P. xylostella*	Interruption of haemocyte recognition	Disruption of cellular immunity	Recombinant CvBV-lectin reduces bacterial attachment to haemocytes.	*In vitro* effect of recombinant protein on host haemocytes	Lee *et al.* (2008)
15b	CvBV, *P. xylostella*			Recombinant CvBV15b: induces impaired spreading of *P. xylostella* and *S. exigua* haemocytes and markedly reduces protein release from haemocytes.	*In vitro* effect of recombinant protein on host haemocytes	Nalini and Kim (2007)
H4 histone	CvBV, *P. xylostella*			Transfection of *P. xylostella* larvae with CvBVH4 recombinant expression vector induces loss of host haemocyte spreading ability.	*In vivo* transfection in natural host using eukaryote expression vector	Gad and Kim (2008)
TnBV1	TnBV, *H. virescens*		Cell death	Infection of Sf21 and Hi5 cell lines with recombinant TnBV1 baculovirus induces apoptosis-like programmed cell death	Recombinant baculovirus expression in insect cell lines	Lapointe *et al.* (2005)
EP1-like	CvBV, *P. xylostella*	Immunosuppressant		Transfection of *P. xylostella* larvae with ELP1 induces reduction in haemocyte numbers.	*In vivo* transfection of natural host	Kwon and Kim (2008)
Egf Smapin	MdBV, Noctuid moths	Inhibition of melanization	Suppression of melanization during parasitism	Recombinant Egf1.0 reduces *M. sexta* PO activity. Conditioned medium from Hi5 cells treated with double-stranded RNAi Egf1.0/1.5 lost antimelanization capacity.	*In vitro* assays using recombinant protein and *M. sexta* plasma	Beck and Strand (2007) Lu *et al.* (2008)
			PO cascade reduces MdBV and wasp survival.	Egf1.0 inhibits *M. sexta* PAP-3 and PAP-1 activity. Recombinant Egf1.0 blocks processing of pro-PAP1, pro-PAP3, proPO, and serine proteinase homologues 1 and 2.		

IκB-like	CsIV, *H. virescens*	Irreversible inhibitors of nuclear factor κB (NF–κB) transcription factors. Disruption of signalling pathways involving NF–κB transcription factors	Increased susceptibility in PDV-infected hosts	IκB nuclear localization post parasitism	Immunofluorescence assays on haemocytes and fat body	Kroemer and Webb (2005)
	MdBV, Noctuid moths *P. includens* and *Trichoplusia ni*	As above		IκB reduces expression of antimicrobial protein (AMP) reporter constructs. IκB binds to *Drosophila* Rel proteins. IκB inhibits Rel binding to κB sites in AMP promoters.	*In vitro* recombinant protein expression and reporter gene assays in *Drosophila* S2 cells Co-immunoprecipitation Electrophoresis mobility shift assays	Thoetkiattikul *et al.* (2005)
	TnBV, *H. virescens*	As above	In *H. virescens* parasitism affects NF-κB nuclear localization.	IκB reduces expression of tumour necrosis factor α (TNFα) reporter-gene constructs. Parasitism affects NF-κB-like protein nuclear localization.	*In vitro* recombinant protein expression and reporter gene assays in Hela cells Immunofluorescence	Falabella *et al.* (2007)
Cystatin	CcBV, *M. sexta*	Immune disruption and/or developmental arrest		Recombinant cystatin 1 is a functional C1A cysteine protease inhibitor *in vitro.* *Cotesia* spp. cystatins are subject to strong diversifying selection.	*In vitro* enzymic assays Molecular evolution models	Espagne *et al.* (2005) Serbielle *et al.* (2008)

been shown using RNAi to be involved in inhibition of cell adhesion. Infection of High Five (*Trichoplusia ni*) cells with MdBV resulted in loss of cell adhesion to culture plates but adhesion was restored when PDV-infected culture cells were treated with double-stranded RNA specifically targeted against Glc1.8 (Beck and Strand, 2003). Furthermore, transient expression of Glc1.8 in High Five cells reduced their ability to adhere to foreign surfaces and to phagocytose *E. coli* in a similar manner to that after MdBV infection, showing that Glc1.8 is an important viral factor involved in disruption of adhesion and phagocytosis in these cell types (Beck and Strand, 2005). Glc1.8 is composed of an extracellular domain with amino acid repeats arranged in tandem, and a C-terminal transmembrane domain. Transient expression of Glc1.8 mutants lacking the membrane anchor had no effect on cell adhesion or phagocytosis. Sequential deletion of the Glc1.8 repeats led to progressive reduction in adhesion blocking activity. Collectively the data indicate that membrane localization is essential for Glc1.8 function, and that PDV mucins form structures which may physically block adhesion by hindering ligand–receptor interactions (Beck and Strand, 2005).

9.4.3 Bracovirus CrV1 and CcV1 glycoproteins

The CrBV glycoprotein CrV1 and its homologue CcV1 from CcBV have also been implicated in altering haemocyte function. Asgari *et al.* (1997) showed that injection of a recombinant CrV1 protein into host *P. rapae* caterpillars caused haemocyte changes which were indistinguishable from those observed after PDV infection. CrV1 was in particular reported to alter actin distribution in haemocytes. Purified recombinant CrV1 was also shown to be endocytosed by host haemocytes after *in vitro* incubation (Asgari and Schmidt, 2002), suggesting that the protein may act intracellularly. Recently a yeast two-hybrid screen and co-immunoprecipitation experiments revealed that the CrV1 homologue, CcV1, interacts with hemolin (Labropoulou *et al.*, 2008). Hemolin is a member of the immunoglobulin superfamily and is considered in insects to play the role of a pattern-recognition molecule capable of binding lipopolysaccharide of Gram-negative bacteria. The interaction between CcV1 and hemolin was shown to interfere with the capacity of hemolin to bind to lipopolysaccharide and induce bacterial agglutination. Furthermore *Bombyx mori* haemocytes, *T. ni* High Five, *Drosophila melanogaster* S2, and *S. littoralis* Sl2b lines all showed dramatic decrease in their ability to phagocytose *E. coli* in the presence of purified CcV1 protein. CcV1 was also shown to be taken up by High Five cells and *B. mori* haemocytes; however; protein co-localization experiments showed that CcV1 and hemolin interact at the cell surface. Therefore, these PDV glycoproteins may affect haemocyte phagocytosis at the level of the cell surface through interaction with hemolin and at an intracellular level, by a mechanism which is not yet characterized.

9.4.4 Cysteine-motif proteins

Genes encoding cysteine-motifs have been identified in ichnoviruses and bracoviruses. Proteins are characterized by one or more cysteine-knot structural motifs (Dupuy *et al.*, 2006; Gill *et al.*, 2006) with conserved cysteine residues spaced with hypervariable residues (Dupas *et al.* 2003). *In vivo* expression of CsIV VHv1.1 protein in *H. virescens* using recombinant baculovirus showed that this protein is involved in reducing the encapsulation response to washed wasp eggs. The localization of the protein on the surface of plasmatocytes and within granulocytes suggests that the effect on encapsulation is mediated via surface receptors (Li and Webb, 1994). Furthermore, injection of recombinant VHv1.1 resulted in increased mortality of *H. virescens* larvae infected with baculoviruses providing further evidence for the role of cysteine-motif proteins in immunosuppressive activities (Fath-Goodin *et al.*, 2006). Interestingly, TSP14, which is a cysteine-motif protein derived from teratocytes, is associated with inhibition of insect growth and development in *H. virescens* parasitized by *M. croceipes* wasps (Rana *et al.*, 2002). Feeding caterpillars with recombinant VHv1.1 also had an impact on larval growth (Fath-Goodin *et al.*, 2006). Since cysteine-motif proteins have been shown to have the capacity to reduce *in vitro* translation of RNAs from different host tissues, it has been postulated that these proteins may

act by inhibiting translation, leading to disruption of immune responses and development (Kim, 2005; Fath-Goodin *et al.*, 2006).

9.4.5 Vinnexins

A gene family encoding proteins homologous to invertebrate gap junctions (innexins) has been described in CsIV and HdIV and represents an excellent candidate to be involved in disruption of cellular immunity (Turnbull *et al.*, 2005). Gap junctions encoded by innexins in insects co-ordinate multicellular processes and are known to form in between haemocytes, and are suspected to be important during encapsulation (Turnbull *et al.*, 2005). Two CsIV viral innexins (Vinnexins) were expressed in paired *Xenopus laevis* oocytes and were shown to form functional gap junctions. Viral innexins may disrupt cellular immunity by altering normal gap junction intercellular communication, by forming hybrid innexin–vinnexin channels or vinnexin channels with modified permeability.

9.4.6 *C. vestalis* proteins involved in haemocyte disruption

A recombinant CvBV lectin was shown to reduce the association between bacteria and non-parasitized *Plutella xylostella* haemocytes suggesting that this secreted protein acts by disrupting haemocyte recognition of pathogens (Lee *et al.*, 2008). In contrast, CvBVH4, a viral histone, and CvBV15b, induce impaired spreading behaviour in haemocytes, and act intracellularly by uncharacterized mechanisms. These may involve inhibition of host gene expression and translation (Nalini and Kim, 2007; Gad and Kim, 2008).

9.4.7 Bracovirus TnBV1 and EP1-like proteins targeting cell viability

Other bracovirus proteins have also been reported to compromise host cell viability. Expression of the TnBV1 protein in Sf21 cells via recombinant baculovirus or in High Five cells using expression vectors led to apoptosis-like programmed cell death (Lapointe *et al.*, 2005). However, in this case the absence of blebbing and apoptotic bodies in the cell lines prevented the characterization of TnBV1 effects as classical apoptosis. The EP1-like protein, ELP1, from CvBV has also been shown to induce a reduction in total haemocyte numbers after transient expression in *P. xylostella* (Kwon and Kim, 2008). EP1 proteins have long been suspected to act as immunosuppressors and it will be exciting to determine the mechanism involved in reducing haemocyte numbers.

9.4.8 MdBV Egf targeting melanization

One of the most striking immunosuppressive effects associated with PDVs is the suppression of the melanization response. So far only one PDV gene, egf1.0, encoded by MdBV, has been shown to block melanization of haemolymph in *Manduca sexta* and other insects *in vitro*. The MdBV Egf family encodes small serine proteinase inhibitor (smapin) homologues (Beck and Strand, 2007) that usually consist of a cysteine-rich trypsin inhibitor-like domain. Egf1.0 was shown to inhibit haemolymph melanization in *M. sexta* by several mechanisms: (a) inhibition of the catalytic activity of proPO-activating protein PAP-1 and PAP-3; (b) inhibition of pro-PAP1 and pro-PAP3 processing; and (c) prevention of proPO and serine proteinase homologue (SPH) 1 and SPH2 processing (Lu *et al.*, 2008; Beck and Strand, 2007). Egf1.0 could not, however, inhibit PO that had been activated already. Since Egf family homologues have not been identified in other PDVs and serine protease inhibitors are also absent, it will be interesting to determine which genes are involved in suppression of melanization in other systems.

9.4.9 PDV IκB

Nuclear factor κB (NF-κB) transcription factors are key regulators in both insect and mammalian innate immune responses. Upon immune stimulation, degradation of the inhibitor of NF-κB (IκB) leads to nuclear localization of the transcription factor and transcriptional activation of antimicrobial peptides in the case of *Drosophila* (see Chapter 2 in this volume). Pathways under NF-κB transcriptional control have also shown to be involved in development and cellular immunity.

The exciting discovery that all PDV genomes sequenced so far encode IκB-like proteins, which lack the regulatory domains for signal-mediated degradation, led to the very attractive hypothesis that PDV IκBs could be acting as irreversible inhibitors of host NF-κB signalling.

So far, the evidence that parasitoid wasps could be affecting NF-κB signalling in Lepidoptera is quite sparse. For instance, double infection experiments involving parasitization followed by bacterial inoculation leads to normal induction of known anti-microbial peptides (Shelby *et al.*, 1998; E. Huguet, unpublished results). However, after bacterial challenge of *H. virescens* larvae parasitized by *T. nigriceps* NF-κB immunoreactive proteins failed to enter the nucleus of host haemocytes and fat body cells (Falabella *et al.*, 2007), suggesting that parasitism can indeed target these pathways. TnBV and MdBV IκB-like proteins were shown to reduce NF-κB-driven expression of reporter gene contructs in Hela cells and S2 cells respectively (Thoetkiattikul *et al.*, 2005; Falabella *et al.*, 2007). Furthermore, co-immunoprecipitation experiments indicated that MdBV H4 and N5 IκBs bound to *Drosophila* Dif and Relish NF-κB proteins. In the presence of these MdBV IκBs electrophoresis mobility shift assays (EMSAs) showed that *Drosophila* Dif and Relish were no longer capable of binding to κB sites (Thoetkiattikul *et al.*, 2005). Collectively these data show that PDV IκBs have the potential to disrupt NF-κB signalling in lepidopteran hosts. The challenge will now be to demonstrate this effect and the immune or developmental consequences *in vivo*. Interestingly, immunofluorescence experiments using antibodies directed against two CsIV IκBs revealed these proteins localized to the nucleus of haemocytes and fat body post-parasitization, suggesting a possible functional role of PDV IκBs in the nuclei of infected lepidopterans (Kroemer and Webb, 2005).

9.4.10 *Cotesia* cystatins

PDVs associated with *Cotesia* spp. and the closely related *Glyptapanteles indiensis* encode cystatins which are tight-binding reversible inhibitors of C1A cysteine proteases, represented in insects by cathepsins B, L, and F, and 26/29 kDa proteases. In insects these proteases have been associated with developmental processes such as embryogenesis, moulting, and metamorphosis (Liu *et al.*, 2006), but there is also growing evidence that they may be involved in the host immune response (Saito *et al.*, 1992; De Gregorio *et al.*, 2001; Levy *et al.*, 2003; Attardo *et al.*, 2006). Several lines of evidence suggest that cystatins are important PDV virulence factors: cystatins are among the most highly expressed genes in the CcBV–*Manduca* interaction and recombinant cystatin 1 proved to be a functional and potent C1 cysteine protease inhibitor *in vitro* (Espagne *et al.*, 2005). Furthermore, approaches combining molecular evolution and three-dimensional modelling have revealed that bracovirus cystatins are subject to strong diversifying selection acting in key active sites which are important for the interaction with target proteases. This particular selection, which is probably imposed by host defences, emphasizes the potential role of cystatins as pathogenic factors and suggests that cystatins co-evolve with host cysteine proteases (Serbielle *et al.*, 2008). The characterization of host targets in this system and their function will enable us to understand the consequences of their inactivation during parasitism.

9.5 Conclusion

Since their first description in the late 1970s considerable efforts have been made to understand the origin, genomic organization, and impact of PDVs on the physiology of their lepidopteran hosts. In recent years, there has been explosive growth in data concerning the functional characterization of PDV virulence genes. Considering that reverse genetics approaches are impossible in these systems, authors have concentrated mainly on functional approaches using highly expressed genes and/or genes presenting conserved functional domains. So far functional characterizations have been performed mainly by *in vitro* biochemical assays and transient expression in non-host and sometimes host cell lines *in vitro*, and more rarely *in vivo*. Although it is debatable whether over-expression of genes in non-host cells is a relevant functional assay, these experiments enabled the identification of targeted pathways in the host. It is likely that *in vivo* certain gene viral products

have a more subtle effect both in time and in space and that in the natural system a blend of genes and their products is necessary to ensure perfect host control. Surprisingly, *in vivo* use of RNAi has not yet been reported in larval parasitoid systems and has only been successfully performed with the egg parasitoid *C. inanitus* (Bonvin *et al.*, 2005). Injection of double-stranded RNA of three CiBV genes into CiBV/venom containing eggs partially rescued last-instar larvae from developmental arrest, indicating that these viral genes are involved in the inhibition of development (Bonvin *et al.*, 2005). In the future, RNAi of viral genes or of their host targets, as has been done in a *Manduca*/parasitic nematode/bacterial system (Eleftherianos *et al.*, 2007) may provide new insights on PDV gene function and host deregulation.

Beyond their impact on host immunity and development PDV also target different aspects of lepidopteran host biology. For example, in a recent study it was also shown that the behaviour of caterpillars can be manipulated by a wasp (*Glyptapanteles* sp.): after the emergence of parasitoid larvae from the host, the caterpillars protect the wasp cocoons against parasites and predators (Grosman *et al.*, 2008). Since this wasp is associated with a bracovirus encoding PTPs it is conceivable that PDV might be involved in this striking effect of parasitism. Indeed a PTP was shown to be responsible for the manipulation of lepidopteran behaviour by baculoviruses, leading the infected caterpillar to climb to the top of the plant, thus favouring virus spread (Kamita *et al.*, 2005). The fact that certain PDV effectors target signalling pathways potentially involved in a multitude of physiological systems could explain the pleiotropic effects of PDVs.

9.6 Acknowledgements

We thank Catherine Dupuy and Appoline Pichon for contribution to the figures and Catherine Dupuy also for critical reading of the manuscript.

9.7 References

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CHAPTER 10

Immune responses and the evolution of resistance

Jacob Koella

10.1 Introduction

Parasites can impose strong selection on their hosts, leading to the evolution of various defence mechanisms. One of the main ones in many organisms is the immune system, which helps to protect against a wide and, to some degree, unpredictable range of parasites. Despite its obvious importance, the immune system's efficacy varies considerably within and among species. The general mechanism underlying the variation is considered to be that evolution balances the benefits and the costs of an immune response and that both vary according to the epidemiological and environmental situations (Schmid-Hempel, 2005). The benefits of the immune system are obvious (at least qualitatively): it protects against debilitating parasites. Its costs are also becoming established in many systems (Sheldon and Verhulst, 1996): mounting an immune response (in the absence of parasitic infection) can increase mortality (Moret and Schmid-Hempel, 2000), decrease fecundity (Schwartz and Koella, 2004), and lead to changes in behaviour (Mallon *et al.*, 2003). Mechanisms leading to such evolutionary costs include the requirement by immune responses for energy and other resources (Lochmiller and Deerenberg, 2000), the risk of auto-immunity (Sadd and Siva-Jothy, 2006), or the risk that they themselves (in particular if they are over-expressed) cause the symptoms of severe disease (Margolis and Levin, 2008). Even if an immune response is not mounted in response to an infection, the immune system can be costly because of the underlying physiological machinery that must be maintained. This can lead to genetic correlations and trade-offs with important life-history parameters, such as fecundity (McKean *et al.*, 2008) or developmental time (Koella and Boëte, 2003a).

Evolutionary biologists have studied the variation of immune responses and their costs and benefits with respect to two main underlying questions. How much should an individual invest in its resistance against parasites? Does immune function indicate an individual's quality, which can, for example, help potential sexual partners to choose their mates?

In this chapter I argue that, although estimates of costs and benefits of immune responses are indispensable to understand the evolution of immune responses and thus resistance against parasitic infection, they are not enough to answer our evolutionary questions. The main point underlying my argument is that the immune system and its interaction with parasites are complex, so the relationship between immune function and resistance (and, thus, a host's quality) is not straightforward. A strong immune response need not always lead to effective resistance or a high cost. It is perhaps such complexity that leads some immune responses to be positively, others to be negatively, related to sexual attractiveness (Rantala and Kortet, 2003).

I discuss two aspects of the complexity underlying immunity and resistance. First, the immune system has many components that interact with and regulate each other to fight infection. Choosing a marker of immune function from this dynamic interplay is problematic. Markers may well be related to resistance of a given parasite, but are not used to resist other parasites (whether these are other species or other genotypes of the

same species) (Adamo, 2004). Indeed, markers of immune function can be associated with increased susceptibility rather than resistance, if branches of the immune system are regulated by trade-offs. In mammals an example of such a trade-off is the reciprocal down-regulation of T-cell types Th1 and Th2 (Abbas *et al.*, 1996). In this case, the Th2 response, say, may be associated with increased susceptibility to a parasite that is resisted via a Th2-type response. Indeed, parasites have evolved to utilize this trade-off. *Leishmania major* parasites, which infect macrophages and are susceptible to the Th1 response, manipulate macrophages to augment the Th2-type T-cell response (Chakkalath and Titus, 1994); they stimulate an immune response that is not only ineffective but that also suppresses the effective immune response. In invertebrates, such trade-offs are less well characterized and results are not yet conclusive. Although some components of the melanization response and an antibacterial response are negatively correlated (Cotter *et al.*, 2004), the phenotypic outcome of the two responses—melanization of beads or clearing bacteria—are positively (genetically) correlated (Lambrechts *et al.*, 2004). Thus, without detailed knowledge of the immune responses that help to resist a specific parasite we risk choosing markers of immune function that are, at best, evolutionarily irrelevant and, at worst (if they are traded off), misleading for our goal to understand resistance (Adamo, 2004; see also Chapter 11 in this volume). To make matters worse, the costs of immune function are also more complex than is generally acknowledged. In particular, they depend not only on the level of the stimulated immune response, but also on the antigen (and thus, in some cases, on the genetic variant the parasite) that stimulated it (Schwartz and Koella, 2004). Second, resistance is the outcome of the interaction between a host and a parasite; each partner can have some genetic control over the outcome and therefore the level and variation of resistance within a population is determined by a co-evolutionary process. That resistance is determined by the interaction of the two partners' genotypes means that any level of investment in immunity can lead to widely differing levels of resistance (which depends on the parasite's traits). A perhaps more important consequence is that considering the evolution of only one of the two partners is not only insufficient to understand resistance, but may well be misleading (Restif and Koella, 2003).

Below I consider these two points with a specific example: the resistance of mosquitoes to malaria parasites. Understanding immune responses and resistance are particularly important for this system, as there is considerable interest in developing techniques to use genetically manipulated mosquitoes for the control of malaria. If mosquitoes can be transformed with genes that make them resistant and if these genes then spread through mosquito populations, it may be possible to block the parasite's transmission (Alphey *et al.*, 2002). This goal has stimulated extensive (mostly molecular) research on the immune responses of mosquitoes (see the reviews in Dimopoulos *et al.*, 2001; Blandin and Levashina, 2004), with the underlying assumption that effective immunity is equivalent to resistance. (Whereas some studies consider artificial peptides that are not part of the natural immune response, e.g. SM-1 (e.g. Ito *et al.*, 2002; Moreira *et al.*, 2004), they do not circumvent questions about their relationship with resistance, their costs, and the parasite's co-evolutionary response.) If, however, immune function is only weakly related to resistance, the approach may be problematic. Although the first problem—that it is difficult to find the immune responses that make mosquitoes resistant against a malaria parasite—may be overcome by detailed studies of immune function, the second appears more critical. If the parasite has some level of genetic control over the level of resistance achieved by a specific immune response, evolutionary pressure is likely to let it avoid any immune response that becomes dominant through the tools of genetic manipulation.

I discuss the relationship between immune function and resistance by reviewing some of the large number of studies on the interaction between malaria and mosquitoes. In particular, I discuss the relationship between effective immune responses in a laboratory setting and in natural situations, and the interaction between the mosquito's and the parasite's genotypes that determine resistance. I then discuss the importance of co-evolution in determining resistance by reviewing a theoretical

model of the interaction between the mosquito and the malaria parasite. The model assumes that the parasite can counter the mosquito's allocation to immunity and that resistance results from the relative strengths of the two partners' responses.

10.2 Immunity and resistance

To begin, I briefly describe the development of malaria parasites in the mosquito (Figure 10.1) and consider possible mechanisms of resistance. Mosquitoes take up infectious gametocytes when they feed on a gametocyte carrier. These exflagellate to produce male and female gametes, which mate to produce a zygote. The zygote transforms to an ookinete, which migrates through the midgut wall and implants as an oocyst. Within the oocyst, rapid replication produces sporozoite precursors. Upon completion of this developmental process, the oocyst bursts to release the sporozoites, which migrate to the mosquito's salivary glands from where they can infect the next victims of the mosquito's bites.

Mosquitoes can block this development with a variety of immune responses: by lysing the ookinetes as they are migrating through the midgut wall (Vernick *et al.*, 1995), by producing nitric oxide (Luckhart *et al.*, 1998) (which impedes the development of the parasite), by melanizing ookinetes and early oocysts (Collins *et al.*, 1986), and by killing parasites with antimicrobial peptides (Dong *et al.*, 2006). Extensive studies in molecular biology have given us an impressive description of the genetic and molecular interactions underlying these processes (reviewed in Dimopoulos *et al.*, 2001; Blandin and Levashina, 2004).

In the context of this chapter, there are two striking features of these molecular studies. First, functional immunity depends on a large number of genes and, in particular, on the interaction between genes. For example, knocking out either one of two receptor genes (CLIP A2 or CLIP A5) roughly halves the number of oocysts in a midgut, knocking out both decreases the number by a factor of close to 10, and knocking out an additional one (CLIP A8) brings the number back up to about a third of the unmanipulated controls (Volz *et al.*, 2006). Second, the efficacy and the function of an immune response associated with several genes differ among mosquito species (Abraham *et al.*, 2005; Dong *et al.*, 2006) and depend on the genetic background of a given mosquito species. For example, Volz *et al.* (2006) studied several genes associated with the melanization of ookinetes. In a malaria-resistant strain of mosquitoes, these genes are responsible for the melanization and disposition of dead ookinetes, but in a malaria-susceptible strain the same genes induce a melanization response that kills the ookinetes directly.

The melanization response is an illustrative example of several important points. The first selection experiment leading to malaria-resistant mosquitoes found that resistant mosquitoes melanize their oocysts (Collins *et al.*, 1986). Mosquitoes

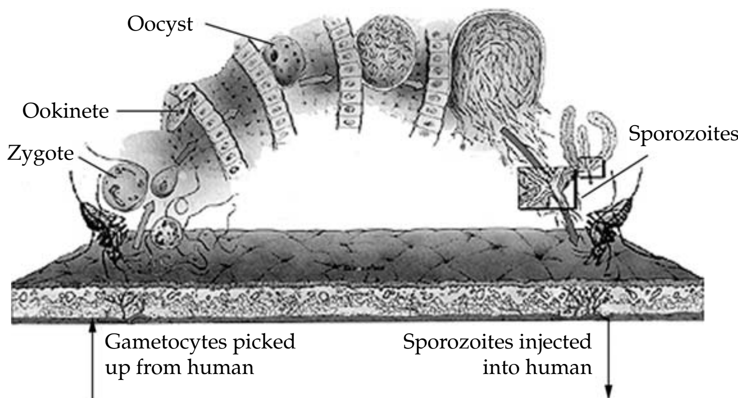

Figure 10.1 The life cycle of malaria parasites in the mosquito.

selected for resistance have a more effective melanization response (estimated by the ability to melanize a Sephadex bead injected into the thorax, a standard and convenient method of assaying this response in mosquitoes; Paskewitz and Riehle, 1994; Chun *et al*., 1995; Suwanchaichinda and Paskewitz, 1998) than susceptible mosquitoes (Voordouw *et al*., 2008a) and the mechanisms underlying malaria resistance and melanization of Sephadex beads share at least part of their genetic determination (Gorman *et al*., 1996; Gorman and Paskewitz, 1997). However, despite the association between resistance and the melanization response, it is unlikely that melanization helps to resist infection in natural populations. Although mosquitoes generally show some resistance to infection (e.g. in a highly endemic region of Kenya, about 70% of mosquitoes that had fed on blood containing the gametocytes of various isolates of the parasite were not infected; Lambrechts *et al*., 2005), malaria parasites are almost never melanized (e.g. one in 200 infected mosquitoes in a Tanzanian study; Schwartz and Koella, 2002). Despite the ineffective melanization of malaria parasites, most mosquitoes can melanize Sephadex beads effectively (85% in the Tanzanian study; Schwartz and Koella, 2002). It is also noteworthy that non-vector species of mosquito use the melanization response to kill malaria ookinetes (Habtewold *et al*., 2008). Thus, mosquitoes have a functional, effective melanization response, but in vector species it cannot be used against malaria parasites. This suggests that the malaria parasite has evolved ways to either avoid being detected by the immune receptors of its vector species or to suppress its melanization response. In either case, it seems clear that at least one of the main branches of the insect immune system—the melanization response—is a bad marker of resistance to malaria. It also begs interesting evolutionary and immunological questions: how and why does the malaria parasite avoid the melanization response? I discuss aspects of these questions below.

To date, despite detailed knowledge about many aspects of the mosquito's immune system, we do not know how mosquitoes resist malaria, in particular in natural populations of malaria vectors. It is likely that nitric oxide and antimicrobial peptides contribute to resistance, as in some populations the allelic variation of the genes underlying the production of nitric oxide synthase and cecropins (an antimicrobial peptide) is associated with the likelihood of infection by malaria in field-caught mosquitoes (Luckhart *et al*., 2003). However, in other studies on the genetic variation of resistance of African mosquitoes, none of the described quantitative trait loci associated with resistance co-localized with the genes that are known to be involved in the immunological processes described above, but suggest a role for a leucine-rich repeat protein that is similar to molecules involved in natural pathogen-resistance mechanisms in plants and mammals. Another recent study describes non-classical immune responses (activation of actin cytoskeleton dynamics and a haemolymph lipid transporter) in the resistance of mosquitoes to field isolates of malaria parasites (Mendes *et al*., 2008).

Overall, resistance of mosquitoes to malaria parasites appears to be the result of complex interactions among several immune processes that may be positively correlated (Lambrechts *et al*., 2004) or traded off (Cotter *et al*., 2004). Dealing with this complexity makes it difficult to reach conclusions about the evolution of the efficacy of individual immune responses. Indeed, it suggests that any immunological marker would be at most a weak marker of resistance and, thus, of the mosquito's evolutionary quality. The best (and only) marker of immune function (as a response to malaria infection) may well be the mosquito's resistance to malaria parasites.

10.3 Interaction between host and parasite

Can we use resistance as a general indicator of a host's quality? Here I argue that this would be useful only if resistance is determined by the host and effective against many parasites, but that resistance is likely to be determined by an interaction between the host and the parasite. I discuss the parasite's influence on resistance first with an empirical approach showing that resistance depends on the interaction between the host's and the parasite's genotypes and then with a theoretical approach describing the co-evolution between the host's immune response and the parasite's counter-response.

10.3.1 Genotype-by-genotype interactions

An individual's resistance depends on the species of parasite that infects it. Clones of *Daphnia magna*, for example, differ in resistance to two bacterial and three microsporidian parasites, and the pattern of resistance differs among the host's clones (Decaestecker *et al.*, 2003). Resistance can also depend on the pathogen's genotype, so that some hosts are resistant against a subset of a parasite's genotypes while other hosts are resistance against other genotypes. An example of such genotype-by-genotype interaction is the gene-for-gene interaction, where resistance is controlled by pairs of matching genes (the resistance gene and the avirulence gene), which give resistance only if the host is homozygous for the resistance allele and the pathogen is homozygous for the avirulence allele. Gene-for-gene interactions are common in plants and their pathogens (Thompson and Burdon, 1992; Jones and Dangl, 2006), and have also been found in, for example, insect–parasitoid interactions (Dupas *et al.*, 2003). Other types of genotype-by-genotype interaction underlie the resistance of snails to their schistosome parasites (Webster and Woolhouse, 1998), bumble bees to their trypanosome parasites (Schmid-Hempel *et al.*, 1999), *Daphnia* to its bacterial parasite *Pasteuria ramosa* (Carius *et al.*, 2001), and *Anopheles gambiae* mosquitoes to the human malaria parasite *Plasmodium falciparum* (Lambrechts *et al.*, 2005). In such a situation—whether resistance varies among parasite species or among parasite genotypes—'resistance' as the host's trait has no meaning; we can only discuss resistance of a given host against a given parasite and average resistance will change as the frequencies of the parasite species or genotypes change.

I will illustrate in more detail host-genotype-by-parasite-genotype interactions with the resistance of mosquitoes to malaria parasites (Lambrechts *et al.*, 2005). The genetic variability of the major African vector *A. gambiae* was assayed by a comparison of the variability of resistance within and among full-sib families, a standard method of quantitative genetics (Falconer, 1989). Individuals of each family were challenged with one of several isolates of the human malaria parasite *P. falciparum*, which were obtained from naturally infected children in Kenya. Resistance was assayed as the likelihood that a mosquito harboured oocysts 8 days after infection and as the number of oocysts if there was at least one. For any given parasite, the mosquito families differed greatly in both measures of resistance (Figure 10.2); in any given mosquito family, the different isolates led to widely

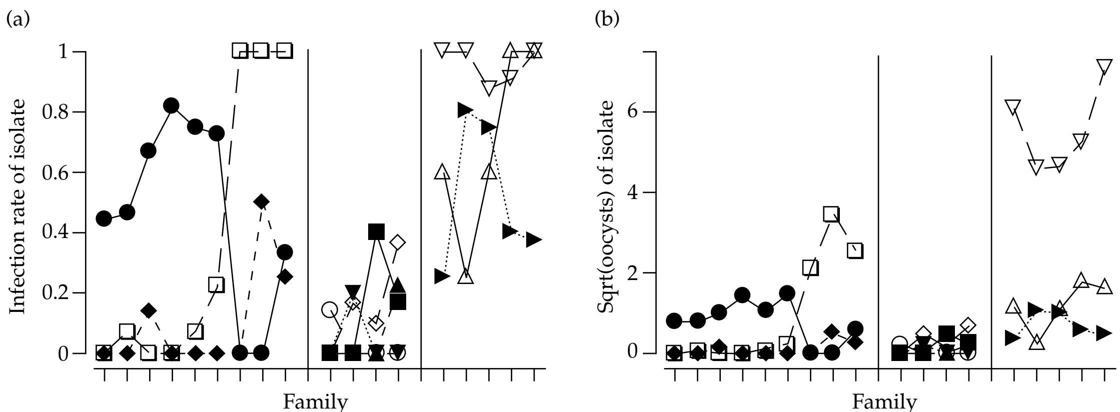

Figure 10.2 Graphical representation of the mosquito-family-by-parasite-isolate interactions underlying (a) the probability and (b) the intensity of infection. Each symbol represents the proportion of infected mosquitoes (in a) or the mean of the square root (Sqrt) of the number of oocysts (in b) for a given combination of family and isolate. The families are indicated on the *x* axes, and are separated into the three blocks of the study with vertical lines. Different symbols represent different isolates (open symbols show isolates containing two clones; closed symbols show isolates containing three clones), and the lines connect points representing the same isolate. Crossing lines give an indication of family-by-isolate interactions (from Lambrechts *et al.*, 2005).

different levels of resistance, ranging in some families from 0% resistance against one isolate to 100% resistance against another. Averaged across all parasites, resistance was similar in all mosquito families, but the pattern of resistance against the isolates differed considerably among the families. In particular, no mosquito family was most resistant to all parasites, and no parasite isolate was most infectious to all mosquitoes. Thus, the level of mosquito resistance depends on the interaction between its own and the parasite's genotype; resistance is not a characteristic of the host that can be used as an indicator of general quality, but depends on the parasite the host is infected with. Any indicator of immune function is bound to give a misleading indication of the host's resistance, and indeed any measure of an individual's resistance (e.g. resistance against specific isolates, average resistance against all isolates) is bound to be a bad measure of its evolutionary success, for the overall resistance of an individual can only be defined for the parasites that happen to infect it and is thus determined by a combination of the host's genotype, the prevalence of each isolate, and chance.

10.3.2 Investment by host and parasite

It is likely that genotype-by-genotype interactions reflect the host's limitations in recognizing a parasite. Once the parasite is recognized and the host mounts an immune response in an attempt to clear it, the outcome of this immune response depends on a further interaction between the host and the parasite, for it is determined not only by the strength of the immune response but also by the ability of the parasite to avoid it. Indeed, as illustrated by the study summarized below, malaria parasites can suppress the mosquito's melanization response. Although this study involves a malaria/mosquito combination that does not occur in nature—the parasite *Plasmodium gallinaceum* in the mosquito *Aedes aegypti*—it is useful to indicate a potential evolutionary response.

10.3.2.1 Immunosuppression

Mosquitoes were blood-fed on an uninfected chicken or on one infected with malaria, and inoculated with a Sephadex bead 1, 2, or 4 days after blood-feeding; that is, when the ookinete is in the process of migrating through the midgut wall, when the oocyst is being established, or when the oocyst has gone through about half of its development (Figure 10.1). About 40% of the uninfected mosquitoes, but less than 25% of the infected ones, melanized the bead (Boëte *et al.*, 2002). The difference between infected and uninfected mosquitoes was most obvious 1 day after infection (at the parasite's ookinete stage), the difference diminished during the early oocyst stage (2 days after infection), and it disappeared at the later oocyst stage (4 days after infection) (Figure 10.3). It is striking that it is the early stages of the parasite that are also most sensitive to the mosquito's immunity (Collins *et al.*, 1986; Vaughan *et al.*, 1992; Gouagna *et al.*, 1998), so that the selection pressure for immunosuppression in these stages should be more intense than in older oocysts. These, in contrast to the ookinetes, appear to avoid recognition by the immune system by incorporating mosquito-derived proteins onto or into their surface capsule (Adini and Warburg, 1999). Such immune evasion by the oocysts would alleviate their need to actively suppress the encapsulation response.

These results suggest that the parasite can either actively suppress the mosquito's immune response or that it modifies the blood of its chicken host in a way that reduces the efficacy of the mosquito's immune system. A later experiment (Boëte *et al.*, 2004) suggests that it uses both mechanisms to suppress the mosquito's melanization response. With either mechanism, resistance against the parasite is determined by the interaction between the host's investment in its immune response and the parasite's ability to suppress immunity, so that resistance will be determined by a co-evolutionary process between mosquitoes and parasites.

10.3.2.2 Co-evolutionary model

Let us therefore consider a mathematical model where resistance is determined by the interaction between the mosquitoes and malaria: the mosquitoes mount an immune response and the parasites suppress it (Koella and Boëte, 2003b). Resistance R, here defined as the probability that a mosquito kills its malaria parasites, is determined by the

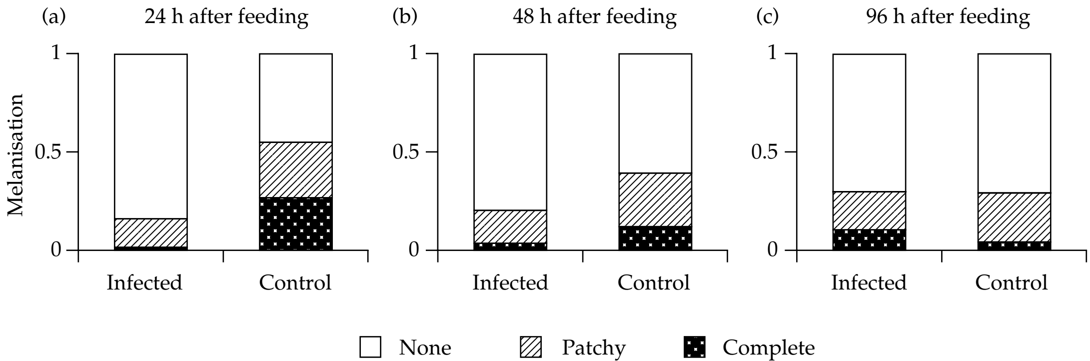

Figure 10.3 Melanization response against inoculated beads. Each panel shows the proportion of infected mosquitoes and uninfected controls that melanize a bead to different degrees (no, patchy, and complete melanization). (a) Response against beads inoculated 24 h after infection (or blood-feeding); that is, when the parasite is in its late ookinete stage and most sensitive to the mosquito's melanization response. (b) Inoculation after 48 h, at an early oocyst stage. (c) Inoculation after 96 h, at a later oocyst stage (from Boëte *et al.*, 2002).

combination of the two strategies—the mosquito's investment x and the parasite's investment y—according to $R = x(1-y)$. (Note that, even if the mosquitoes invests all of their resources to the melanization response (i.e. $x = 1$), strong suppression by the parasite can make the immune response ineffective.)

Details of the mathematical model (which combines evolutionary approaches with the epidemiological dynamics of malaria) and its analysis can be found in Koella and Boëte (2003b). The analysis involves three steps: (a) finding the host's optimal investment in resistance as a function of the parasite's strategy, (b) finding the parasite's optimal investment in immunosuppression as a function of the host's strategy, and (c) finding the co-evolutionary equilibrium of the two.

(a) Host's investment. As any other trait, the mosquito's evolutionary response to being parasitized balances costs and benefits. The benefits of an immune response are clear: they reduce the probability of being infected or increase the likelihood of clearing the parasite and thus reduce the detrimental effects of malaria infection. These can be substantial, at least in natural situations (as opposed to laboratory studies of unnatural host–parasite combinations; Ferguson and Read, 2002). Early stages of infection (oocysts) decrease fecundity, in particular if the infection is intense (Hogg and Hurd, 1995, 1997). Late stages (sporozoites) increase mortality (Anderson *et al.*, 2000), most probably because the sporozoites manipulate mosquitoes to increase the biting rate (and thus the rate of transmission) (Koella *et al.*, 1998, 2002). As the ability to manipulate the biting rate increases with the intensity of infection (Koella, 1999), it is likely that the rate of mortality also increases with the number of sporozoites. Thus, there is considerable evolutionary pressure for the mosquito to invest in being resistant to malaria.

On the other hand, any costs of the immune response and resistance would constrain the evolution of resistance. Such costs include that adult mosquitoes in lines selected to resist malaria are smaller, take smaller bloodmeals, and lay fewer eggs than unselected mosquitoes (Yan *et al.*, 1997), although such costs are not found in all experiments (Hurd *et al.*, 2005). The melanization response itself is also costly in that it can reduce fecundity (Schwartz and Koella, 2004); again, this cost is not observed in all experiments (Voordouw *et al.*, 2008b) or for all immune-stimulating antigens (Schwartz and Koella, 2004).

In our model, we assumed that the benefit of resistance is proportional to the probability that a mosquito becomes infected, which in turn is determined by the epidemiological dynamics of malaria (more specifically, the infected proportion of the human population). We assumed that the cost of

resistance increases with the mosquito's investment in its immune responses. Finally, we assumed that evolution maximizes the mosquito's reproductive success.

The model's predictions for the host's investment are shown in Figure 10.4a. Consider first low potential of transmission (thin curve). (Potential of transmission is defined as the basic reproductive number of malaria that would be achieved in a population of mosquitoes with no resistance. It is essentially determined by the number of mosquitoes and their epidemiological parameters: biting rate and longevity.) At low levels of immunosuppression, the host invests more in its immune response as immunosuppression increases. If immunosuppression passes a threshold, however, the cost of the immune response that would lead to a high level of resistance becomes prohibitive. Rather than paying the high cost of a very effective immune response, the host evolves less resistance at a lower cost, and uses the spare resources to reproduce before it is killed by the parasite. At the extreme, if immunosuppression is complete, there is of course no point in investing in immunity, as any level of immunity can only lead to complete susceptibility. As the potential of transmission increases (increasing thickness of lines), the host's optimal investment increases at low levels of immunosuppression, but decreases at high levels. At intermediate levels, the host can have two strategies that maximize its fitness (locally). Very high investment ensures that the parasite is cleared rapidly; low investment enables the host to reproduce efficiently. Therefore, if the parasite's investment is fixed, the host's evolutionary response depends on the initial conditions and can take it to either a very strong or a very weak immune response against malaria.

(b) Parasite's investment. The benefit of immunosuppression is that the parasite is less likely to be killed by the host's immune response. Whereas there is no evidence for a cost of immunosuppression, the model assumes that it increases with investment. (Varying the shape of the cost function makes only minor differences to the outcome.) Following most models of parasite evolution, we assume that evolution maximizes the basic reproductive number of the parasite. The mathematical model predicts that immunosuppression should increase with increasing immune-efficacy (Figure 10.4b).

(c) Co-evolution. The co-evolutionary equilibrium is the intersection of these two curves. As at this point both partners are at their optimal strategies: the two strategies are co-evolutionarily stable. Figure 10.4c shows the co-evolutionary equilibria at the potentials of transmission given in Figure 10.4a. Several aspects of the pattern are noteworthy. First, the investment in immunity is only weakly related to resistance (Figure 10.4d). Although at the co-evolutionary equilibrium, increased investment in immunity generally implies increased resistance, over large ranges of investment resistance is almost independent of investment. Second, in the cases where the host has two locally optimal strategies, the co-evolutionarily stable strategy is at the host's lower investment, whereas the higher investment is never co-evolutionarily stable (Figure 10.4c). Third, the mosquito's investment in its immune responses is fairly low at any potential of transmission. Furthermore, as the potential of transmission increases, the mosquito's investment *decreases*. This is associated with a decrease of the parasite's ability to suppress the immune response and a *decrease* in overall resistance (Figure 10.4e).

10.3.2.3 Evidence

One of the most striking predictions of this co-evolutionary mathematical model is that, for a wide range of parameter values, mosquitoes should invest the least in their immune response in the areas with the most intense transmission (and therefore there is little evolutionary pressure for parasites to invest in suppressing the immune response). Although there are no data available to test this prediction, a recent study (Lambrechts *et al.*, 2007) corroborates it. As mentioned above, malaria parasites (as ookinetes or young oocysts) can suppress the melanization response of their mosquito vector. Both of the described experiments were done with a malaria–mosquito combination that does not occur in nature: *P. gallinaceum* and *A. aegypti*. When a similar experiment—a comparison of the melanization response against Sephadex beads of malaria-infected and -uninfected mosquitoes—was done with a natural system (*P. falciparum* and *A. gambiae*) in an area with intense transmission, no

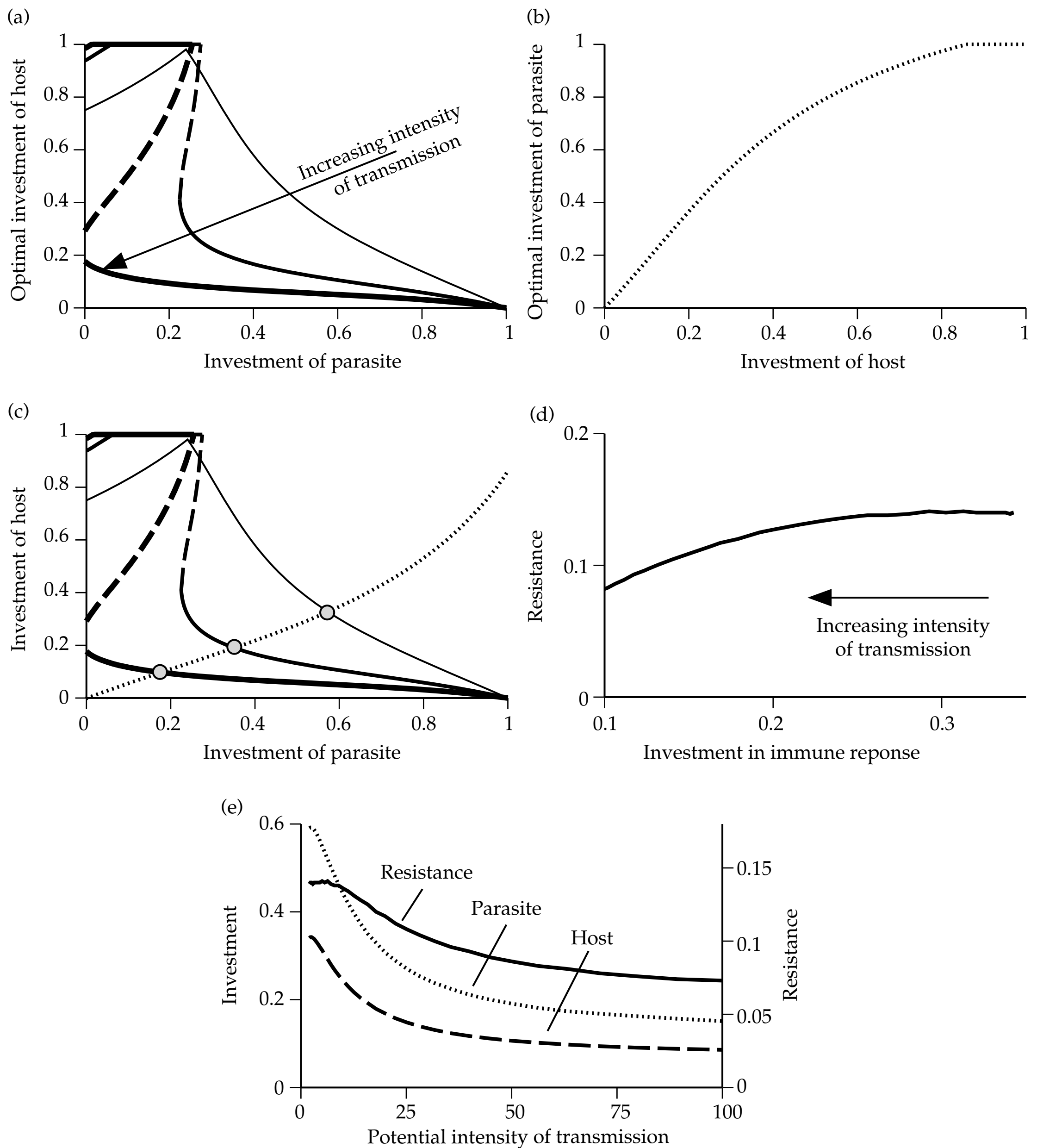

Figure 10.4 Evolutionary and co-evolutionary equilibria of the host's investment in its immune response and the parasite's investment in immunosuppression. (a) The host's optimal level of investment as a function of the parasite's investment, for various potentials of transmission (defined as the basic reproductive number of malaria that would be achieved in a population of mosquitoes with no resistance). It is essentially determined by the number of mosquitoes and their epidemiological parameters: biting rate and longevity. The solid lines give the optimal response and the dashed line shows the level of investment that minimizes reproductive success. The potential of transmission increases with the thickness of the lines. (b) The parasite's optimal level of investment as a function of the host's immune response. (c) The intersects of the host's and the parasite's optimal responses give the co-evolutionary equilibrium. The isoclines of the host and parasite show the levels of investment that maximize the host's success (solid lines), that minimize the host's success (dashed lines), and that maximize the parasite's success (dotted line). The co-evolutionary equilibria are given by the intersection of the isoclines. (d) Relationship between the host's investment in its immune response and resistance against the parasite. (e) Investment by the host and the parasite and level of resistance as a function of the potential of transmission (modified from Koella and Boëte, 2003b).

immunosuppression was observed (Lambrechts *et al.*, 2007). Although the difference in results may be due to many factors, one possibility is that the lack of immunosuppression in this natural system is the co-evolutionary equilibrium. Indeed, this might also help us to understand the general lack of a melanization response against malaria parasites in natural populations.

10.4 Conclusions

My discussion of the evolution of immune function emphasized two points. First, the immune system is complex, with many responses that may act together or inhibit each other to determine the outcome of an infection. Using an immune response as an indicator of the host's resistance (or, more generally, its quality) is therefore problematic, as increased investment in a given immune response may well indicate increased susceptibility to a parasite. Second, resistance is a product of the interaction between a host and a parasite. Thus, we cannot understand the evolution of immune function without considering the co-evolution of the host's and the parasite's contributions to resistance. Indeed, as found in a more general context (Restif and Koella, 2003), mathematical models of the evolution of the host that do not consider the co-evolutionary response by the parasite can be misleading as their predictions can differ qualitatively from the co-evolutionary dynamics and equilibrium. An example of a surprising result from a co-evolutionary model is that, as the potential of transmission increases, the host's investment in immunity and its resistance to a parasite do not increase, but rather decrease (Koella and Boëte, 2003b). Thus, simplistic interpretations of immune function are dangerous; co-evolutionary dynamics can give counter-intuitive outcomes.

Although these points may not be surprising, they are often neglected in studies of immune function. Evolutionary biologists, for example, try to understand the variability of the efficacy of the immune response among individuals and among populations. We have studies, for example, on the genetic underpinning of the melanization response (Cotter and Wilson, 2002), on the cost of inducing an immune response (Robb and Forbes, 2006), and on the tendency for females to choose males with effective immune responses as mating partners (Rantala *et al.*, 2002). Both of these questions implicitly assume that the measured immune response is related to resistance. But, little effort has been put into estimating the relationship between immune responses and resistance against the predominant parasites in natural populations, so that it is difficult to reach strong conclusions. Indeed, immune responses can be negatively related to sexual attractiveness (Rantala and Kortet, 2003), and a recent overview of sexual selection of immune responses (Lawniczak *et al.*, 2007) emphasizes that any correlation between immune response and partner choice can be expected and the correlation is influenced by the trade-offs within the immune system and those between immune function and other traits. Of course, I am not arguing that immune responses are not associated with resistance, and a given immune response may well reflect the host's quality in some circumstances. But we need detailed description of immune function and how it is linked to resistance and, ultimately, reproductive success and we need a better understanding of co-evolutionary dynamics before we can reach strong conclusions. Because of these problems in interpreting immune function, it is reassuring that many evolutionary studies continue to focus on explicit measures of resistance rather than immune function (e.g. studies on *Drosophila* and it parasites and parasitoids; Kraaijeveld and Godfray, 1997; Kraaijeveld *et al.*, 2001b; Rolff and Kraaijeveld, 2003; Lazzaro *et al.*, 2006; Vijenravarma *et al.*, 2008), including co-evolutionary aspects of the host and its parasite. (Kraaijeveld and Godfray, 1999; Kraaijeveld *et al.*, 2001a).

Acknowledging co-evolution and the complexity of immunity is also critical in more applied contexts, for example the genetic manipulation of mosquitoes for the control of malaria. A key step is to identify the genes most relevant in determining resistance to malaria. While considerable progress has been made in the past decade or so, most efforts have considered mosquito–parasite associations that are neither natural nor relevant for human health. Will the identified genes be important in natural systems, against all parasite genotypes,

in all genetic backgrounds of the mosquito? Will the parasite have the ability to counteract the mosquito's resistance? Our limited knowledge, some of which is reviewed above, suggests that the answer to the first question is no and to the second may be yes (Boëte and Koella, 2003; Lambrechts *et al.*, 2006, 2008). If so, attempts at manipulating the mosquito's immune response for malaria control may be futile.

Overall, this chapter argues that to understand the evolutionary pressures on immune function, we must understand in much more detail its complex relationship with resistance. On the one hand, the immune system that helps to influence resistance is complex and involves trade-offs among its components. On the other hand, resistance is partly determined by the parasite, so that the evolutionary patterns of resistance can only be understood with a co-evolutionary approach.

10.5 Acknowledgements

I thank Jens Rolff, Lex Kraaijeveld, and an anonymous reviewer for comments.

10.6 References

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CHAPTER 11

The impact of physiological state on immune function in insects

Shelley A. Adamo

11.1 Introduction

Insect immune systems are dynamic. Their response to infection is altered by both internal and external conditions (Stoks *et al.*, 2006; Adamo, 2008a, 2008b). This plasticity exists because of a complex web of interconnections between the immune system and other physiological systems (e.g. Adamo *et al.*, 2008; Schmidt, 2008). For example, activities such as flight-or-fight (i.e. the acute stress response), reproduction, and development all lead to co-ordinated changes in multiple physiological systems (Chapman, 1998; Nation, 2002). These shifts in physiological state result in concomitant changes in immune function (acute stress, Adamo and Parsons, 2006; reproduction, see Lawncizak *et al.*, 2007; development, e.g. Meylaers *et al.*, 2007).

Changes in physiological state can alter immune-system function directly via neural/neuroendocrine/immune connections (Adamo, 2008a, 2008b). Such direct effects may adapt the immune system to changing resource availability and/or changing immunological needs. Changes in physiological state can also alter immune system function indirectly by reducing the resources needed for an immune response (e.g. Adamo *et al.*, 2008).

In this chapter, I discuss how and why short-term changes in physiological state (i.e. the acute stress response) alter immune responsiveness in insects. I also explore the ramifications of these effects for ecological immunologists.

11.2 The puzzle of acute stress-induced immunosuppression in animals

When responding to danger, animals shift into a new physiological state: the acute stress response. Most of the resulting alterations in physiological function optimize the animal's ability to perform flight-or-fight behaviours (Figure 11.1; insects, Roeder, 2005; mammals, Charmandari *et al.*, 2005). However, the effects of acute stress on immune function seem maladaptive. Acute stress is immunosuppressive in animals from three different phyla (Chordata, Mollusca, and Arthropoda) (Adamo, 2008b). It results in a transient decline in resistance to infection in insects (Figure 11.2), molluscs (Lacoste *et al.*, 2001), and vertebrates (Davis *et al.*, 1997). Some of the effects of acute stress on immune function are induced directly via neural/neuroendocrine/immune connections (Adamo, 2008b).

Some of these direct connections to the immune system appear to be conserved across phyla, suggesting that they serve an important function (Ottaviani and Franceschi, 1996). For example, vertebrates, molluscs, and insects use chemically similar derivatives of the amino acid tyrosine to implement their acute stress responses (Ottaviani and Franceschi, 1996). Vertebrates (Cooper *et al.*, 2003) and molluscs (Lacoste *et al.*, 2001) release noradrenaline (norepinephrine) during acute stress, whereas insects release noradrenaline's chemical cousin, octopamine (Orchard *et al.*, 1993). The chemical similarity between octopamine

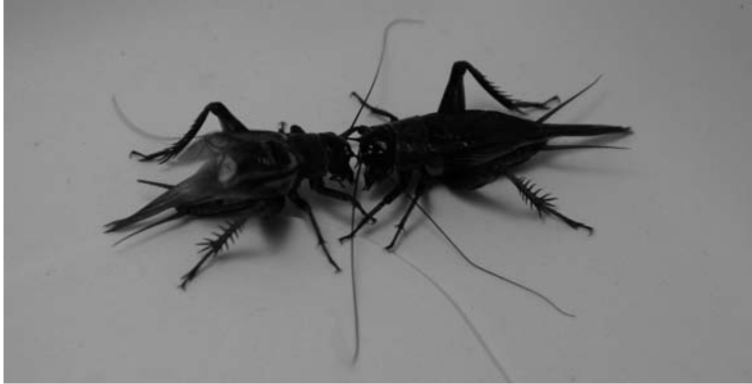


Figure 11.1 Fighting crickets (*Gryllus texensis*).

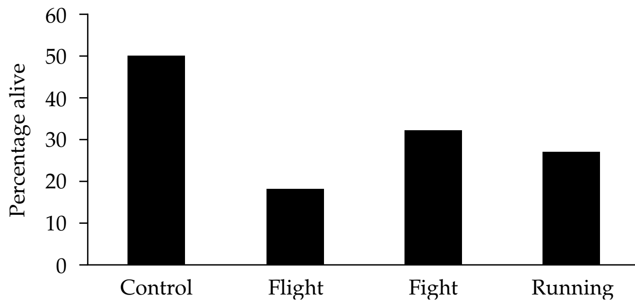


Figure 11.2 Flying, fighting, and forced running result in a decline in resistance to the bacterium *Serratia marcescens* compared with resting control crickets (*Gryllus texensis*) ($Z = 3.3$, $P < 0.001$; test for trends and contrasts for frequency data; Meddis, 1984). Data are normalized and taken from Adamo and Parsons (2006) and unpublished data. Sample sizes: control $n = 225$, flight $n = 94$, fight $n = 24$, running $n = 120$.

and noradrenaline (Figure 11.3), the similarity between the enzymes involved in their synthesis, and the similarities between the sequences of their receptor and transporter molecules support the argument that octopamine and noradrenaline pathways arose from the same ancestral pathway (Evans and Maqueira, 2005; Roeder, 2005; Caveney *et al.*, 2006).

Both octopamine (Table 11.1) and noradrenaline are involved in preparing the body for flight-or-fight behaviours (Roeder, 2005), suggesting that this is an ancient, conserved function of these compounds (Gerhardt *et al.*, 1997; Roeder, 1999). In vertebrates, noradrenaline also mediates a connection between the nervous system and the immune system that is active during acute stress (Emeny *et al.*, 2007; Nance and Sanders, 2007). In insects, the evidence suggests that octopamine performs a similar function (Table 11.2). Octopamine is released as a neurohormone in insects (e.g. orthopterans) by the dorsal unpaired medial cells (DUM neurones) during an acute stress response (Orchard *et al.*, 1993; Pflüger and Stevenson, 2005; Roeder, 2005). DUM neurones also have extensive peripheral processes (Pflüger and Stevenson, 2005). Therefore, octopamine has the potential to reach both circulating immune cells (i.e. haemocytes) and immune organs such as the fat body.

Noradrenaline and octopamine can influence immune function because immune cells in vertebrates (e.g. Webster *et al.*, 2002; Madden, 2003), molluscs (Lacoste *et al.*, 2002), and insects (Gole *et al.*, 1982; Orr *et al.*, 1985) have receptors for these compounds. In insects, octopamine may mediate some of the decline in disease resistance after acute stress. Injections of octopamine prior to a bacterial challenge result in increased mortality (Adamo and Parsons, 2006). However, octopamine also has immunoenhancing effects (Table 11.2; Brey, 1994). Octopamine can increase resistance to infection

when the pathogen is co-incubated with it (Baines *et al.*, 1992; Baines and Downer, 1992; Dunphy and Downer, 1994). However, this effect may be non-specific. When bacteria were incubated with octopamine plus an octopamine antagonist, cockroaches still exhibited increased disease resistance (Baines and Downer, 1994). This is opposite to the result that would be expected if the effect were specific for octopamine. Dunphy and Downer (1994) suggest that octopamine may act as an opsonin, due to the surface charge on the molecule. If octopamine could act as an opsonin, this would explain why co-incubating bacteria or other pathogens with octopamine prior to injection increases pathogen clearance from the haemocoel (Dunphy and Downer, 1994) and increases host survival (e.g. Baines *et al.*, 1992). Other octopamine effects on the immune system, however, appear to be mediated by specific octopamine receptors (Baines and Downer, 1994).

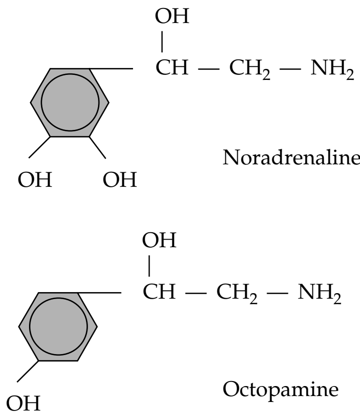


Figure 11.3 The chemical structures of octopamine and noradrenaline (norepinephrine). Adapted from Cooper *et al.* (2003).

Like octopamine in insects, noradrenaline in vertebrates produces a mix of immunosuppressive and immunoenhancing effects (Nance and Sanders, 2007). As in insects, the overall effect of noradrenaline in vertebrates is an increase in susceptibility to pathogens (e.g. Cao and Lawrence,

Table 11.1 Effects of octopamine in insects. Not all effects occur in all species (Orchard *et al.*, 1993; Roeder, 1999, 2005).

Function	Direction of change
Respiratory rate	Increased
Heart rate	Increased
Lipid release (direct and/or indirect effects)	Increased
Responsiveness to sensory stimuli	Increased
Muscle tension	Increased (some muscles)
Energy metabolism	Increased glycolysis
Feeding	Decreased

Table 11.2 Effects of octopamine on insect immune function.

Immune function	Change	Reference
Susceptibility to bacterial infection	Increased	Adamo and Parsons (2006)
Haemocyte phagocytic ability	Increased	Baines *et al.* (1992)
Haemocyte motility	Increased	Diehl-Jones *et al.* (1996)
Nodule formation	Increased	Baines *et al.* (1992)
Number of circulating haemocytes	Increased (pharmacological dose) Decreased (physiological dose)	Dunphy and Downer (1994)
Phenoloxidase activity	No effect	Dunphy and Downer (1994) S.A. Adamo (unpublished results)

2002). The complexity of the effects of noradrenaline and other stress hormones on vertebrate immune function has prevented a clear adaptive explanation for these changes (Sternberg, 2006). Madden (2003), Maestroni (2005), and Kin and Sanders (2006) suggest that these complex effects are a result of noradrenaline playing a role in maintaining immune homeostasis (i.e. normal immune function). Octopamine may play a similar role in invertebrates. Octopamine is present in the haemolymph of resting insects (e.g. Adamo *et al.*, 1995). Although this may reflect the difficulty of taking blood from insects without stressing them, it may also indicate that octopamine is chronically present in the haemolymph. Octopamine has a half-life of 15 min or less in insect haemolymph (Goosey and Candy, 1982; Adamo, 2005). Therefore, it should be undetectable unless it is being released constantly. A background level of octopamine in non-stressed animals would be consistent with the hypothesis that octopamine helps to maintain normal immune function in invertebrates. However, if octopamine (in insects) and noradrenaline (in mammals) help to maintain immune homeostasis, why do the levels of both compounds increase dramatically during acute stress (e.g. Orchard *et al.*, 1993; Kin and Sanders, 2006)? In other words, how does an increase in the octopamine or noradrenaline concentration help to maintain optimal immune function in animals during acute stress?

11.2.1 Why does acute stress-induced immunosuppression exist?

Increased susceptibility to disease during flight-or-fight should reduce survival. As Dhabhar (2002) has pointed out, during fighting or fleeing, animals run a real risk of injury and, therefore, exposure to pathogens. Although it might make good adaptive sense to delay copulation, digestion, and egg-laying until the predator has passed, the immune response may not be dispensable during flight-or-fight behaviours, because of the increased risk of injury (Dhabhar, 2002).

Nevertheless, animals from three different phyla exhibit this pattern, suggesting that immunosuppression provides some benefit, despite its costs. Below I review some of the hypotheses about why animals display acute stress-induced immunosuppression. I assess which of these hypotheses fit the available data on insects. The hypotheses are not mutually exclusive.

11.2.1.1 The energy crisis hypothesis

One common hypothesis for the existence of acute stress-induced immunosuppression is that it allows animals to channel more energy into flight-or-fight behaviour (e.g. see Råberg *et al.*, 1998; Segerstrom, 2007). The increased energy is hypothesized to raise the odds of escaping a predator successfully, or of winning a fight. These benefits are thought to outweigh the increased risk of developing wound infections. However, if the suppression of energetically expensive physiological processes enhances the success of flight-or-fight, then other phenomena, such as red-blood-cell production in mammals, should also be suppressed. Red blood cells have a half-life of approximately 120 days in humans and take 7 days to form from precursor cells (Ganong, 1983, p. 422). Therefore, suppressing their production for a few hours would probably be less costly in terms of reduced survival than depressing immune function during flight-or-fight. Nevertheless, stress hormones such as glucocorticoids appear to increase erythropoiesis under some conditions (e.g. Kolbus *et al.*, 2003).

Furthermore, it is unclear whether stress-induced immunosuppression saves energy over the short term. For example, some mechanisms of stress-induced immunosuppression in vertebrates (e.g. apoptosis of precursor lymphoid cells leading to reduced lymphopoiesis; see Trottier *et al.*, 2008) require an initial increase in energy expenditure (Dhabhar, 2002). The need to suppress entire physiological systems to decrease energy demand may be more important for longer-term changes in energy expenditure (e.g. egg production) than for short-term flight-or-fight demands. Over the short term (e.g. minutes) insects do not seem to be energy-limited, even during intense activities such as flight (Chapman, 1998, p.220).

At present, there is little direct evidence supporting the energy-crisis hypothesis in insects.

11.2.1.2 The over-excitation hypothesis

Acute stress-induced immunosuppression may be beneficial because it prevents the immune system

from becoming too active and harming the animal during flight-or-fight. In vertebrates, intense exercise produces minor damage to tissues such as muscle, increasing the risk of an autoimmune reaction (Råberg *et al.*, 1998). Therefore, the vertebrate immune system shifts towards a less inflammatory state (Elenkov and Chrousos, 2006). This shift leads to a decrease in inflammation, but also leads to an increased susceptibility to bacterial and viral pathogens. The increased risk of infection is thought to be less than that of an autoimmune reaction. However, this key assumption remains untested.

Animals also run the risk of having an overactive immune response during an immune challenge. As would be predicted by the over-excitation hypothesis, an immune challenge also activates the acute stress response in vertebrates (Elenkov and Chrousos, 2006).

Like vertebrates, insects show some aspects of the acute stress response when they respond to an immune challenge. For example, some larval lepidotopterans (i.e. caterpillars) release octopamine when challenged with bacteria (Dunphy and Downer, 1994), although the source of this octopamine is uncertain (Adamo, 2005). However, in insects immune cell activity appears to be upregulated during acute stress (Table 11.2). Such upregulation does not support the over-excitation hypothesis.

11.2.1.3 The shift-in-focus hypothesis

This hypothesis suggests that during flight-or-fight behaviours animals are not immunosuppressed *per se*, but that they shift the focus of their immune effort from protection against systemic invaders to protection against opportunistic organisms that might gain entry through a wound (Dhabhar, 2002; Trottier *et al.*, 2008). However, the shift-in-focus hypothesis may not apply to insects. Octopamine does enhance haemolymph clotting in some arthropods (e.g. Battelle and Kravitz, 1978), although this effect has yet to be demonstrated in insects. Regardless of the effects of octopamine on haemolymph clotting in insects, flight-or-fight behaviour results in an increase in the risk of infection after wounding (Adamo and Parsons, 2006). With their stiff exoskeletons, insects may have less need of increased peripheral defence during flight-or-fight than do vertebrates. However, more studies on this issue are required to determine definitively whether insects express a shift in focus.

11.2.1.4 The resource crunch hypothesis

A number of physiological changes are needed to make flight-or-fight possible (Orchard *et al.*, 1993; Charmandari *et al.*, 2005). The resource crunch hypothesis suggests that some of these changes will result in a shift in resources away from the immune system, in order to optimize the flight-or-fight response. This hypothesis differs from the energy crisis hypothesis because it is not energy *per se* that is limiting, but specific molecules that are required for both immunity and some other physiological function.

The resource crunch hypothesis explains, at least in part, acute stress-induced immunosuppression in insects. In crickets, conflicts between immune function and lipid transport can lead to acute stress-induced immunosuppression (Adamo *et al.*, 2008). Crickets release octopamine during flight-or-fight behaviours (Adamo *et al.*, 1995). Octopamine, directly and/or indirectly, induces the mobilization of lipid from the fat body in order to fuel flight-or-fight behaviours (Orchard *et al.*, 1993). As lipid levels in the haemolymph increase, the protein apolipophorin III (apoLpIII) changes its conformation and combines with high-density lipophorin (HDLp) to form low-density lipophorin (LDLp), which has an increased lipid-carrying capacity (Figure 11.4; see Weers and Ryan, 2006 for review). However, in the unlipidated form, apoLpIII acts as an immune-surveillance molecule (Weers and Ryan, 2006). Once apoLpIII becomes part of LDLp, it appears to lose that ability. This loss results in a decline in immune surveillance (Adamo *et al.*, 2008). The decline in immune surveillance probably explains the increase in disease susceptibility that occurs immediately after flying and fighting (Adamo *et al.*, 2008). In crickets, intense activity leads to transient immunosuppression because apoLpIII is co-opted into lipid transport and becomes unavailable as an immune-surveillance molecule (Adamo *et al.*, 2008). Therefore, crickets become immunosuppressed during flight-or-fight, even if they have abundant energy stores (Adamo *et al.*, 2008).

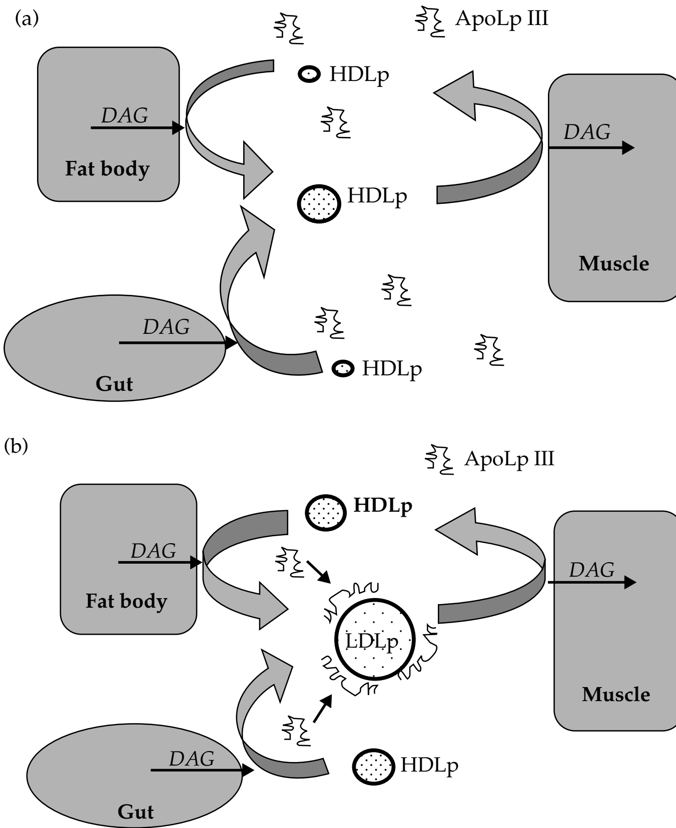


Figure 11.4 Lipid transport in Orthoptera. (a) Under normal conditions (e.g. when the insect is at rest), high-density lipophorin (HDLp) transports lipid (diacylglycerol, DAG) from the fat body and gut to the muscle. Apoliphorin III (apoLpIII) remains in the unlipidated form. (b) Under flight-or-fight conditions, apoLpIII undergoes a conformational change and combines with HDLp to form low-density liphophorin (LDLp). The amount of free apoLpIII in the haemolymph declines. Adapted from Weers and Ryan (2003).

The ability of octopamine to mobilize lipid probably explains why octopamine produces immunosuppression when it is injected into crickets. The injection of octopamine results in the release of lipid (Woodring *et al.*, 1989), which would lead to a decrease in immune surveillance as the amount of free apoLpIII in the haemolymph declines. However, octopamine also enhances the ability of haemocytes to respond to pathogens (Table 11.2). I hypothesize that octopamine helps maintain immune system function as some of the components of the immune system are being siphoned off into lipid transport. In other words, octopamine helps to liberate lipid stores (needed to fuel flight-or-fight behaviour) while simultaneously reconfiguring the immune system to maintain maximal function under the new physiological conditions. I predict that without the effects of octopamine on immune function, disease resistance would decline even more precipitously during flying or fighting. This hypothesis, if correct, would explain why octopamine can have both immunosuppressive and immunoenhancing effects.

Why do crickets not make enough apoLpIII to support both immune surveillance and increased lipid transport? First, it would be energetically expensive to do so. ApoLpIII is already a very abundant protein in the haemolymph of many adult insects (Weers and Ryan, 2006). To produce more of this protein would decrease the energy available for reproduction and other activities. Second, as the concentration of apoLpIII increases, it may begin to bind more promiscuously, initiating inappropriate immune responses. Such autoimmunity could be costly (e.g. Sadd and Siva-Jothy, 2006). Therefore, the most adaptive response may be to shuttle apoLpIII between immune surveillance and lipid transport, even though it results

in transient immunosuppression during flying or fighting.

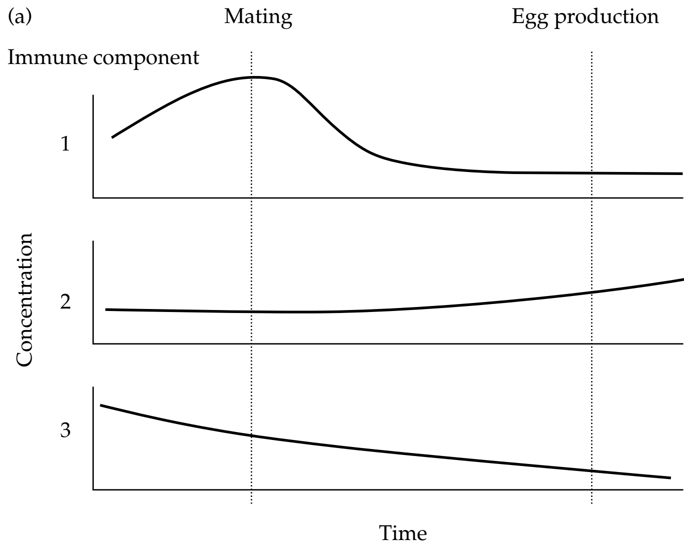
However, this particular resource crunch may not exist in all insects, because not all species use lipid to fuel flight-or-fight. For example, stressed cockroaches exhibit hypertrehalosaemia, not hyperlipidaemia, and injections of octopamine increase trehalose, not lipid, in the haemolymph (Downer, 1980). Therefore, I predict that cockroaches will not show a decline in apoLpIII during acute stress. Whether an immune response during flight-or-fight behaviours leads to other physiological conflicts in cockroaches remains unexplored.

Determining whether acute stress-induced immunosuppression in crickets is the result of a lack of energy or a lack of resources might seem superficially unimportant to evolutionary considerations about immune function (i.e. ecological immunology). However, an understanding of the physiological mechanisms responsible for a change in immune function is often critical for the design and the interpretation of experiments in ecological immunology. For example, if a researcher did not know that acute stress-induced immunosuppression is mediated by a conflict between lipid transport and immune function, it might be assumed that this immunosuppression was caused by insufficient 'energy' to fuel both flying and mounting an immune response. This false assumption would lead to the prediction that increased energy intake will decrease stress-induced immunosuppression. However, feeding a bolus of high-lipid (i.e. high-energy) food to crickets leads to increased immunosuppression (S.A. Adamo, unpublished results). The increased immunosuppression probably occurs because eating high-lipid foods increases the amount of lipid in the haemolymph (S.A. Adamo, unpublished results). The increased lipid, in turn, reduces the amount of free apoLpIII in the haemolymph, resulting in reduced immune surveillance and resistance to bacterial infection. Conversely, food deprivation would be expected to increase stress-induced immunosuppression. But the effects of food deprivation are likely to depend on whether it raises haemolymph lipid levels (e.g. by inducing the breakdown of fat stores) or lowers them. Therefore, whether a researcher finds evidence of an energetic constraint on immune function can depend on the experimental details (e.g. type of food used, duration of food deprivation), resulting in confusion in the literature.

11.3 The effects of physiological state on immune function can mask, mimic, or mediate trade-offs

Animals have limited resources. Selection should lead to the allocation of these resources in such a way as to maximize fitness. Following this logic, it has been suggested that insects, like other animals, increase the energy available for reproduction by depressing immune function (see Siva-Jothy *et al.*, 2005; Lawniczak *et al.*, 2007). Therefore insect ecological immunologists have searched for trade-offs between reproduction and immune function (Siva-Jothy *et al.*, 2005). Trade-offs occur when two traits are functionally connected, such that an increase in one trait leads to a reduction in the other (Zera and Harshman, 2001). Unfortunately, two traits can be negatively correlated even when there is no functional connection between them (Zera and Harshman, 2001). Without knowing the physiological mechanisms connecting two traits, it is impossible to know whether they are functionally linked (Harshman and Zera, 2007). This observation is especially true for trade-offs involving the immune system. Although a number of studies find the predicted negative correlation between reproduction and immune function (see Siva-Jothy *et al.*, 2005), other studies find no correlation (e.g. Schwarzenbach *et al.*, 2005). Some studies even find a positive correlation (e.g. Schwarzenbach and Ward, 2006; Shoemaker *et al.*, 2006; Wilfert *et al.*, 2007). Although it is possible to have a positive correlation between two traits connected by a trade-off (Zera and Harshman, 2001), the more plausible interpretation may be that immune function and reproduction are not functionally connected in some species.

Below I demonstrate how the shift in physiological state that occurs during reproduction can complicate the search for trade-offs between reproduction and immune function (Figure 11.5). In some cases, these shifts can mask or mimic trade-offs. In other cases, they may play a role in mediating them. For



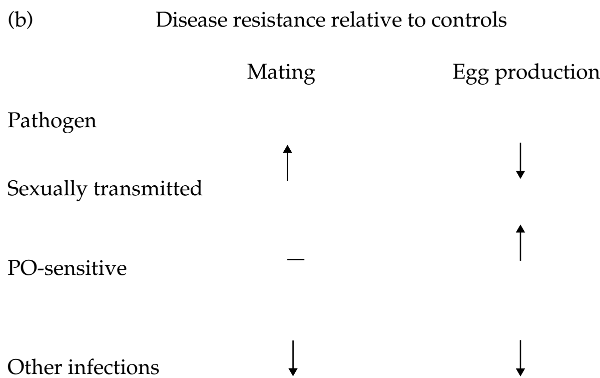


Figure 11.5 A schematic outline of possible relationships between immune function and reproduction. (a) In this model insect there are three immune components: 1, 2, and 3. Component 1 represents changes in local immunity within the female reproductive tract. Component 2 represents a factor like phenoloxidase (PO) that is used by both immune function and reproduction. Component 3 represents other immune functions. (b) The predicted effects on disease resistance due to changes in the concentrations of immune components 1–3 induced by matching and egg production as shown in panel (a). The changes in concentrations of 1, 2, and 3 result in changes in disease resistance. Note the mixed effect of reproduction on disease resistance.

example, if a reproductive hormone inhibits immune cells via specific receptors, then reproduction will be accompanied by a decline in immune function (eg. French *et al.*, 2007). In that case, reproductive physiology mediates the trade-off between reproduction and immune function. However, many interactions between reproduction and immune function may be less straightforward. For example, phenoloxidase (PO) is involved in the tanning of some insect eggs (Chapman, 1998; Kim *et al.*, 2005), and it is also an important component of the immune system (Kanost and Gorman, 2008). Superficially, PO activity would seem to be a good candidate for the study of potential trade-offs between reproduction and immune function in females, because it is required by both processes. However, prophenoloxidase (proPO) is synthesized only in haemocytes (e.g. in mosquitoes; Cho *et al.*, 1998; Kim *et al.*, 2005), and it is thought to be transported through the haemolymph to the ovary (Kim *et al.*, 2005). If the hypothesis of Kim *et al.* (2005) is correct, egg production will not reduce PO activity in the haemolymph unless its uptake by the ovary is faster than its production and release into the haemolymph. In fact, there could be a positive correlation between reproduction and immune function (i.e. PO activity) if the amount of PO in the haemolymph increases in order to supply the ovary with PO during egg production. Moreover, both haemolymph volume and protein content are altered during egg production in some species (Chapman, 1998). These changes make meaningful comparisons of enzyme activity per microlitre of haemolymph between egg-producing and non-egg-producing females difficult.

11.3.1 Assessing immune function across different physiological states

As discussed above (sections 11.2.1.4 and 11.3), researchers should be sensitive to the underlying physiology of the immune system and interconnected physiological systems when they design and interpret immune assays. Knowledge of the physiological details will help experimenters to determine the suite of immune measures they need to test their hypotheses. Single measures of immune function are known to give a poor overview of immune system health (Luster *et al*., 1993; Keil *et al*., 2001). For example, sexually active male *Drosophila melanogaster* remove non-pathogenic bacteria from their bodies more slowly than do males that lack mating opportunities (McKean and Nunney, 2001). McKean and Nunney (2001) concluded from these results that there is a negative trade-off between male sexual behaviour and disease resistance. However, Corby-Harris *et al.* (2007) found that the ability to clear bacteria from the haemolymph does not correlate with the ability to survive a bacterial challenge in *D. melanogaster*. Therefore, bacterial clearance appears to be a poor measure of disease resistance in this species (Corby-Harris *et al*., 2007). Nonetheless, the studies of McKean and Nunney (2001, 2008) suggest that male mating activity has some effect on immune function. There are at least three possibilities. First, there could be a trade-off between male sexual behaviour and disease resistance, as suggested by McKean and Nunney (2001, 2008). Bacterial clearance may decline concurrently with several other immune functions, resulting in reduced disease resistance. Second, male mating activity may lead to a reconfiguration of the immune system. Male mating behaviour is an energetic behaviour that probably activates the acute stress response. This response could lead to a reconfiguration of the immune system to compensate for the physiological changes that occur during active behaviour. Bacterial clearance rate declines, but other immune functions may be upregulated, such that disease resistance is maintained. Third, male mating activity could induce a shift in focus within the immune system, leading to increased resistance to pathogens that may be acquired during mating (i.e. sexually transmitted diseases). Note that these three possibilities run the range of possible functional connections between male sexual activity and disease resistance (i.e. a negative relationship, no relationship, and a positive relationship, at least for resistance to some pathogens). Moreover, the three explanations are not mutually exclusive. Mating activity could exert an immunosuppressive effect via an acute stress response. At the same time, mating activity could induce a shift in immune resources towards the reproductive tract and away from systemic immunity, enhancing resistance to sexually transmitted pathogens. An understanding of the ways in which mating alters immune function is required to disentangle these various possibilities (Figure 11.5). To start, it would be helpful to show that sexually active males are less disease-resistant by using a host-resistance test (i.e. that there is a change in the dose required to kill 50% of the flies using an ecologically valid pathogen).

11.3.2 Interpretation of assays of immune function

The correct interpretation of measurements of immune function (e.g. PO activity, haemocyte count, etc.) remains a problem for ecological immunologists (Adamo, 2004a; Martin *et al*., 2006). One complexity rarely noted is that an animal's physiological state can alter the relationship between an immune measure and disease resistance. For example, immediately after acute stress, an *increase* in some immune assays can correlate with a *decrease* in resistance to pathogens (Adamo, 2008b). Similarly, *D. melanogaster* females *increase* expression of immune-related genes after mating (Domanitskaya *et al*., 2007; Fedorka *et al*., 2007), but show a transient *decrease* in resistance to bacterial infection (Fedorka *et al*., 2007). The contradictory results found among studies on trade-offs between reproduction and immune function may be partly due to uncontrolled variations in the physiological state of the subjects (Harshman and Zera, 2007).

Another difficulty in measuring immune function arises because stressors, such as handling, can bias the result of immune assays (Ewenson *et al*.,

2003). For example, handling stress induces the release of octopamine in crickets (Woodring *et al.*, 1988). Therefore, studies that examine how different treatments influence immune function need to consider whether each group, including the control group, is exposed to the same level of stress. Otherwise, the observed differences in immune function may be due to non-specific stress effects, as opposed to the treatment being tested.

Not all immune responses are altered by acute stress in crickets (Table 11.2). Because individual immune responses are more important against some pathogens than others (Adamo, 2004b), changes in disease resistance induced by shifts in physiological state may be pathogen-specific. For example, the enzyme PO, a commonly assayed immune parameter, is critical for resistance against some pathogens such as viruses and multicellular parasites (Kanost and Gorman, 2008). However, *D. melanogaster* mutants that lack PO activity are as resistant as wild-type flies to bacteria and fungi (Leclerc *et al.*, 2006). Therefore, decreases in PO activity may not lead to decreases in resistance to those pathogens. During shifts in physiological state, animals may selectively suppress some immune functions while maintaining others to retain resistance against the pathogens that are the most serious threat to their fitness. This possibility may explain some of the inconsistencies in the literature (see Lawncizak *et al.*, 2007) regarding the relationship between immune function and reproduction.

The health status of an animal can also bias the results of an immunological test. Vertebrate immunologists reduce this problem by having strict quarantine regulations for bringing new animals into a colony, maintaining animals under almost antiseptic conditions, and periodically killing 'sentinel' animals and testing them for pathogens. Conditions are much less stringent in studies using insects. However, insect colonies commonly harbour pathogens (Fuxa and Tanada, 1987), and every laboratory has its own policies on bringing in new animals and their attendant diseases. It is not easy to recognize when an insect is suffering from a sublethal infection. The uncontrolled nature of pathogen exposure in a typical insect colony means that immune assay results are likely to show high variability within groups. Also, if one group has been exposed more to pathogens than another, this could cause large increases in some immune responses that might appear to be due to the treatment.

In addition, prior pathogen exposure can have long-term consequences on commonly monitored immune functions (Jacot *et al.*, 2005). For example, immune challenges in early life result in increased levels of PO and lysozyme-like activity in adult crickets; however, the encapsulation response is unaffected (Jacot *et al.*, 2005). The unequal effect of prior infection on different components of the immune system could lead to immune assay results that mimic a trade-off. For example, crickets from a crowded colony may be exposed to multiple immune challenges during development. These challenges would produce elevated lysozyme-like activity relative to the encapsulation response, creating the illusion of a trade-off between lysozyme-like activity and encapsulation.

It can be difficult to differentiate between a decline in immune function and a shift in focus within the immune system. However, some immune measures are frequently negatively correlated with one another (e.g. Rantala and Roff, 2005), and assays assessing both measures can help to make this distinction. If different functional assays all show a substantial decline with a treatment, then there is support for the hypothesis that there has been a decrease in disease resistance. However, discordant changes in immune parameters probably imply a reconfiguration of the immune system. Reconfiguration of the immune system does not necessarily signal a change in disease resistance (Keil *et al.*, 2001). If a researcher is faced with a lack of concordance among different immune measures, it may be necessary use live pathogens (i.e. a host-resistance test) to determine whether there has been a change in disease resistance. The pathogen selected for the host-resistance test should present a challenge to the host immune response thought to be most affected by the treatment or change in physiological state. The advantage of a host-resistance test is that it demonstrates unequivocally that a shift in immune function has resulted in a decrease in resistance to at least some pathogens. Unfortunately, a negative result from

a host-resistance test does not mean that disease resistance is unchanged. The wrong pathogen or the wrong dose of a pathogen might have been used (Luster *et al.*, 1993).

11.4 Conclusions

An understanding of the impact of physiological state on immune function requires detailed information on the immune system and interconnected physiological systems. Fortunately, our understanding of insect molecular biology and physiology continues to grow. For example, the hormonal links between reproduction and immune function in insects are becoming clearer (Flatt *et al.*, 2005; Flatt and Kawecki, 2007). These advances will help to refine our understanding of the proximate and ultimate causes behind immune-system responses to changes in physiological state.

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CHAPTER 12

Costs and genomic aspects of *Drosophila* immunity to parasites and pathogens

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12.1 Introduction

Virtually every organism will be attacked by parasites and/or infected by pathogens at some point in its life. The ubiquitous nature of parasites and pathogens means that selection pressures to evolve resistance mechanisms, in one form or another, will be common. The benefits of a resistance mechanism against parasites and pathogens (be it a physiological immune system or a behavioural mechanism) are obvious, as it will alleviate or prevent the fitness loss caused by the parasite or pathogen. However, resistance mechanisms can also bear costs and in such cases, the relative magnitudes of costs and benefits determine the strength of the overall selection pressure for, or against, a strong resistance mechanism.

Drosophila and its parasites and pathogens have been proven to be a valuable model system for understanding opposing selection pressures on resistance to parasites and pathogens. Most of the work has focused on the wide range of parasites and pathogens infecting *Drosophila melanogaster*, both in the field and in the laboratory, but other species (as detailed below) have also been included in several studies. Microbial pathogens such as fungi and bacteria are tackled by the humoral immune system, in which various antimicrobial peptides play a key role (Lemaitre *et al.*, 1997). The cellular immune system does play a role against microbial pathogens via phagocytosis, but is mostly used against macro-parasites which are too large to be phagocytosed (such as parasitoids). Macroparasites are encapsulated by a two-stage process consisting of envelopment of the parasite by blood cells, followed by the deposition of melanin (Lavine and Strand, 2002).

Costs of resistance come in two forms. First, when a host is parasitized or infected and launches an immune response, this will require energy and resources. Assuming energy and resources are limiting, once used in the activation of the resistance mechanism, they cannot be used for other purposes and this may have fitness consequences to the individual. Second, energy and resources will be necessary for constructing and maintaining the resistance mechanism (e.g. the immune system) in anticipation of future parasitism or infection. These are the costs of the *ability* to resist parasites or pathogens. Whereas the first form of cost is only paid when the individual is actually parasitized or infected, the second is paid by the individual irrespective of parasitism or infection. These two forms of cost are analogous to the costs of maintaining a standing army (second form), and the costs of taking this army to war (first form).

The approach mostly used for identifying and quantifying the costs of actual resistance, involves comparing unparasitized hosts with those parasitized hosts which have successfully combated the parasite or pathogen, in a range of fitness parameters. One problem here is that it is often difficult (if not impossible) to separate the effect that activating the resistance mechanism has on the host's fitness

from the pathogenic effect the parasite or pathogen may have on the host before being dealt with by the resistance mechanism. A powerful way to quantify and identify the costs of the resistance mechanism itself is artificial selection. Typically, a base population is subject to replicated selection for increased resistance. Selected lines are then compared, in the absence of parasitism, with the appropriate control lines in a range of fitness parameters. Differences observed are likely to be linked to the resistance mechanism, as the genetic background of control and selected lines coming from the same base population, are identical. Replication at the line level is essential in such experiments, to rule out an association between focus trait and correlated responses occurring due to hitchhiking or chance.

In the first section of this chapter, we focus on the parasites and pathogens known to attack or infect *D. melanogaster* (or other *Drosophila* species), from macro-parasites, such as parasitoids and mites to microbial (fungal and bacterial) pathogens to viruses. We concentrate on the main selection pressures for and against resistance against these parasites and pathogens. High resistance is selected for when the benefits it incurs outweigh its costs. For each parasite/pathogen, we summarize the existing knowledge on abundance in the field, fitness effects of parasitism/infection, and costs of resistance, where we will distinguish between costs of actual resistance and costs of the resistance mechanism.

In the second section of this chapter, we shift our focus to the genomic level. Over the last few years several papers have been published where microarrays have been used to investigate *D. melanogaster* resistance to parasites and pathogens. We ask what these studies are telling us about *Drosophila* resistance mechanisms and about the associated costs.

12.2 Parasites and pathogens of *Drosophila*

12.2.1 Macro-parasites

12.2.1.1 Parasitoids

In Europe, the most common parasitoid species attacking larvae of *D. melanogaster* (and related species) are the braconid *Asobara tabida* and the figitids *Leptopilina boulardi* and *Leptopilina heterotoma* (Carton *et al.*, 1986). In addition, the most common species attacking *Drosophila* pupae is *Pachycrepoideus vindemmiae* (Carton *et al.*, 1986). As, by definition, parasitoids kill their host as part of their normal development, the fitness loss for a *Drosophila* not defending itself against parasitoid attack and encapsulating the parasitoid egg is very high: death in the pupal stage; that is, before becoming reproductively active. Rates of parasitism by parasitoids can reach very high levels (up to 70%) in field populations of *Drosophila* (Carton *et al.*, 1991; Fleury *et al.*, 2004). Despite parasitoids being so common, and the fitness consequences of parasitoid attack being potentially severe, field populations of *D. melanogaster* show a considerable amount of variation in resistance against parasitoids, both between and within populations (Carton and Boulétreau, 1985; Kraaijeveld and van Alphen, 1995).

As explained above, costs of resistance, coupled with temporal and/or spatial variation in rates of parasitism, could explain the maintenance of this genetic variation in resistance. In *D. melanogaster*, surviving parasitoid attack has been shown to bear costs. Parasitized larvae have a lower competitive ability (Tiën *et al.*, 2001) than unparasitized larvae. After pupation, larvae which have successfully encapsulated the parasitoid egg have an increased risk of being attacked by pupal parasitoids (Fellowes *et al.*, 1998b). Adult flies which succeeded in encapsulating the parasitoid egg as larvae are smaller than flies which were not parasitized, with females having lower fecundity and males having lower mating success (Carton and David, 1983; Fellowes *et al.*, 1999a). Presumably, these costs are a result of the larva redirecting resources to its immune response, although pathogenic effects cannot be ruled out.

Investment in an immune system, in anticipation of being parasitized, is also costly in *D. melanogaster*. Using replicated artificial selection, Kraaijeveld and Godfray (1997) and Fellowes *et al.* (1998a) showed that high resistance against both *A. tabida* and *L. boulardi* can be selected for. Larvae from lines selected for increased parasitoid resistance, have higher levels of circulating haemocytes (Kraaijeveld *et al.*, 2001). However, this high

resistance is correlated with a reduced feeding rate and a reduction in competitive ability (Kraaijeveld and Godfray, 1997; Fellowes *et al.*, 1998a, 1999b). Interestingly, replicated selection for increased competitive ability leads to an *increase* in parasitoid resistance (Sanders *et al.*, 2005), possibly as a result of selection for increased wound-healing ability under crowded conditions. This shows that the trade-off between resistance and competitive ability is potentially asymmetrical. The trade-off appears more complex than one involving just haemocyte numbers and feeding rate, and traits other than these two may be involved.

As the pupal parasitoids attacking *Drosophila* are ectoparasitoids, their eggs do not come into contact with the host's immune system. The only barrier that parasitoids need to breach, the puparial wall, does not appear to be able to act as a 'resistance mechanism', as variation in the thickness of the puparial wall is not correlated to variation in risk of parasitism (Kraaijeveld and Godfray, 2003). Therefore, the only 'defence' that *Drosophila* pupae have against pupal parasitoids is to reduce the probability of being parasitized in the first place. The size of a pupa plays a role in its probability of being found and attacked by a searching parasitoid female (Kraaijeveld and Godfray, 2003), so a population under attack from pupal parasitoids is expected to evolve towards a smaller pupal size. Pupal and adult sizes are strongly correlated, so the cost of avoiding attack by pupal parasitoids is a reduction in adult size (Kraaijeveld and Godfray, 2003), leading to a decrease in a range of fitness parameters such as mating success, fecundity, and dispersal.

12.2.1.2 Mites

Drosophila nigrospiracula, which feeds on rotting cactus tissue, is susceptible to parasitism by the facultative ectoparasitic mite *Macrocheles subbadius*. The mites use flies as a means of dispersal, but also consume host haemolymph (Polak and Markow, 1995; Polak, 1996, 2003; Luong and Polak, 2007a, 2007b). The prevalence of mites in field populations is variable, but can reach levels of over 30% of flies infected (Polak and Markow, 1995). Infected flies suffer a reduction in longevity, fecundity, and mating success, with the size of the reduction depending on the number of mites attached (Polak and Markow, 1995; Polak, 1996).

Resistance of flies against the mites is not immunological, but behavioural: sudden movements are used to prevent mites getting a hold and tarsal flicking dislodges mites that have taken a hold (Polak, 2003). Genetic variation for this behavioural resistance exists in natural populations (Polak, 2003), which, as in the case of parasitoid resistance discussed above, suggests that behavioural resistance bears a cost.

The energetic costs of the movements and flicks are unknown, but selection for increased behavioural resistance leads to a decrease in larval competitive ability, adult body size, and fecundity (Luong and Polak, 2007a, 2007b), suggesting that the resistance mechanism is indeed costly.

12.2.1.3 Nematodes

Several species of nematodes are obligate parasites of *Drosophila*. Species of the *quinaria* and *testacea* groups feeding on decaying mushrooms are primarily attacked by nematodes in the genus *Howardula*, whereas species of the *obscura* group feeding on fermenting fruits are attacked by *Parasitylenchus diplogenus* (Welch, 1959; Montague and Jaenike, 1985; Jaenike, 1992). Parasitism starts with a single free-living worm entering a *Drosophila* larva, which is then followed by the production of one or two generations in the fly as it reaches adulthood. The worms then leave the fly abdomen in search of new host larvae to parasitize (Welch, 1959). In the case of *Parasitylenchus*, the abdomen of an infected fly can contain thousands of worms (A.R. Kraaijeveld, personal observation).

The abundance of nematodes in field populations can be as high as 35% of individuals of *quinaria* group species parasitized by *Howardula* (Montague and Jaenike, 1985). In the case of *Parasitylenchus*, rates of parasitism in flies of *obscura* group species have been reported as 3% (Gillis and Hardy, 1997), but can go up to 10% (A.R. Kraaijeveld, unpublished results). *D. melanogaster* is susceptible to infection by *Parasitylenchus* in the laboratory (Welch, 1959), but there are no records of infection of *D. melanogaster* by nematodes in field populations.

Infected flies suffer from increased mortality and, in the case of females, from being effectively

sterilized. This female sterilization occurs after infection with *Howardula* (Jaenike, 1992; Jaenike *et al.*, 1995) and *Parasitylenchus* (A.R. Kraaijeveld, unpublished results).

Resistance of *Drosophila* against parasitism by nematodes has not been recorded, although encapsulation is recorded in other Diptera (Stoffolano, 1973). As such, nothing is known about any resistance mechanism against nematodes that *Drosophila* may employ, or about the costs of resistance to nematodes.

12.2.2 Microbial pathogens

12.2.2.1 Fungi

In the laboratory, adults of *D. melanogaster* and related species are susceptible to entomopathogenic fungi such as *Beauveria bassiana*. Nothing is known about the rate of infection of *Drosophila* by *Beauveria* or other such fungi in the field, although genetic variation for resistance against *Beauveria* does exist both among and within natural populations (Tinsley *et al.*, 2006).

Typically, infected flies die 5–28 days after exposure to fungal spores (Fytrou *et al.*, 2006; Tinsley *et al.*, 2006; Kraaijeveld and Godfray, 2008). Once infected, flies continue to produce eggs for several days (Kraaijeveld and Godfray, 2008). The costs of launching an immune response against fungal infection are unknown, and it can not be ruled out that the use of resources for the production of the relevant antimicrobial peptides contributes to the early death of infected flies.

Artificial selection for increased resistance to *Beauveria* results in evolutionary change in *D. melanogaster*, but in an unexpected way. Interestingly, there is no difference in life span after infection between selection and control flies. Instead, infected selection flies continue laying eggs for longer than infected control flies (Kraaijeveld and Godfray, 2008). This results in the fecundity of infected selection flies, from infection to death, being higher than that of control flies, despite both types of flies succumbing to the fungus after the same amount of time. Thus, it seems that the selection regime selects for some kind of tolerance more than actual resistance. Even though the precise mechanism underlying this tolerance is still unknown, comparison of uninfected control and selection flies suggests a cost of tolerance: selection flies have lower lifetime fecundity than control flies when uninfected (Kraaijeveld and Godfray, 2008).

12.2.2.2 Microsporidia

Laboratory populations of *D. melanogaster* have been reported to be infected by a few species of Microsporidia, of which *Tubulinosema kingi* (formerly *Nosema kingi*) is the best studied (Armstrong and Bass, 1989a, 1989b; Franzen *et al.*, 2006; Futerman *et al.*, 2006; Vijendravarma *et al.*, 2008, 2009). Typically, larvae (younger instars are especially susceptible) ingest spores coming from infected cadavers; pathogen load remains low in subsequent larval instars, but increases rapidly in adult flies (Futerman *et al.*, 2006; Vijendravarma *et al.*, 2008). Nothing is known about the prevalence of *T. kingi* or other Microsporidia in natural *Drosophila* populations, and the only two infected flies reported from the field are likely to have been escapees from the nearby laboratory (Futerman *et al.*, 2006).

Infected flies show decreases in several fitness parameters, from developmental rate to survival probability to adult size (Armstrong and Bass, 1989a, 1989b; Futerman *et al.*, 2006). The fitness parameter which seems to suffer most after infection by *T. kingi* is fecundity, which is reduced by 33–66%, depending on *Drosophila* species (Armstrong and Bass, 1989b; Futerman *et al.*, 2006). Very little is known about the resistance mechanism of *Drosophila* against Microsporidia, although both cellular and humoral mechanisms appear to play a role in other insect species (Hoch *et al.*, 2004; Tokarev *et al.*, 2007). Nothing is known about the costs of launching an immune response (whatever this response may be) against Microsporidia.

Experimental evolution, in which fly populations were exposed to microsporidian spores on their food, resulted in evolutionary changes. As the resistance mechanism is unknown, fecundity was taken as a proxy measure of resistance in these experiments. Flies from selection lines suffer less of a reduction in fecundity after exposure to microsporidian spores than flies from control lines, and spore loads are lower in infected selection flies than in infected control flies (Vijendravarma *et al.*,

2009), suggesting that they indeed evolved higher levels of resistance.

Comparison of uninfected selection and control flies suggests that this increase in resistance bears costs: larvae from the selection lines are poorer competitors for food than larvae from the control lines (Vijendravarma *et al.*, 2009). On top of this, adult flies from the selection lines have lower early-life fecundity than adult flies from the control lines (Vijendravarma *et al.*, 2009).

12.2.2.3 Bacteria

Very little is known of the abundance of pathogenic bacteria in *Drosophila* populations in the field, but the overall bacterial community associated with *D. melanogaster* in natural populations appears to be quite variable (Corby-Harris *et al.*, 2007). Most work in the laboratory has used pricking with bacteria-infected needles as the means of inoculation, and this leads to high levels of mortality within a few days (Lazarro *et al.*, 2004). However, when natural (oral) means are used to inoculate flies with such seemingly highly pathogenic bacteria (e.g. *Serratia marcescens*), the bacterium does not appear to have any negative fitness effects (Lazarro *et al.*, 2004). However, one species of pathogenic bacterium has been identified which causes death to larvae and adults 1–4 days after oral inoculation: *Pseudomonas entomophila* (Vodovar *et al.*, 2005).

Genetic variation in resistance against bacteria after inoculation by 'dirty needles' has been found in natural populations (Lazarro *et al.*, 2004; Corby-Harris and Promislow, 2008). Using a genetic correlation approach, McKean *et al.* (2008) showed that resistance to the bacterium *Providencia rettgeri* has maintenance costs. In the absence of infection, there was a negative correlation among families between fecundity and resistance. Interestingly, this cost is environment-dependent, as it is only found when food is limited and not when food is plentiful. They also reported a cost of actual resistance, but this seems to be a cost of wounding the fly during the infection process rather than the cost of the immune system launching a response against the bacterial infection itself.

Wolbachia is an unusual bacterium in that it is intracellular. It is a widespread symbiont of arthropods and nematodes, and induces male-killing, feminizing, and/or cytoplasmic incompatibility (Werren, 1997) in its host in order to further its own spread in the host population. Here we will focus solely on the bacterium as a potential pathogen of the individual it has infected. *Wolbachia* infection in *D. melanogaster* populations is common and widespread (Fry *et al.*, 2004; Riegler *et al.*, 2005). The fitness effects it has on *D. melanogaster* and related species (e.g. *Drosophila simulans*) are variable, but reductions in size, fecundity, sperm competitiveness, and immune response to parasitoid eggs have all been reported (Fry *et al.*, 2004; Champion de Crespigny and Wedell, 2006; Fytrou *et al.*, 2006). Whether *Drosophila* launches an immune response to combat *Wolbachia* infection is unknown, and so nothing is known about any resistance mechanism nor about any costs of resistance.

12.2.2.4 Viruses

At least half a dozen RNA viruses are known from natural populations of *D. melanogaster*, with up to 40% of flies infected (Carpenter *et al.*, 2007). C virus is not pathogenic after natural (oral) infection, although it is highly pathogenic when injected (Thomas-Orillard *et al.*, 1995). An additional effect of the virus on its host is to *increase* fecundity (Thomas-Orillard *et al.*, 1995). Infection by X virus renders flies very sensitive to lack of oxygen (death occurs when flies are exposed to pure carbon dioxide; Zambon *et al.*, 2005). Sigma virus is host-specific to *D. melanogaster* and widespread in natural populations (Carpenter *et al.*, 2007). Unlike the other viruses, it is vertically transmitted. Flies infected by Sigma virus suffer the same effect as those infected by X virus (extreme sensitivity to anoxia) and, in addition, infected eggs have a lower viability and infected adults a lower survival (Carpenter *et al.*, 2007; Tsai *et al.*, 2008).

The resistance mechanism of *D. melanogaster* against viruses is not fully understood, although several immune pathways (Toll, Janus kinase/signal transduction and activators of transcription (JAK/STAT)) appear to be involved (Dostert *et al.*, 2005; Zambon *et al.*, 2005; Tsai *et al.*, 2008). Nothing is known about the costs of mounting an immune reaction against viruses or of the costs of the antiviral resistance mechanism itself.

12.2.3 Summary: similarities and differences

It is clear that *Drosophila* in natural populations is subject to attack from a range of parasites and pathogens. Of the macro-parasites, parasitoids are common and widespread, and *D. melanogaster* will often have to face parasitoid attack. Abundance data on mites are known from one species of *Drosophila*, but how often other species, especially *D. melanogaster*, are subject to mite attack is unclear at present. Nematode parasitism is relatively common in *quinaria* and *testacea* group species on mushrooms, but data on rates of parasitism on fermenting fruits are much more scarce; no records exist of *D. melanogaster* parasitized by nematodes in the field.

Although *Drosophila* in general, and *D. melanogaster* specifically, is susceptible to a range of microbial pathogens in the laboratory, knowledge is limited as to how often they are infected with microbial pathogens in the field. *Wolbachia* and viruses appear to be common and widespread, but little or nothing is known about rates of infection by entomopathogenic fungi, microsporidia, and bacteria in natural populations.

The fitness consequences of being attacked or infected by parasites or pathogens vary widely. At one end of the scale, infected individuals face either quick death (as in the case of pathogenic bacteria, although this is often linked to unnatural methods of inoculation) or, if they do not die soon after infection, genetic death (as in the case of parasitoids and nematodes, who either kill their host before it becomes reproductively active or sterilize it). Reductions in a range of fitness parameters, including fecundity, longevity, and mating success, occur after parasitism by mites and infection by fungi and microsporidia; fitness effects of infection by *Wolbachia* appear to be variable. At the other end of the scale are viruses, which appear to have little or no negative effects on the fitness of their hosts (the sensitivity to anoxia reported in laboratory circumstances is unlikely to be important in the field).

Once an individual is attacked or infected, it launches an immune response. Only in the case of parasitoids do data exist to show that this actual resistance is costly (Table 12.1, first column), in that individuals that successfully encapsulate the parasitoid egg are poorer larval competitors, more likely to be attacked by a pupal parasitoid, and smaller adults (with negative knock-on effects on other fitness parameters). The nature and level of costs of actual resistance against mites, nematodes,

Table 12.1 Summary of fitness parameters identified in *D. melanogaster* associated with costs of actual resistance and costs of ability to resist against a range of parasites and pathogens; see text for references.

Parasite/pathogen	Cost of *actual* resistance	Cost of *ability* to resist
Larval parasitoids	Larval competitive ability Susceptibility to pupal parasitoids Adult size Fecundity Male mating success	Larval competitive ability
Pupal parasitoids	?	Adult size
Mites	?	Larval competitive ability Adult size Fecundity
Nematodes	?	?
Fungi	?	Fecundity
Microsporidia	?	Larval competitive ability Fecundity
Bacteria	?	Fecundity
Viruses	?	?

fungi, microsporidia, bacteria, and viruses are as of yet unknown.

Resources need to be spent on having a resistance mechanism, and ready to launch an immune response when parasitism or infection actually takes place. Table 12.1 (second column) summarizes the fitness traits identified as costs of the ability to resist. Interestingly, very similar costs are found in the ability to resist very different parasites/pathogens, which involve very different immunological pathways (and in one case is not even an immunological mechanism at all, but a behavioural one). Larval competitive ability is found as a cost of a resistance mechanism against parasitoids, microsporidia, and mites. A reduction in adult fecundity is a cost of the resistance mechanism against fungi, microsporidia, bacteria, and mites. These similarities suggest that re-allocation of resources at a very basic level plays a key part in the costs of resistance in *D. melanogaster*.

12.3 Genomics of the immune response

So far we have discussed resistance traits and costs at the phenotypic level. To better understand the mechanisms of resistance and its costs, we can also investigate the genetic regulation of these traits and trade-offs. The molecular and genetic mechanisms underlying *Drosophila* immunity have been studied extensively. An important reason is that the signalling pathways and immunity genes in the *Drosophila* defence responses against bacteria, fungi, and viruses are highly conserved in vertebrates, making *Drosophila* an excellent model system for studying innate immune responses (Brennan and Anderson, 2004; Wang *et al.*, 2006). Since the completion of the genomic sequencing of *Drosophila* in 2000, the application of several post-genomic techniques has contributed considerably to our insight into the genes associated with immunity and defence. Moreover, genomic tools make it possible to study whole genetic networks in parallel, and to search for indications of interactions between signalling pathways. The costs incurred by launching an immune response may be due to genetic interactions with other genes and pathways, where (the change of expression in) one gene can be responsible for a range of phenotypic effects. In this section, we summarize the advances that post-genomic tools have provided in *Drosophila* immunity, and the genetic interactions that likely reflect some of the associated costs.

12.3.1 Genomic approaches to studying immunity signalling pathways

The innate immune responses in *Drosophila* against parasites and pathogens are regulated through several signal transductions pathways, in particular the Toll, Imd, and JAK/STAT pathways. These pathways are activated in a parasite-/pathogen-specific manner, and it was recognized before the genomic era that the pathways interact for several types of infection (e.g. Lemaitre *et al.*, 1996). Post-genomic technology has been successful in further elucidating components of these pathways, the interactions, and other genes and pathways involved in the regulation of the immune response.

12.3.1.1 Transcriptomics

Transcriptomic data describe the levels of gene expression by measuring the relative abundances of all mRNAs within a biological sample. Comparing infected and uninfected samples yields information on the genes for which expression is up- or downregulated in response to the immune challenge. Microarrays have been used to capture the genome-wide transcriptomic changes in *Drosophila* during the immune responses against most of the pathogens and parasites that can infect them (bacteria and fungi, De Gregorio *et al.*, 2001; bacteria and fungi, Irving *et al.*, 2001; bacteria, Boutros *et al.*, 2002; bacteria, fungi, microsporidia, and viruses, Roxström-Lindquist *et al.*, 2004; viruses, Dostert *et al.*, 2005; parasitoids, Wertheim *et al.*, 2005; parasitoids, Schlenke *et al.*, 2007; *Wolbachia*, Xi *et al.*, 2008).

One of the primary strengths of transcriptomic data is that they enable identification of a variety of genes that are involved with, affected by, or associated with an immune response, and their sequence of action. Although a change in expression is insufficient evidence for involvement in immunity without experimental validation, transcriptomic data are valuable as a first step in identifying novel genes

with a putative role in the genetic control of immunity. The first transcriptomic studies focused on the antimicrobial responses, which involve primarily the humoral immune response. These studies yielded several hundred novel putative immunity genes that are still being characterized in follow-up studies (e.g. Maillet *et al*., 2008). Parasitism induces the cellular immune response, which is less well studied than the humoral immune response. Comparing the expression in parasitized and control larvae at nine time points after attack by the braconid parasitoid wasp *A. tabida*, revealed approximately 160 genes as being differentially expressed after parasitoid attack, most of which had not previously been associated with immunity functions (Wertheim *et al*., 2005).

To get an indication of the functional roles of (novel) genes in the immune responses, comparisons with the simultaneous expression patterns of all other genes may provide an additional benefit of microarrays. Several suites of genes responded similarly across time after parasitoid attack, and shared functional annotations to a larger degree than expected by chance. For example, a group of genes involved in proteolysis and peptidolysis was upregulated during the encapsulation/melanization phase of the immune response (Wertheim *et al*., 2005). For unannotated genes within such suites of co-expressed genes, the similarity in their expression pattern to genes with annotations provides a starting point for putative functional annotations.

We can also use expression data to screen for candidate proteins with specific functional domains that are relevant during a certain stage of the immune response. This may provide information on the key genes for particular processes, but may also reveal new insights in the nature of the processes itself. For example, to recognize invading organisms the *Drosophila* genome codes for various types of pattern-recognition receptors (PRRs), such as lectins and receptors for microbial peptides. Non-self recognition by PRRs leads to triggering of immune-signalling pathways. This avoids the costs of constitutive immune defences and ensures the production of the appropriate defence molecules for a particular type of infection. During the first few hours after parasitoid attack, two out of the 20 peptidoglycan-recognition proteins (PGRP-LB and PGRP-SB1) were differentially expressed, while during the encapsulation phase one of the 30 C-type lectins in the *Drosophila* genome (*lectin-24A*) showed a striking increase in expression (Wertheim *et al*., 2005). The PGRP molecules recognize a component in the bacterial cell wall, and their increased expression may be a response to low-level microbial infections following the puncturing of the cuticle by the parasitoid. Lectins not only function in recognition, but are also thought to be important in changing cell-adhesion properties. It is therefore plausible that lectin-24A is a key player in recruiting the haemocytes to the parasitoid egg, and the subsequent formation of the multilayered cellular encasing of the egg. Another transcriptomic study compared the expression in *Drosophila* larvae after attack by two different parasitoid species (the figitids *L. boulardi* and *L. heterotoma*; Schlenke *et al*., 2007). Both species can successfully suppress encapsulation by *Drosophila*, but *L. boulardi* appears to invoke a complete immune response that is only sabotaged at the final stage, whereas *L. heterotoma* appears to achieve a near-complete lack of a transcriptional immune response. Interestingly, *L. boulardi* induced a similar massive upregulation of the same *lectin-24A* gene during the first 2–5 h after infection, while the lectin was not upregulated at all after attack by *L. heterotoma*.

Another merit of transcriptomic data is that it can help unravel genetic networks and interactions. These network interactions can be crucially important in understanding the regulation of immune responses and the associated costs. By applying bioinformatic approaches to the transcriptomic data, suites of genes with simultaneously changing expression patterns can be investigated for shared regulatory elements (e.g. promoter or enhancer sites), which could indicate that the genes are under control of the same (co-)transcription factors. Several suites of genes that were differentially expressed genes at various stages after parasitoid attack, harboured a significant over-representation of three transcription factor DNA-binding motifs (TFBMs) in their upstream regions (for *Stat92E*, *NFκB*-like, and *serpent* (*srp*)) (Wertheim *et al*., 2005). In many cases, the upstream regions contained several replicates of two or three of these TFBMs,

suggesting that the pathways could jointly regulate the expression of target genes. Transcriptomic studies after microbial infection in single and double mutants for the Toll, Imd, and JAK/STAT pathways also showed that regulation of target genes during an immune response can be compensated for by the other pathway (redundancy), or can partially or wholly depend on both pathways (co-regulation, cross-regulation, or synergism; De Gregorio *et al.*, 2002; Brun *et al.*, 2006). Transcriptomic studies also identified the c-Jun N-terminal kinase (JNK) pathway as a separate branch of the Imd pathway, and its involvement in immune responses (Boutros *et al.*, 2002; Silverman *et al.*, 2003; Park *et al.*, 2004). These findings all support the existence of extensive genetic interactions among the various immunity pathways.

12.3.1.2 Proteomics

This technique compares and describes all proteins (and their modified varieties) among biological samples (e.g. infected and uninfected). Proteomic studies have focused mainly on the peptides in the haemolymph after microbial or fungal challenge (Levy *et al.*, 2004; Loseva and Engstrom, 2004; Vierstraete *et al.*, 2004a, 2004b; de Morais Guedes *et al.*, 2005). Proteins are the final gene products, and proteomics thus provides a more direct measurement of the 'actors' in the immune response. Proteomics is complicated by the need to analytically determine the identity of each expressed protein in the sample while relying on incompletely developed databases for doing so, it requires minimized variation in cell types, necessitating tissue-specific analyses, and it is less sensitive to small changes in abundance. However, in contrast to transcriptomics, these measurements also incorporate the effects of post-transcriptional regulation and post-translational modifications. Therefore, this technique provides important additional information on the regulation of the innate immune response, including proteolytic cascades.

A proteomic study identified 37 instantly released peptides after immune challenge that were not induced after sterile injury (Vierstraete *et al.*, 2004b). Insects can stockpile proteins in a pre-active stage that can be immediately deployed in the event of an invasion, through, for example, phosphorylation and cleavage. Proteolytic cascades often form the start of signalling pathways, and the activity of such peptides cannot be detected by transcriptomic studies (although the subsequent replenishment of the proteins may be measured).

12.3.1.3 RNA interference (RNAi) screens

Finally, RNAi screens have been used for the elimination of specific genes, to investigate their roles in the immune response. With this technique, genes are silenced post-transcriptionally by introducing double-stranded RNA for a target gene, resulting in the degradation of the targeted mRNAs. The effect of the loss of function for each gene is then measured in a screen for phenotype or signalling pathway activity (e.g. using reporter constructs to visualize whether the target genes of the pathway are switched on). One large advantage of this technology is that it already includes a degree of experimental validation. In *Drosophila*, it has been used to investigate immunity signalling pathways or functional groups of molecules (Imd pathway, Foley and Farrell, 2004; JAK/STAT pathway, Muller *et al.*, 2005; serine proteases, Kambris *et al.*, 2006). The RNAi constructs can be introduced most easily to cell cultures, but can also be used *in vivo* in whole organisms (reviewed in Boutros and Ahringer, 2008).

A genome-wide RNAi screen was used on a *Drosophila* haemocyte-like cell line and identified approximately 90 novel genes that were under influence of the JAK/STAT pathways (Muller *et al.*, 2005). To identify the components and negative regulators of the Imd pathway and their relative position in the pathway, an RNAi screen was performed on a reporter cell line that was exposed to lipopolysaccharide, a bacterial cell-wall component known to trigger the Imd pathway. This study not only revealed several novel components, but also many inhibitors that either keep the pathway inactive in the absence of infection, or downregulate the response to infection (Foley and Farrell, 2004). RNAi was also applied *in vivo* against 75 serine proteases in the *Drosophila* genome to study the serine protease cascade upstream of the *Toll* receptor. The study identified five serine proteases that are required for activation of the pathway (Kambris *et al.*, 2006).

12.3.2 Comparison of the genome-wide studies

The various genome-wide studies all used different assays and control treatments, statistical criteria for inclusions in gene lists, and a range of pathogens or parasites. These differences between assays also provided important insights. For example, in the bacterial and viral infection assays, the pathogenicity as well as the number of differentially expressed genes were markedly less when infection was induced by feeding, compared to injury with a septic needle (Irving *et al.*, 2001; Roxström-Lindquist *et al.*, 2004; Dostert *et al.*, 2005). This marked difference suggested that the epithelial barrier was largely influential as a first line of defence, which has indeed been confirmed in other studies (reviewed in Lemaitre and Hoffmann, 2007).

A comparison of the differentially expressed genes across the various studies also illustrates the degree of specificity for *Drosophila* immune responses. Although some immunity genes change expression after most types of immune challenge (*dorsal*, *Spätzle*, *Relish*, *IM2*, *attacin-A* and *-B*, *Metchnikowin*, *Jonah 25Bii*, *CG6687*), the majority of genes are only reported as induced after one or few immune challenges (Table 12.2). For the PRRs, we find the expected specificity, but some overlap too: many different immune challenges induce the expression of PGRP-SA, which can activate the Toll pathway in response to Gram-positive bacteria, but not to fungi (Michel *et al.*, 2001). The Gram-negative-binding proteins (GNBPs) appear to be induced after various types of microbial infection, while lectins are upregulated after infection with various macro-parasites. A large family of serine-protease-like proteins in the *Drosophila* genome (with catalytic site (serine proteases) or without (serine protease homologues, SPHs)) and their inhibitors (serpins) form proteolytic cascades. An extensive comparison of the 201 trypsin-like serine proteases also included the published transcriptomic data after infection with either bacteria/fungi or parasites/parasitoids (Shah *et al.*, 2008). The authors reported that half of the serine protease-like proteins were differentially expressed during immune responses, with a subset induced upon infection by either microbes or parasites and a subset affected by only one type of immune challenge (Shah *et al.*, 2008). Some serine proteases and serpins were massively upregulated after various immune challenges (*CG6639*, *CG6687*, *CG18563*), while others responded to one challenge only (*CG4793*, *CG7219*, *CG18477*, *Jonah 99Fi*). Among the serine proteases with monophenol mono-oxygenase activity, the overlap seems to be larger across immune challenges (*CG3066*, *Cyp4p3*, and *SPE* are induced by three different immune challenges). The intracellular infections (viral and *Wolbachia*) appear to barely change the expression in any of the serine proteases or prophenoloxidase (proPO) genes, although they do show upregulation of the antimicrobial peptides. Additionally, they show changes in the expression for genes related to temperature stress (various heat-shock proteins, *Dna-J*, and *Frost*). The latter molecules bind to unfolded proteins or ATP, and are important for refolding or decondensing (loosening) of chromosomal sites. It is unclear whether these changes in expression reflect a stress response to the infection or an immunity reaction. Finally, the *Drosophila* genome contains six thioester-containing protein (Tep) genes, three that are upregulated after infection under the control of the JAK/STAT pathway and are thought to act as opsonins (Lagueux *et al.*, 2000). The transcriptomic data seem to suggest that *Tep1* is only upregulated in parasitized larvae. This is misleading, as *Tep1* is also upregulated after microbial challenge in larvae (Lagueux *et al.*, 2000), but the antimicrobial microarray experiments were all performed on adults.

One caveat of these studies is that they were performed on whole organisms or cell lines, while enormous differences in expression exist among tissues. Although tissue-specific analyses in expression data are not as crucial as in proteomic data, expression differences within a tissue can be completely obscured in whole-fly analyses (Chintapalli *et al.*, 2007). This problem has implications for both the sensitivity of the analysis, as well as for the detection of tissue-specific effector genes. For example, the Toll pathway is required for the production of antimicrobial proteins in the fat body, whereas in the lymph gland it induces the production of haemocytes (Qiu *et al.*, 1998). One example where tissue-specificity was taken into

Table 12.2 Genes with changed expression after infection. The table was composed from the published gene lists, using the criteria of the respective authors. Listing implies that the gene was considered to be differentially expressed in at least one study, although other studies may not have found this. Genes with a highest reported fold change of more than 8-fold are shown in bold.

Molecular function	Bacteria[1,2,3,4]	Fungi[1,2,4]	Viruses[4,5]	*Wolbachia*[4,6]	Microsporidia[4]	Parasitoids[7,8]
Pattern-recognition receptors (PPRs)	**GNBP-like (CG13422)**	GNBP-like (CG12780)	GNBP-like (CG12780)		CG12780	CG2736
	GNBP-like (CG12780)	**GNBP-like (CG13422)**	PGRP-SA		Lectin-33A	GNBP-like (CG13422)
	GNBP-like	PGRP-SA			Lectin-37Da/Db	**Lectin-24A**
	PGRP-LB	PGRP-SC2				PGRP-LB
	PGRP-LC	**PGRP-SD**				PGRP-SA
	PGRP-LF					PGRP-SB1
	PGRP-SA					PGRP-SD
	PGRP-SB1					αPS4
	PGRP-SC2					Santa-maria (CG12789)
	PGRP-SD					
Immunity signal transduction pathways	Cactus	Cactus	Dome	Dorsal		Cactus
	Dif	Dorsal	Dorsal	dJun		CG14225
	Dorsal	Kayak	Relish	Ird5		Dome
	Necrotic	Necrotic	Thor	Puckered		Embargoed
	Pelle	Pelle	Spätzle	Relish		Hop
	Relish	Relish		Spätzle		Necrotic
	Spätzle	Spätzle				Nup214
	Thor	Stat92E				Pelle
	Toll	Thor				Relish
		Toll				Stat92E
						Toll
Antimicrobial peptides	Andropin	Andropin	Attacin-A	Attacin-A		Attacin-A
	Attacin-A	Attacin-A	Attacin-B	Attacin-B		Attacin-B
	Attacin-B	Attacin-B	Attacin-C	Attacin-C		Attacin-D
	Attacin-C	Cecropin-A1	Cecropin-A1	Attacin-D		Cecropin-C
	Attacin-D	Cecropin-A2	Cecropin-A2	Diptericin-B		CG15065
	Cecropin-A1	Defensin	Diptericin-B			**CG15066 (IM23)**
	Cecropin-A2	**Drosomycin**	Drosomycin			**CG18279 (IM10)**
	Cecropin-B	Drosomycin-5	IM2			**IM1**
	Cecropin-C	**IM1**	IM3			IM2
	CG15066 (IM23)	IM2	Metchnikowin			IM3
	CG18279 (IM10)	IM2-like				**IM4**
	Defensin	**Metchnikowin**				Metchnikowin
	Diptericin					

Table 12.2 *Cont.*

Molecular function	Bacteria[1,2,3,4]	Fungi[1,2,4]	Viruses[4,5]	*Wolbachia*[4,6]	Microsporidia[4]	Parasitoids[7,8]
	Diptericin-B					
	Drosocin					
	Drosomycin					
	Drosomycin-5					
	IM1					
	IM2					
	IM3					
	IM2-like (CG15065)					
	IM4					
	Metchnikowin					
Defence/stress response	Hsp26	CG4164	**Frost**	Hsp70Aa/Ab		Hemolectin
	Hsp68	**Frost**	Hsp70Bc	Hsp70Ba/b/Bc/Bbb		Hsp60
	Hsp70Bc	Peroxidasin		Hsp68		Hsp83
	Peroxidasin	**TepII**		Hsp67Bc		mthl2
	TepII	TepIV		Hsp27		Peroxidasin
	TepIV	Tollo		Hsp22		**TepI**
	Tollo	**TotM**		DnaJ-1		TepII
	TotM	Transferrin 1				TepIV
	Transferrin 3	Transferrin 3				**TotA**
						TotB
						TotC
Trypsin and serine protease-like (SP and SPH)/serpins	Acp67A	CG2045 (Ser7)	CG6687		Acp67A	CG2056 (spirit)
	CG2045 (Ser7)	CG2105 (Corin)	Jonah 25Bi		CG2229	CG2105 (Corin)
	CG2056 (spirit)	CG2145	Jonah 25Bii		CG6289	CG3117
	CG2105 (Corin)	CG3505	Jonah 99Ci		CG6663	CG3344
	CG2229	CG3604			CG7542	CG3505
	CG3505	CG5246			CG8952	CG3916
	CG3604	**CG5909**			CG9564	CG4053
	CG5909	**CG6639**			CG10477	CG4259
	CG6361	CG6687			CG16749	CG4653
	CG6467	CG7219			CG17571	**CG4793**
	CG6639	CG8738			CG18180	CG5246

CG6687
CG7219
CG7695
CG8215
CG8571 (smid)
CG8952
CG9631
CG9645
CG11459
CG11836
CG11841
CG11842
CG12558
CG15046
CG16030
CG16713
CG18180
CG18563
CG31326/
CG33109
CG33836
Jonah 25Bii
Jonah 25Biii
Jonah 99Cii
Spn27A (CG11331)
Spn42C
Spn88E
Spn43Ad
Spn3
Spn4
Spn5

CG8952
CG9372
CG9645
CG9649
CG10031
CG10586
CG10882
CG11459
CG11841
CG11842
CG11843
CG11911
CG16704
CG16712
CG16713
CG16997
CG18563
CG31199/
CG31200
CG31326/
CG33109
Spn43Ad
Spn4
Spn5

\Jonah 25Bi
Jonah 25Bii
Jonah 25Biii
Jonah 65Aiii
Jonah 65Aiv
Jonah 74E
Jonah 99Cii
Jonah 99Ciii
Jonah 99Fi
Trypsin 29F

CG6041
CG6639
CG6687
CG9240
CG9673
CG9675 (spheroide)
CG11912
CG12951
CG16704
CG16712
CG16713
CG17278
CG17475
CG17477
CG17572
CG18477
CG18478
CG18563
CG30414
CG30086
CG30090
CG30371
CG31266
CG31269
CG31780
CG31827
CG32374
CG32376
CG32483
CG33127
Jonah 25Bii
Jonah 65Aii
Jonah 65Aiii
Tequila
Trypsin (CG18681)
λTrypsin (CG12350)

Table 12.2 *Cont.*

Molecular function	Bacteria[1,2,3,4]	Fungi[1,2,4]	Viruses[4,5]	*Wolbachia*[4,6]	Microsporidia[4]	Parasitoids[7,8]
Prophenoloxidase cascade (proPO): mono/diphenol oxidase activity and melanin intermediates (including some serine proteases)	CG1102	CG3066 (Sp7)	Cyp4p3		Cyp4d21	CG3066 (Sp7)
	CG3066 (Sp7)	CG16705 (SPE)				CG9733
	CG9733	Cyp4p3				CG11313
	CG16705 (SPE)	Ddc				CG16705 (SPE)
	Cp19	pale				Cyp4e3
	Cyp26d1					Cyp12e1
	Cyp4p3					Cyp309a1
	Cyp-like					Cyp6a17
	Ddc					Cyp12a5
	Dihydropteridine reductase (Dhpr)					Cyp12a4
	Laccasse-like (CG3759)					Cyp9f2
	Pale					Cyp9c1
	Punch					Dox-A3
	yellow-f					Dihydropteridine reductase (Dhpr)
						Fmo-2
						yellow-c
						yellow-f
						yellow-f2
						yellow-g

[1]DeGregorio *et al.* (2001), Table 1; [2]Irving *et al.* (2001), Tables 1 and S2; [3]Boutros *et al.* (2002), Figure S4; [4]Roxström-Lindquist *et al.* (2004), Tables 1 and S2; [5]Dostert *et al.* (2005), Table S1; [6]Xi *et al.* (2008), Figures 4 and 5; [7]Wertheim *et al.* (2005), Tables 2 and S1, excluding cluster 9; [8]Schlenke *et al.* (2007), Tables S2a and S3, exclusing Lh vs Lb.

account was a genome-wide transcription study on the different types of haemocytes in larvae (Irving *et al.*, 2005). This study showed that after infection some immunity genes are differentially expressed in the haemocytes but not in the fat body (e.g. *Spätzle*, *attacin-D*, *cecropins B* and *C*), some are not differentially expressed in haemocytes (e.g. *necrotic*), and some are expressed exclusively in a particular type of haemocyte (e.g. *DoxA3* and *αPS4* in lamellocytes).

Another important aspect of genomic studies is the use of appropriate experimental controls. As the technique (both transcriptomics and proteomics) relies on thousands of comparisons of relative abundances, rather than on actual quantification, it is vitally important to ensure that the only difference between a treated and a control sample is the treatment. For example, gene expression follows diurnal rhythms; it changes strongly over the course of a life (even at a day-to-day scale); and it may respond to handling and/or measurements (McDonald and Rosbash, 2001; Pletcher *et al.*, 2002). In the case of a zero-hour control sample compared to a 12 h post-infection sample, for example, it becomes impossible to distinguish between genes that changed expression due to the infection and due to diurnal rhythms. To avoid such confounding effects, ideally control and infected samples should be collected in parallel and treated exactly the same throughout handling, except for the actual infection.

12.3.3 Microarrays can indicate costs of immunity

In many of the transcriptomic experiments, gene expression is measured along a time course after infection to include various stages of the immune response. In addition to the putative immunity genes, this can also provide information on genes that reflect the costs of launching an immune response.

Most genes with differential expression after parasitoid attack were not exclusively expressed (or switched off) during the immune response, but the expression was changed relative to the (unparasitized) controls (Wertheim *et al.*, 2005). The continuous expression of all these genes in unparasitized samples implies that the immune response consists at least partially of a modulation of other ongoing developmental and metabolic processes. These expression data provided some evidence that such modulation may incur some costs. A suite of metabolic genes was downregulated during the immune response, and these genes shared the same over-represented TFBM in the upstream regions as some suites of the upregulated 'immunity' genes (Wertheim *et al.*, 2005). The authors proposed that the immune response redirected these transcription factors away from their normal function, at the cost of metabolic processes. In addition, a group of genes involved in puparial adhesion proteins showed a significant reduction in their expression in infected larvae relative to the controls at the very last time point. This likely reflected the known delay in pupation that parasitized flies incur relative to their controls.

Some of the other transcriptomic studies also found indications for costs associated with the immune response. After parasitoid attack, a large group of genes for the generation of energy were upregulated in parasitized larvae, while developmental genes were downregulated (Schlenke *et al.*, 2007). Among the genes that are upregulated after infection with a microsporidium, several had annotations related to carbohydrate metabolism, including the transcription factor *Sugarbabe* (*CG3850*), a carbohydrate transporter (*CG7801*), and a phosphatase involved in trehalose biosynthesis (*CG5171*) (Roxström-Lindquist *et al.*, 2004).

Once activated, the immune response needs to be kept in check to avoid the deleterious effects on fitness. The costs of an activated (or overactive) immune system include reduced fecundity, hypersensitivity to infection, cancer, (auto)inflammatory diseases, or developmental defects (Zerofsky *et al.*, 2005; Bischoff *et al.*, 2006; Aggarwal and Silverman, 2008). Multiple negative regulators of the immunity pathways have been identified in *Drosophila*, including feedback loops, degrading agents of the triggering molecules of the immunity pathways, and possibly a repressosome that binds to the promoter regions of effector genes (reviewed in Aggarwal and Silverman, 2008).

All these costs discussed so far reflect the costs of actual resistance: mobilizing the immune

system in the event of an infection. But what about the costs of having the ability to produce a strong immune response? To identify the genes that are involved in a strong immune system, we selected for increased parasitoid resistance in the laboratory, and compared the expression of selected and control flies during their development before they were exposed to parasitoids. Hence, we only measured the changes in expression in flies with increased resistance in anticipation of parasitism, and we validate these genes in replicate control and selection lines to rule out genetic drift. In addition to a number of genes with strong differences in expression between the control and selection line flies, we find evidence for hundreds of genes with small but very consistent changes in expression (Wertheim *et al*., unpublished work). This suggests that the investment in a stronger immune system is not a simple allelic trait, but results in a significant reorganization of many developmental processes in the growing flies.

Crucially, the Toll and JAK/STAT pathways are not functioning exclusively in the immune response, but are also important for the proper differentiation and morphogenesis of multiple tissues during development (Kambris *et al*., 2002). Similarly, most of the serine protease molecules are important in both development and immunity (Shah *et al*., 2008). This dual function of the pathways and molecules supports the notion that an investment in immune responses is likely to harbour side effects in other processes. It also links in to costs of having increased resistance, as identified by the selection experiments discussed above. These showed that increased resistance to parasitoids, mites, and microsporidia all resulted in a lower larval competitive ability, which was tentatively linked to reduced head musculature (Kraaijeveld *et al*., 2002).

12.4 Conclusion

Genome-wide studies are uniquely powerful to detect genetic networks and interactions, and to identify novel putative immunity genes and pathways. They provide a snapshot of all genes with changed expression at a specific time point in an infected sample when compared to an appropriate control, without the need for *a priori* assumptions or knowledge. Using mutants can aid in the dissection of signalling pathways. Moreover, time-course experiments in particular can provide very useful new insights in the variety of genes that are associated with the immune responses and their sequence of action, providing an overview of the direct effects and (potentially costly) side effects of immune challenge on genes and pathways.

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CHAPTER 13

Population genetics of insect immune responses

Punita Juneja and Brian P. Lazzaro

13.1 Introduction

The immune system that we can observe and measure today is but a snapshot of a dynamic and evolving process, a moment in an ongoing genetic battle between hosts and their pathogens. Indications of this conflict are etched in the genome as signatures of adaptive evolution in the host immune system. These evolutionary signatures can also be read experimentally to give insight into the nature of host–pathogen interactions. This chapter will examine the evolutionary genetics of insect immune systems over both short and long timescales. In several instances, comparisons and contrasts will be drawn between species with distinct ecologies to elucidate commonalities and idiosyncrasies of insect immune evolution.

Adaptive evolution can manifest in evolutionarily favoured amino acid substitutions within genes as well as in genomic diversification of gene families. Both processes can be measured by comparing homologous genes and gene families across related species. Adaptive amino acid evolution is generally detected as a significantly elevated rate of amino acid substitution relative to an expectation based on the evolutionary rate at genetically silent positions (Box 13.1; Anisimova and Liberles, 2007). Adaptive gene family expansion can be inferred from an increased rate of duplication relative to that of other gene families in the genome (Hahn *et al.*, 2005). The recent availability of whole-genome sequences from several insect species allows such comparisons to be made on a wide scale.

Innate immunity, which is shared by homology between vertebrates and insects, is hardwired within the genome and lacks the antibody production that characterizes the adaptive immune response of higher vertebrates. The insect innate immune system is capable of recognition and subsequent eradication of microbes and multicellular parasites through humoral and cellular defence mechanisms (reviewed in Lemaitre and Hoffmann, 2007). Humoral immunity is mediated by production of microbicidal peptides, enzymes, oxidative free radicals, and other compounds that are secreted directly into the insect haemolymph (blood). The humoral defence against microbial infection is genetically well understood in *Drosophila melanogaster*. Invading microbes are detected by recognition molecules performing surveillance, signal is transduced through two primary signalling pathways, and defence is effected in part by abundantly produced antimicrobial peptides (AMPs). The two signalling pathways, termed the Toll and Imd pathways, are conserved between invertebrates and vertebrates. Cellular immunity is defined by encapsulation or engulfment of infective agents by circulating haemocytes. It has been less well characterized at the genetic level, although some genes that mediate cellular recognition and trigger phagocytic engulfment of microbes have been identified. A distinct process, RNA silencing (RNA interference, RNAi), allows specific detection and eradication of RNA viruses (Wang *et al.*, 2006). It is expected that functional diversity within the immune response will translate into variation in the selective pressures on different components of the defence response. This chapter will examine the evolutionary genetics of immune defence, interpreting molecular evolutionary patterns in light of protein function to

Box 13.1 Detecting adaptation in the genome

Evidence for natural selection can be revealed through examination of rates of DNA sequence evolution (reviewed in Anisimova and Liberles, 2007). The null model for these studies is that genes evolve by neutral evolutionary processes. Neutral, selectively equivalent mutations arise by chance and, in the absence of natural selection, occasionally become fixed by random genetic drift. The rate with which this happens is the neutral substitution rate. Many mutations that arise within functional genes cause deleterious changes to protein structure or function. These mutations are constrained from rising to high frequency by negative, or purifying, selection and are assumed to rarely fix between species. In contrast, mutations that are advantageous, such as those that confer resistance to disease, may rapidly rise in frequency by positive, or directional, selection. Positive selection leads to a short-term reduction in genetic diversity as the favoured allele replaces existing variation in a population. A sufficiently high number of recurrent adaptive fixations may also increase long-term divergence between species. Alternatively, multiple polymorphisms can be maintained in populations by balancing selection, which increases genetic diversity. In very rare cases, balanced polymorphism can occur when there is a heterozygote advantage, or overdominance, where heterozygote combination of two alleles has a higher fitness than homozygotes of either allele. More frequently, temporal or spatial variation in selection can maintain multiple alleles if each variant is advantageous in a different time or place.

Adaptive evolution can be detected by comparing DNA sequence of homologous genes from closely related species. This is generally achieved by comparing the rate of non-synonymous, amino acid-replacing, substitutions (d_N) to the rate of synonymous substitutions (d_S), which do not affect amino acid sequence. Synonymous substitutions are assumed to be invisible to selection and thus reflect neutral evolution. If all non-synonymous mutations were also selectively neutral, d_N would equal d_S, and the ratio d_N/d_S would equal one. Positive selection on amino acid substitutions would result in an increase in the rate of non-synonymous substitutions, or d_N being greater than d_S. The ubiquity of purifying selection, however, means that the empirically observed rate of non-synonymous substitutions over whole genes is much smaller than the rate of synonymous substitution, and d_N/d_S is almost always much less than one across entire genes. A more sophisticated implementation of this test, phylogenetic analysis by maximum likelihood (Yang *et al.*, 2000), uses gene sequences from multiple species to test the hypothesis that d_N/d_S varies among codons in a gene, allowing localization of the target of selection to particular residues or gene regions. Another test for natural selection, the McDonald–Kreitman test (McDonald and Kreitman, 1991), uses information about polymorphism in species and divergence between species. It tests the null hypothesis that the ratio of non-synonymous and synonymous substitutions segregating within species is the same as the corresponding ratio between species. In this test, positive selection is detected as a proportional excess of non-synonymous fixed differences between species. Selection favouring allelic diversification within species, in contrast, would lead to an excess of non-synonymous polymorphisms. These tests, among others, allow inference of natural selection acting on specific genes and gene regions.

draw insight into how the immune response adapts to pathogen pressures.

13.2 Evolutionary patterns in the antimicrobial immune response

Immune genes tend to evolve more quickly and adaptively than non-immune genes in vertebrates and insects (Murphy, 1991; Schlenke and Begun, 2003; Nielsen *et al.*, 2005; Sackton *et al.*, 2007; Waterhouse *et al.*, 2007). This adaptive evolution is shown by elevated rates of amino acid substitution between species and by elevated rates of duplication within gene families. The availability of whole-genome sequences allows for quantitative contrasts to be made between immune and non-immune genes, as well as for comparisons between functional classes of immune response genes. The recent complete genome sequencing of 12 species of fruit flies in the genus *Drosophila* has allowed particularly fine measurement of rates of substitution and genomic rearrangements between

closely related species. More distant comparative genomic analyses can be achieved by comparing genome sequences of *Drosophila*, the mosquitoes *Anopheles gambiae* and *Aedes aegypti*, the honey bee *Apis mellifera*, and the red flour beetle *Tribolium castaneum* (Figure 13.1).

Genome comparisons between species reveal the distinct selective pressures acting on each species through its unique life history. For example, the honey bee *A. mellifera* has apparently reduced copy number in immune-related gene families, perhaps reflecting decreased emphasis on immunological defence due to hygienic behaviour in the hive (Evans *et al.*, 2006). Mosquitoes have expansions in gene families thought to play defensive roles against pathogens borne in vertebrate blood (Christophides *et al.*, 2002; Waterhouse *et al.*, 2007). Interpretation of these comparisons is often limited, however, because identification of most immune genes in insects stems from functional characterization in only a few species, and primarily in *D. melanogaster*. Novel defence mechanisms in functionally uncharacterized organisms will not be detected through homology searching of genome sequences if they are too divergent to be detected by similarity at the DNA sequence level. Additionally, genes that are evolving extremely rapidly may diverge too quickly to be identified in comparisons between distantly related species. Genomic comparisons will gain power with

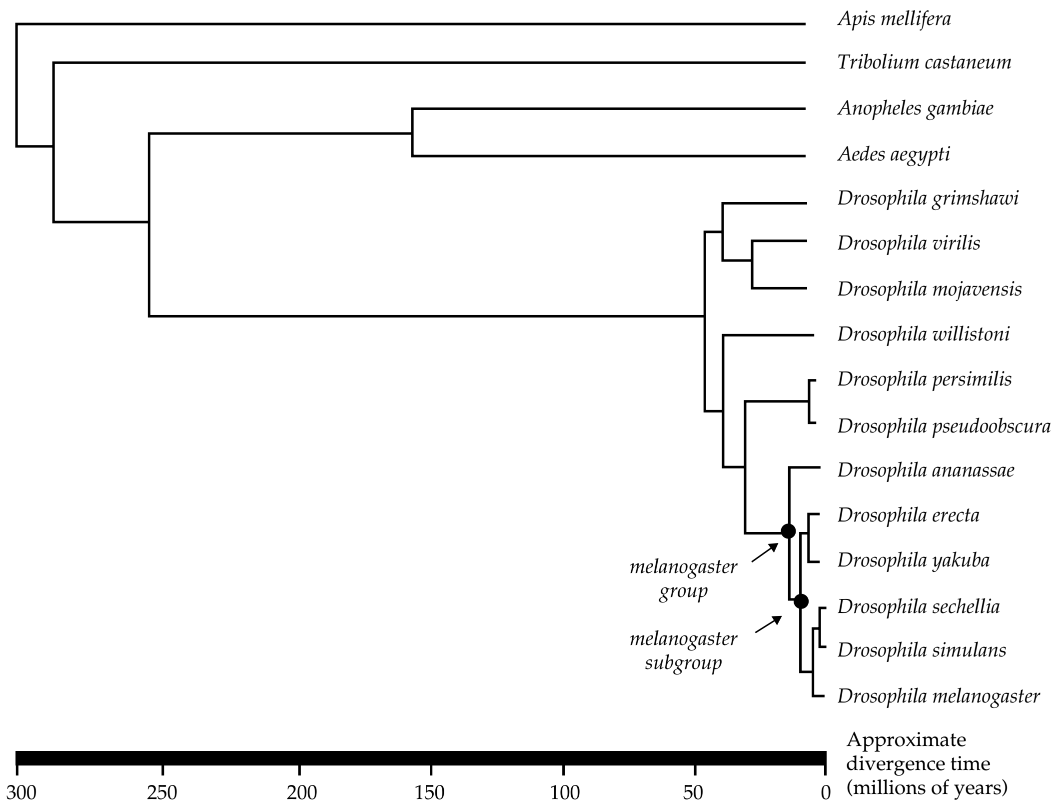

Figure 13.1 Phylogeny of select insect species with sequenced genomes. The *melanogaster* species group and *melanogaster* species subgroup are indicated. Gene-family expansions and contractions were evaluated among *Drosophila* (fruit flies), *Anopheles gambiae* (African malaria mosquito), *Aedes aegypti* (yellow fever mosquito), *Apis mellifera* (honey bee), and *Tribolium castaneum* (red flour beetle) and within the genus *Drosophila*. Adaptive amino acid evolution measurement, which requires shorter phylogenetic distances, was performed primarily in the *melanogaster* species group of *Drosophila* (Tamura *et al.*, 2004; Savard *et al.*, 2006; *Drosophila* 12 Genomes Consortium, 2007; Waterhouse *et al.*, 2007).

increasing functional characterization of non-model systems and the accumulation of whole-genome sequences for phylogenetically dispersed organisms.

Comparative genomic and molecular evolutionary analyses have revealed that not all genes in the immune system evolve along the same trajectories. Genes in broadly defined functional categories differ in evolutionary mode, suggesting contrasting selective pressures based on gene function. The supporting data and potential selective pressures that drive these evolutionary patterns will be considered in detail.

13.2.1 Toll and Imd signalling pathways

Nearly all core signalling proteins in the Imd and Toll pathways are maintained as strict orthologues among *Drosophila* species (Sackton *et al*., 2007) and between *Drosophila* and mosquitoes (Christophides *et al*., 2002; Waterhouse *et al*., 2007), honey bees (Evans *et al*., 2006), and *Tribolium* (Zou *et al*., 2007). Despite this maintenance of orthology, however, these signalling genes show unexpectedly high levels of amino acid divergence between *D. melanogaster* and mosquitoes and considerable evidence of adaptive evolution within *Drosophila* (see Figure 13.2; see also Figure 6.3 in this volume; Schlenke and Begun, 2003; Jiggins and Kim, 2007; Sackton *et al*., 2007; Waterhouse *et al*., 2007).

The adaptive evolution of innate immune signalling pathways is illustrated dramatically by proteins in the Relish cleavage complex of the Imd signalling pathway (Figure 13.3). Relish is a nuclear factor κB (NF-κB) family transcription factor that is cytoplasmically bound in the absence of infection. Activation of the Imd signalling pathway leads to phosphorylation of Relish, caspase-mediated cleavage of the Relish inhibitory domain, and translocation of the activated transcription factor to the nucleus. Several proteins in the cleavage complex (Dredd, dFADD, IKK_{β}, IKK_{γ}, and Relish itself) appear to be evolving adaptively in *D. melanogaster*, *Drosophila simulans*, and/or the *melanogaster* species group. Adaptive mutations are disproportionately located in protein domains important for the release of activated Relish: the Relish autoinhibitory domain and cleaved linker, the Dredd caspase domain, the dFADD death domain, and the IKK_{β} kinase domain (Figure 13.3; Begun and Whitley, 2000; Schlenke and Begun, 2003; Jiggins and Kim, 2007; Sackton *et al*., 2007). Adaptive evolution of the Relish complex is not universal among *Drosophila*, but is restricted to certain species in the *melanogaster* group (Levine and Begun, 2007; Sackton *et al*., 2007). In an interesting parallel, the *Relish* gene of *Nasutitermes* termites also evolves adaptively, again with positively selected mutations localized in and around the caspase cleavage site and linker (Bulmer and Crozier, 2006), suggesting convergence of selective pressures in these distantly related insects. Nor is adaptive evolution in *Drosophila* restricted to the Relish complex. Many other signal transduction genes in the Imd and Toll pathways (*imd*, *spirit*, *persephone*, *Toll*, *dorsal*, *necrotic*) also show evidence of rapid evolution in *Drosophila* (Schlenke and Begun, 2003; Jiggins and Kim, 2007; Sackton *et al*., 2007).

One hypothesis to explain the preponderance of adaptive mutations in signalling genes is that at least some pathogens may actively interfere with host immune signalling (Begun and Whitley, 2000). Such pathogens could include bacteria that inject immunomodulatory molecules into host cells, immunosuppressive fungi and parasitoid mutualistic polydnaviruses (reviewed in Schmid-Hempel, 2008). In the Relish example, pathogen interference with the assembled cleavage complex could drive co-evolutionary adaptation in several proteins. Alternatively, interference with a single important member of the complex could drive adaptation in that member while promoting compensatory adaptations in the interacting proteins to retain host function. Such compensatory mutations may occur throughout the signalling pathway, amplifying the evidence of natural selection in this gene set (DePristo *et al*., 2005). The convergence of adaptive evolution of genes within the Relish complex in different insect species suggests that some of these genes are common targets of pathogens.

13.2.2 Antimicrobial peptides (AMPs)

The humoral immune response culminates in the production of effector molecules that kill invading microbes. One well-studied class of effector

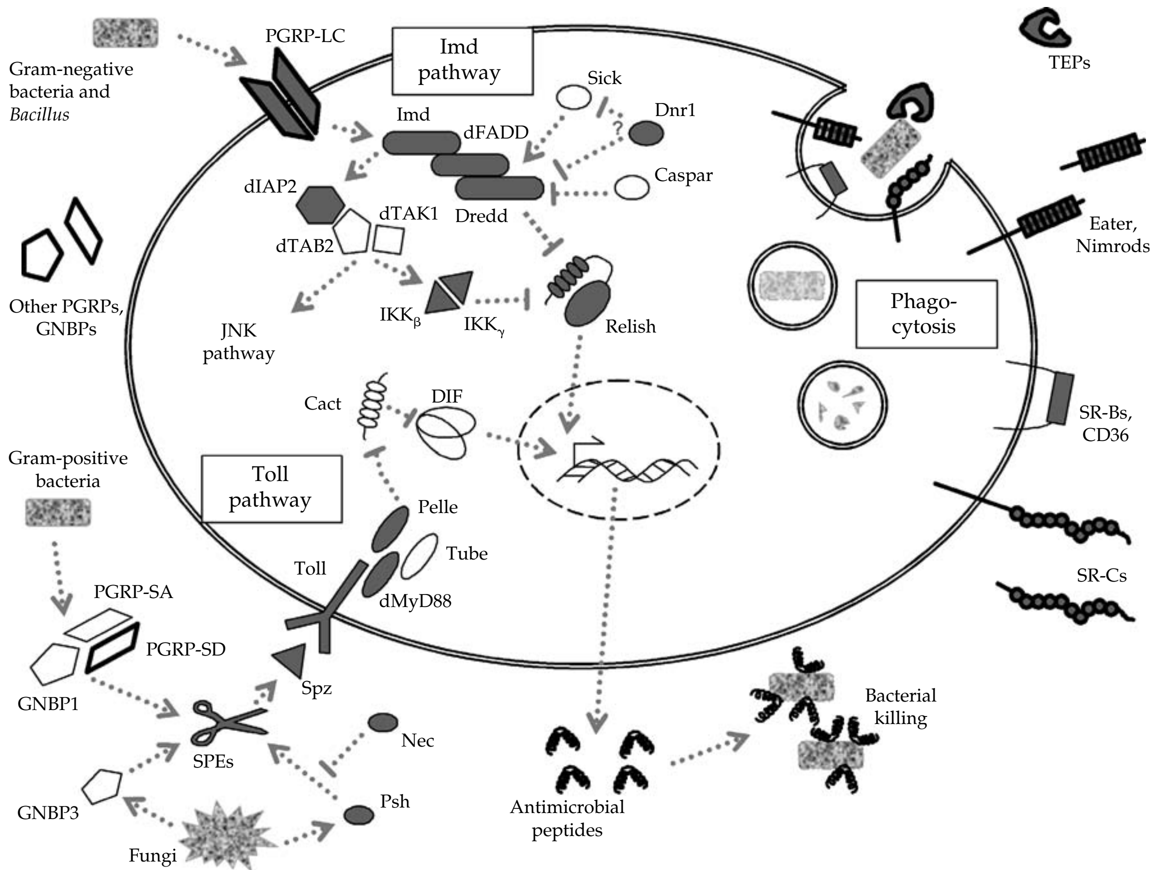

Figure 13.2 A schematic illustration of an idealized *D. melanogaster* immune-responsive cell illustrating prominent proteins required for the activation of a humoral immune response and receptors involved in defensive phagocytosis. Proteins whose gene families have experienced considerable genomic turnover within the genus *Drosophila* and among *Drosophila*, *Anopheles*, *Aedes*, *Apis*, and *Tribolium* are outlined in heavy black. Grey-shaded proteins have been implicated as evolving adaptively at the amino acid sequence level in *D. melanogaster* and/or *D. simulans*. Reproduced with permission from Lazzaro (2008). Cact, cactus; DIF, Dorsal-related immune factor; GNBP, Gram-negative-binding protein; IKK, I-κB kinase; JNK, c-Jun N-terminal kinase; Nec, Necrotic; PGRP, peptidoglycan-recognition protein; SPE, Spätzle-processing enzyme; Spz, Spätzle; TEP, thioester-containing protein.

molecules is AMPs. Most AMPs are short cationic peptides whose microbicidal activity is mediated by direct interaction with the negatively charged lipid membranes of bacteria and fungi (Zasloff, 2002; Lemaitre and Hoffmann, 2007; Yeaman and Yount, 2007). AMPs drew early attention as potential sites of host–pathogen co-evolution (Clark and Wang, 1997; Date *et al*., 1998; Ramos-Onsins and Aguadé, 1998) because of their direct role in the lysis and targeted killing of pathogens. However, systematic study of AMP genes, first in *D. melanogaster* and more recently across six *Drosophila* species, has failed to uncover evidence of adaptive evolution at the amino acid level (e.g. Lazzaro and Clark, 2003; Jiggins and Kim, 2005; Sackton *et al*., 2007). *Drosophila* AMP genes do, however, show extremely high rates of gene family expansion and contraction (Sackton *et al*., 2007). This high rate of genomic turnover extends to other taxa and is characteristic of most AMPs (Figure 13.2). In fact, the majority of *Drosophila* AMPs have no identifiable homologues in the genomes of mosquitoes, honey bees, or *Tribolium* (Christophides *et al*., 2002; Evans *et al*., 2006; Waterhouse *et al*., 2007; Zou *et al*., 2007).

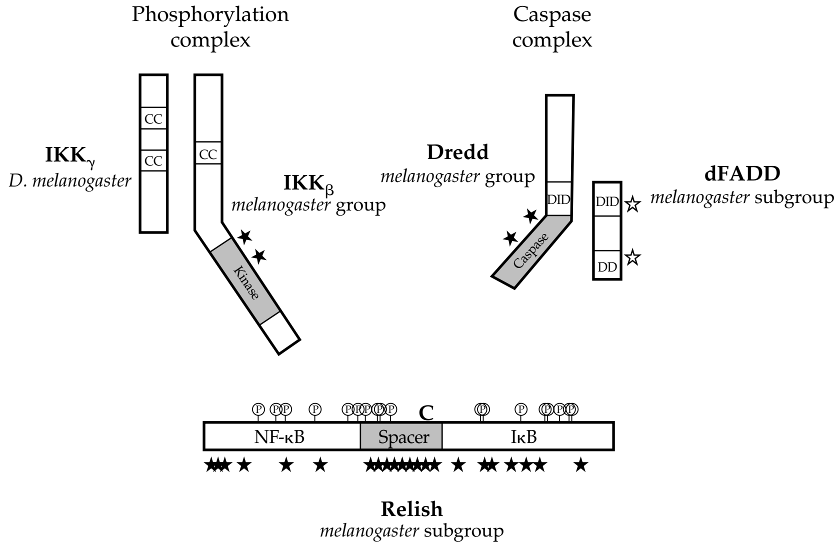

Figure 13.3 Adaptive evolution in the Relish complex. Caspase cleavage of the phosphorylated Relish spacer region allows the nuclear factor κB (NF-κB) domain to be translocated to nucleus, where it drives expression of immune response genes. IKK_γ and IKK_β form a complex through interaction at coiled-coil domains, and IKK_β phosphorylates Relish. The caspase Dredd is activated by dFADD via interaction at death-inducing domains and forms a complex with Relish. Putative Relish activation domains are indicated in grey. Positively selected sites (posterior probability >0.75) are indicated (★=significant at $P<0.01$; ☆=significant at $P<0.02$) and reflect selection along the *D. melanogaster* branch (*Relish*, *IKK*$_\beta$, or *dFADD*) or across the *melanogaster* (*Dredd*) species group (Sackton *et al.*, 2007). Taxonomic lineages where these genes appear to have evolved adaptively are indicated beneath each gene name (Begun and Whitley, 2000; Schlenke and Begun, 2003; Jiggins and Kim, 2007; Sackton *et al.*, 2007). C, caspase cleavage site; CC, coiled-coil domain; DD, death domain; DID, death-inducing domain; IKK, I-κB kinase; IκB, inhibitory κB; P, phosphorylation site.

Instead, these insects each have their own unique peptide families (Bulmer and Crozier, 2004; Evans *et al.*, 2006; Waterhouse *et al.*, 2007; Zou *et al.*, 2007). In some cases, AMP families in different species converge independently on similar tertiary structures and presumably functions (Broekaert *et al.*, 1995). Thus, whereas AMPs as a functional class of protein are ubiquitous among higher eukaryotes, there appears to be little homologous retention of peptides over evolutionary time.

The levels of sequence constraint seen in *Drosophila* do not characterize AMP evolution in all taxa. Genomic duplication of AMP genes is occasionally coupled with adaptive diversification at the amino acid level, presumably reflecting functional divergence (Tennessen, 2005; Yeaman and Yount, 2007). Genes encoding a termite-specific class of AMPs, *termicins*, have independently duplicated or triplicated in several termite species, with one duplicate typically sustaining mutations that decrease the polarity of the peptide (Bulmer and Crozier, 2004). These changes, which are driven by positive selection on amino acid sequence, result in peptides with divergent charges. Similarly, the mosquito *A. gambiae* has duplicated members within the *defensin* family (Dassanayake *et al.*, 2007). Again, expansion is coupled with elevated rates of amino acid substitutions that change polarity, suggesting adaptive value to having two defensins with slightly different polar affinities. Previous studies in vertebrate AMP families have also found evidence of duplication coupled with positive selection, although in these cases peptide charge is maintained (reviewed in Tennessen, 2005; Yeaman and Yount, 2007). There is compelling evidence from insects and vertebrates that gene-family expansion can sometimes allow adaptive diversification of peptide function (Tennessen, 2005).

AMPs are remarkably efficient at combating infection. Resistance in microbes is seldom observed in nature, and, when it is, it tends to arise in specialized pathogens that are likely to be under

strong selective pressure to resist this form of defence (see Samakovlis *et al*., 1990; Zasloff, 2002). There are several possible explanations for why it may be difficult for most bacteria to evolve resistance. One common AMP mechanism is to disrupt membrane integrity though biochemically simple mechanisms, such as forming open pores (Zasloff, 2002; Yeaman and Yount, 2007). The ability of microbes to evolve resistance to such activities may be limited. However, heritable variation for resistance can be created and selected upon in microbial populations in the laboratory (Perron *et al*., 2006). In natural contexts, hosts simultaneously produce an array of AMPs that differ in charge, hydrophobicity, structure, and activity, probably ensuring that most pathogens are susceptible to at least a subset of them. This is conceptually similar to the application of multiple antibiotics in clinical settings and may serve to delay or eliminate the evolution of resistance (Yeaman and Yount, 2007). If pathogens are slow or fail to evolve resistance to peptides, there may be little selective pressure on insect hosts to adapt their AMPs at the amino acid level over modest evolutionary time. However, divergent bacteria and fungi display a range of susceptibilities to individual peptides (Zasloff, 2002), so diversification in AMP function may be selectively favoured in instances when a host shifts to a new ecological niche and is immediately presented with a novel and distinct set of pathogen pressures.

13.2.3 Recognition molecules in the humoral response

The humoral immune response is activated when circulating recognition factors are stimulated by highly conserved microbial compounds. Gram-negative-binding proteins (GNBPs) and peptidoglycan-recognition proteins (PGRPs) activate the humoral response after recognizing microbial cell-wall peptidoglycans and β-glucans. Some members of the PGRP family downregulate the immune response by degrading free peptidoglycan into non-immunogenic monomers (Lemaitre and Hoffmann, 2007).

PGRP and *GNBP* gene families generally evolve under purifying selection over short evolutionary time, but have undergone substantial genomic turnover on the lineages that separate *Drosophila* from mosquitoes, honey bees, and *Tribolium* (see section 6.4.4 in this volume; Evans *et al*., 2006; Waterhouse *et al*., 2007; Zou *et al*., 2007). Most *GNBPs* and *PGRPs* do not appear to have experienced recent adaptive evolution in *Drosophila* (Jiggins and Hurst, 2003; Schlenke and Begun, 2003; Jiggins and Kim, 2006; Sackton *et al*., 2007), mosquitoes (Little and Cobbe, 2005), or the crustacean *Daphnia* (Little *et al*., 2004). A notable exception, however, is a *Drosophila PGRP* which shows strong indications of adaptive evolution. *PGRP-LC*, an alternatively spliced gene that sits atop the Imd signalling cascade, has sustained a two amino acid insertion in the PGRP-LCa isoform in species of the *melanogaster* subgroup. This insertion is predicted to alter the binding specificity of that isoform, and appears to have been positively selected in conjunction with several additional adaptive substitutions (Sackton *et al*., 2007). Interestingly, the alternatively spliced binding domains of *PGRP-LC* show evidence of either recent independent duplication or concerted evolution in *D. melanogaster* and *A. gambiae* (Christophides *et al*., 2002). These patterns potentially reflect lineage-specific selection for recognition of distinct microbes. In another exception, limited positive selection was also detected in *GNBP* genes of *Nasutitermes* termites (Bulmer and Crozier, 2006). In this case, it was hypothesized that adaptation of recognition capability was driven by a shift in ecology as previously herbivorous termite species adapted to feed on decaying matter, exposing them to a novel community of pathogens.

One potential explanation for the observation that *PGRPs* and *GNBPs* tend to exhibit little indication of adaptive amino acid evolution is that these proteins recognize highly conserved pathogen sugar moieties. The cell-wall components recognized by these proteins are indispensable for most microbes, and, generally speaking, may not be easily modifiable. There thus may be little pressure on these genes to adapt over short time periods. Additionally, these recognition proteins are active against molecules that are conserved across a wide range of microbial taxa. There are, however, a limited number of examples of positive selection on *PGRPs* and *GNBPs*. Coupled with the observations of gene family duplication and divergence among

species, instances of positive selection may reflect bursts of diversification as recognition function is fine-tuned to species-specific selective pressures.

13.2.4 Recognition molecules in the cellular response

Recognition is also a necessary prerequisite for pathogen clearance via cellular immunity, and several gene families have been identified that encode membrane-bound phagocytic receptors. Phagocytosis is also promoted by 'tagging' of microbes with extracellularly secreted opsonins. Several genes encoding both phagocytic receptors and opsonins show evidence of adaptive amino acid evolution within the genus *Drosophila* (Sackton *et al*., 2007) and frequent genomic turnover within *Drosophila* and between *Drosophila* and other insects (Figure 13.2; Evans *et al*., 2006; Sackton *et al*., 2007; Waterhouse *et al*., 2007; Zou *et al*., 2007). In *Drosophila*, recognition genes are significantly more likely to show evidence of positive selection than genes with signalling or microbicidal functions (Sackton *et al*., 2007). This difference is largely driven by recognition genes that trigger the cellular response, with nine of 10 recognition genes that yield significant evidence of positive selection having been either experimentally confirmed to be involved in phagocytosis or homologous to known phagocytosis genes. Specifically, these are genes encoding thioester-containing proteins (TEPs) (Jiggins and Kim, 2006; Sackton *et al*., 2007), the Eater and Nimrod families (Sackton *et al*., 2007), the class C scavenger receptors (Lazzaro, 2005), and the CD36 homologue epithelial membrane protein (emp) (Sackton *et al*., 2007).

TEPs have been directly implicated as opsonins mediating the cellular clearance of microbes including bacteria and malaria-causing *Plasmodium* in *Drosophila* and *Anopheles* (Levashina *et al*., 2001; Blandin *et al*., 2004; Stroschein-Stevenson *et al*., 2006). Proteolytic cleavage of a hypervariable spacer, or 'bait', domain exposes the thioester motif, which then covalently binds microbes and labels them for phagocytosis. TEPs appear to be hotspots of adaptation in several species. In *D. melanogaster*, there are six *Tep* genes, four of which have intact thioester domains and thus are likely to function as opsonizing agents (Blandin and Levashina, 2004). One of four of the intact *Teps* show evidence of adaptive divergence between *D. melanogaster* and *D. simulans* and three show evidence for directional selection in the *melanogaster* species group (Table 13.1; Jiggins and Kim, 2006; Sackton *et al*., 2007). Interestingly, one of the adaptively evolving *Tep* genes is constitutively expressed at higher levels in European than African populations of *D. melanogaster*, suggesting that expression of this *Tep* may be locally adapted (Hutter *et al*., 2008). *Tep* genes in mosquitoes and the more distantly related crustacean *Daphnia* also show evidence of adaptive amino acid evolution (Little *et al*., 2004; Little and Cobbe, 2005). In all cases, positively selected amino acid mutations are overrepresented in the bait domain that is cleaved to expose the thioester motif. It is unknown whether the proteases that cleave TEPs are produced by host or pathogen, so it is not yet possible to say whether adaptation in this domain is due to co-evolution with pathogen proteases or with pathogen molecules that interfere with host proteolysis.

Tep gene families are expanded in mosquitoes, with 13 *Tep* genes found in the *Anopheles gambiae* genome and eight in the *Aedes aegypti* genome (Christophides *et al*., 2002; Waterhouse *et al*., 2007). The expansions in size of the *Tep* gene family appear to have been independent in each of these two taxa and potentially reflect elevated pressure on cellular immunity. The *A. gambiae Tep1* gene is segregating for two sharply divergent alleles, one of which, when homozygous, confers absolute resistance to experimental infection with the rodent malaria *Plasmodium berghei* (Blandin *et al*., 2004; Baxter *et al*., 2007). Individuals homozygous for the susceptible allele sustain robust *P. berghei* infections. These two alleles differ by multiple amino acid substitutions, including several that are clustered around the thioester domain. It is currently unclear which substitutions cause the phenotypic differences in susceptibility, or whether it is an epistatic phenotype involving substitution in multiple domains of the protein. Both alleles are found at high frequencies in natural populations (Obbard *et al*., 2008), suggesting selective forces maintain these two alleles in the wild. This system provides a tantalizing opportunity to understand the mechanisms

Table 13.1 Evolutionary genetics of the *Tep* gene family of phagocytic recognition molecules in *Drosophila*.

	Tep 1	*Tep 2*	*Tep 3*	*Tep 4*	*Tep 5*	*Tep 6* (*Mcr*)	Reference
Functional data							
Overview	Upregulated in response to infection	Upregulated in response to infection		Upregulated in response to infection	Not expressed; likely to be a pseudogene	Lacks a thioester domain	Reviewed in Blandin and Levashina (2004)
Phagocytic activity		Required for efficient phagocytosis of the bacterium *Escherichia coli*	Required for efficient phagocytosis of the bacterium *Staphylococcus aureus*			Required for efficient phagocytosis of the fungus *Candida albicans*	Stroschein-Stevenson *et al.* (2006)
Species divergence:							
d_N/d_S (Box 13.1)	Exceptionally elevated d_N/d_S between *D. melanogaster* and *D. simulans* clustered around the bait domain; elevated d_N/d_S in the *melanogaster* species subgroup	Elevated d_N/d_S in the *melanogaster* species subgroup	Not significant[a]	Not significant			Jiggins and Kim (2006)
	Elevated d_N/d_S in the *melanogaster* group with trend towards an excess of positively selected sites at the bait domain	Elevated d_N/d_S across the entire gene in the *melanogaster* species group	Not significant	Elevated d_N/d_S in the *melanogaster* species group with an excess of positively selected sites at the bait domain		Not significant	Sackton *et al.* (2007)
McDonald–Kreitman test (Box 13.1)	Elevated amino acid replacements across entire gene in *D. melanogaster*	Not significant		Not significant			Jiggins and Kim (2006)
Population divergence							
Differential expression		Expression levels significantly higher in European than in African *D. melanogaster* populations	Not significant	Not significant			Hutter *et al.* (2008)

[a] Not significant indicates genes that were included in the referenced studies but not found to depart from the null expectation. Empty cells indicate that no information has been obtained.

that lead to the maintenance of immune response polymorphisms in a natural context.

Whole-genome comparisons within the genus *Drosophila* indicate that, in striking contrast to recognition molecules that trigger the humoral response, recognition molecules that initiate the cellular response show abundant evidence of adaptive evolution. Deeper investigation of the *Tep* gene family reveals that adaptive evolution extends beyond *Drosophila* to include mosquitoes and *Daphnia*, and demonstrates extant functional variation in a mosquito *Tep* gene. The signals of adaptive evolution suggest that these recognition molecules interact with evolutionarily labile pathogen motifs or that, like signalling molecules in humoral defence, they are potentially subject to interference by pathogen-produced proteins.

13.2.5 Summary

The diverse evolutionary trajectories of various genes in the insect immune response (Figure 13.2) can be interpreted in light of their molecular functions and interactions with pathogens. Pathogen-recognition molecules that stimulate the humoral response interact with highly conserved microbial cell-wall components. Although obligate pathogens are sometimes able to reduce their cell walls to escape detection, most microbes are evolutionarily constrained because they must also be able to persist in non-infectious environments. Similarly, there may be few ways in which microbes can evolve resistance to AMPs, especially when host insects simultaneously employ multiple peptides with distinct activities. If there is little adaptation in pathogens to escape host humoral recognition and antibiotic killing, then it may be expected that there would be little indication of adaptive amino acid evolution in the host genes over short evolutionary time. Both humoral recognition factors and AMPs exhibit rapid rates of genomic duplication and deletion, and in some taxa duplication is coupled with a burst of amino acid diversification that presumably increases breadth of function.

In contrast, signal transduction proteins in the humoral immune response are largely maintained in strict orthology across insect species, but frequently show indications of adaptive amino acid evolution within species. A hypothesized explanation is that the strong maintenance of orthology in these pathways makes them attractive targets for immune suppression by generalist pathogens. This may be a particularly successful strategy for microbes that are unable to evade or resist the recognition and microbicidal stages of humoral immunity. Gene duplication and diversification are not commonly observed here, perhaps because this is not a successful strategy for escaping pathogen interference. Genomic retention of a duplicated gene that can be manipulated by pathogens would be detrimental because host signalling function would be impaired. Instead, rapid fixation of amino acid 'escape' variants in signalling genes seems to be the most effective host strategy, and coordinate compensatory mutation in physically interacting proteins may amplify the signal of adaptive evolution in this functional category.

Recognition factors and opsonins in the cellular immune response evolve by adaptive amino acid evolution and frequent genomic turnover. In general, little is known about the specific activities and recognition profiles of these genes, making it difficult to interpret the evolutionary patterns in a functional context. The evolutionary genetics, however, do lead to functional predictions, including that the cellular recognition factors bind evolutionarily labile pathogen epitopes or are subject to pathogen interference, both of which could drive rapid amino acid evolution. At the moment, virtually nothing is known about the molecular evolution or population genetics of host genes that drive phagocytosis after pathogen recognition. Microbes are capable of manipulating host cells both to promote and inhibit phagocytic uptake (Schmid-Hempel, 2008), leading to the prediction that genes encoding the machinery of phagocytosis will, like genes in humoral signalling pathways, show abundant evidence of adaptive evolution.

13.3 Evolutionary patterns in the antiviral immune response

Early characterization of the immune response focused primarily on antimicrobial defence. Antiviral defence is at least partially distinct from

that against microbes, and currently is only poorly understood. Both the Toll and Imd pathways are activated during the course of some viral infections; however, only the Toll pathway seems to confer protection (Lemaitre and Hoffmann, 2007). RNAi provides an independent mechanism of defence that is specific against RNA viruses (Wang *et al.*, 2006). Viruses are formidable opponents for the immune system. They are capable of rapid evolution owing to their fast generation times, large population sizes, high mutation rates and obligate pathogen lifestyles. These factors hint that the evolutionary patterns of antiviral defence genes will be different from those described previously for the antimicrobial defence.

Short-term evolution of an antiviral defence gene has been studied at the *D. melanogaster* locus *ref(2)P*, which is proposed to function in the Toll pathway (Avila *et al.*, 2002). This locus is polymorphic for alleles that explain a large component of the variation in susceptibility or resistance to the rhabdovirus Sigma (Contamine *et al.*, 1989; Bangham *et al.*, 2007, 2008). A single domain, termed PB1, of *ref(2)P* is required for viral replication (Carré-Mlouka *et al.*, 2007). Sigma is infective if a permissive allelic variant of this domain is present, but not with a restrictive allele or genetic knock-out of the domain. This domain has an excess of amino acid polymorphisms (Wayne *et al.*, 1996), consistent with natural selection acting to maintain allelic diversity. A random sample of 10 phenotypically random alleles identified six amino acid polymorphisms in the PB1 domain (Wayne *et al.*, 1996). A single complex mutation, with a single glycine residue substituted for glutamine and asparagine residues, was found on restrictive but not permissive alleles. The remaining polymorphisms are shared by both restrictive and permissive alleles. The frequency of the complex mutation varies between populations, ranging from absent in some African and European populations to 23% in some North American populations (Bangham *et al.*, 2007). There is greatly reduced variation in the restrictive haplotype in a North American population, suggesting that it has recently risen to high frequency by directional selection (Box 13.1). This indicates that selection is acting on localized spatial scales, likely in concert with Sigma virus, which also varies in frequency and genotype between populations (Carpenter *et al.*, 2007).

The fact that there is an excess of non-synonymous polymorphism in *ref(2)P* PB1 domain but that only a single complex mutation separates restrictive and permissive alleles suggests that current Sigma virus populations have become adapted to some of the remaining polymorphisms. Indeed, analysis of all combinations of polymorphisms on the restrictive allele in artificially generated constructs indicates that no fewer than two of the three mutations are required to create a restrictive allele (Carré-Mlouka *et al.*, 2007). These data suggest a model wherein novel mutations have been driven to high frequency by directional selection, but that the sweeps are incomplete because the virus quickly adapts to the increasingly common allele before it fixes in the population. Host resistance then requires the repeated reintroduction of novel restrictive mutations. The most escalated rates of evolution are expected when host and pathogen are co-evolving, such that host adaptations to escape infection are met by a gene-for-gene pathogen adaptation to maintain virulence (Dawkins and Krebs, 1979). Over the evolutionary long term, there is evidence for elevated amino acid substitution at this domain, with more adaptive mutations becoming fixed in *D. melanogaster* when compared with *D. simulans*, a species in which Sigma infection is rare or absent (Wayne *et al.*, 1996). Restrictive polymorphisms that are driven to high frequencies during partial selective sweeps will fix by genetic drift more often than mutations that are selectively neutral over their entire evolutionary history, which may lead to elevated amino acid divergence between species.

A distinct pathway using RNAi presents an important defence against RNA viruses. In *D. melanogaster*, double-stranded viral RNA (dsRNA) is recognized and cleaved into small interfering RNA (siRNA) by Dicer-2 (Wang *et al.*, 2006). These siRNAs then guide cleavage of matching RNA via formation of an RNA-induced silencing complex (RISC). Some viruses produce proteins that suppress RNA silencing. For example, *Drosophila* picornavirus C produces a dsRNA-binding protein that interferes with Dicer-2 activity and promotes viral establishment and proliferation (van Rij *et al.*, 2006). *Dicer-2*, along with RISC genes *R2D2* and *Argonaute-2*,

are among the most rapidly evolving genes in the *D. melanogaster* genome. These antiviral genes, but not their paralogues with housekeeping regulatory function, show indications of adaptive evolution by recurrent fixation of novel amino acid mutations (Obbard *et al*., 2006).

The unique patterns of evolution of antiviral defence yield a useful system for integrating measures of short- and long-term evolution. In the case of *ref(2)P* in *D. melanogaster*, rapid evolution is driven by a gene-for-gene interaction between host and virus, and is evidenced by reduced genetic variation within the selectively favoured allele in the short term and increased amino acid divergence in the long term. Rates of long-term evolution in RNAi antiviral genes in *D. melanogaster* are dramatically higher than the genome average. Evidence suggests that the selective pressures are different from those that act on antimicrobial defence, leading to elevated rates of evolution. This may reflect either rapid viral evolution or high host specificity in viruses, either of which would facilitate co-evolution. Like humoral signalling pathways in the antimicrobial defence, RNAi pathways are also subject to pathogen interference to overcome host defences, indicating that they too are a potential site of direct conflict. Thus, evidence from both types of defence suggests that sites of pathogen interference display elevated evolutionary rates. As antiviral defence becomes better characterized at the molecular level, this system will yield further insights into genetic adaptation to pathogen pressures and serve as a comparison for evolutionary patterns observed in antimicrobial defence.

13.4 From genotype to phenotype

All the patterns discussed thus far have pertained to the long-term evolution of the immune system. It is important to remember, however, that all adaptive evolution is based on phenotypic polymorphism that segregates in populations at some point in time. Indeed, extant natural populations harbour considerable genetic variation for immunocompetence. This segregating phenotypic variation is the substrate for short-term evolution. Understanding its genetic basis and the forces governing its persistence is essential for predicting the evolutionary response to natural or artificial perturbations in infectious pressure in natural populations.

In organisms with well-characterized genomes, it is possible to directly test the phenotypic effects of allelic variation in pre-chosen 'candidate' genes though genotype–phenotype association mapping. These studies have been employed most effectively in *D. melanogaster*. For instance, natural allelic variation in the *ref(2)P* gene clearly determines resistance to the vertically transmitted Sigma virus in *D. melanogaster* females in an almost purely Mendelian fashion (Contamine *et al*., 1989; Bangham *et al*., 2008). Genetic variation in Sigma viral transmission through males, however, does not map to *ref(2)P* (Bangham *et al*., 2008). Variation in the ability of *D. melanogaster* to suppress bacterial infection has been mapped to polymorphisms in pathogen-recognition factors and signalling genes within the Toll and Imd pathways (Lazzaro *et al*., 2004, 2006). Expression levels, but not polymorphisms, of AMPs are also associated with resistance to infection (T.B. Sackton, B.P. Lazzaro, and A.G. Clark, unpublished data). These observations, coupled with evaluation of transcriptional activity of the immune system, indicate that signalling flux through the Toll and Imd pathways is a tremendously important determinant of resistance to bacterial infection. In contrast to the antiviral resistance determined by *ref(2)P*, polymorphisms mapped in the antibacterial association studies each make relatively small contributions to variance in the resistance phenotype, suggesting that resistance to bacterial infection is a combinatorial function of multiple genes of individually small effect. Even in sum, the mapped antibacterial factors do not explain the entirety of the genetic variance, indicating that other unstudied genes also contribute to variation in resistance.

If pathogen infection can be so detrimental to the condition of the host, and host alleles that confer high resistance to infection exist in natural populations, why then does resistance not spread to all individuals? Genetic trade-offs, whereby immunocompetence comes at a cost to another phenotype within an organism, can constrain natural selection from fixing resistant genotypes (Roff and Fairbairn, 2007). Potential costs of resistance include direct damage to host tissues due to immune activity and correlated reduction in investment in

other physiological traits, including alternative immune functions, metabolism, and reproduction. Which investment strategy is most favourable will depend on the strength of pathogen pressures and on selection acting on other fitness traits of the organism.

An experimental approach that has been used to study genetic trade-offs is artificial selection for increased resistance to infection and subsequent measurement of correlated changes in other fitness traits. This method identifies costs of resistance, defined as changes in traits that reduce fitness in selected lines compared with unselected lines. Artificially selecting the Indian meal moth, *Plodia interpunctella*, for increased resistance to granulosis virus infection led to correlated increases in larval development time and pupal weight and a decrease in egg viability in selected lines (Boots and Begon, 1993). Selection in *D. melanogaster* for resistance to parasitoid or fungal infection led to a correlated decreases in larval competitive ability and adult fecundity, respectively, in the absence of infection (Kraaijeveld and Godfray, 1997, 2008). Costs that are measured in artificial selection lines should be interpreted with caution, however, as selection experiments can sometimes result in the fixation of rare alleles with large phenotypic effects that are not representative of functional genetic variation in natural contexts. For example, *A. gambiae* mosquitoes selected for refractoriness to *Plasmodium* infection achieve this through an increased melanization response (Collins *et al*., 1986) and high levels of cellular oxidative free radicals that are extremely damaging to host cells (Kumar *et al*., 2003). Natural resistance in wild populations of *A. gambiae*, however, is generally accomplished with a melanization-independent mechanism (Riehle *et al*., 2006), and is likely to be less costly or damaging than mechanisms seen in laboratory-selected lines.

A more relevant, but much subtler, measurement of genetic trade-offs is obtained by measuring genetic correlations between traits in naturally occurring, unselected genotypes. This is commonly done by measuring phenotypes in genetic clones or in individuals' with known genetic relatedness and estimating the genetic contribution to the phenotype. In *D. melanogaster*, genotypes with high resistance to bacterial infection had low fecundity in the absence of infection in a food-limited environment (McKean *et al*., 2008). In the pea aphid *Acyrthosiphon pisum*, clonal lines with high resistance to attack by the parasitoid wasp *Aphidius ervi* had reduced fecundity (Gwynn *et al*., 2005). However, in this case, resistance to parasitoids can be conferred by bacterial endosymbionts, so the genetic basis for this trade-off may be mediated by factors outside the host genome. In both examples, the cost of resistance is a decrease in reproductive fitness.

The ultimate goal is to identify the genetic architecture underlying trade-offs. Quantitative trait locus (QTL) mapping has been used to locate these genetic regions. This approach relies on contrived crosses between chosen parents to establish phenotypically variable recombinant progeny. Genetic markers are then genotyped at periodic intervals across the genome, allowing the localization of genomic regions encoding the phenotypic variation without relying on *a priori* candidate genes. QTL mapping, however, lacks the resolution to identify specific genes or alleles. In the red flour beetle *T. casteneum* and in the bumble bee *Bombus terrestis*, simultaneous mapping of immune and fitness traits found that loci associated with immune phenotypes occasionally co-localized with QTLs involved in fecundity, viability, and body size (Zhong *et al*., 2005; Wilfert *et al*., 2007a). There are two potential genetic mechanisms that could cause genetic correlations between immune and fitness traits. Genetic correlations can be caused by pleiotropy, where a single gene influences multiple traits. Trade-offs are due to antagonistic pleiotropy, where a single allelic variant of a gene has a positive effect on one trait but a negative effect on the other. Alternatively, allelic variants of distinct genes affecting the two traits may be in linkage disequilibrium due to physical proximity on a chromosome, and thus these variants are coordinately passed to the offspring. Selection acts simultaneously on traits that are correlated by either pleiotropy or linkage disequilibrium. However, only antagonistic pleiotropy places a long-term constraint on selection because recombination can degrade correlations based on linkage disequilibrium. QTL mapping relies on experimentally generated linkage disequilibrium that spans much

greater physical distances than are observed in natural populations, so it is relevant to follow QTL-based studies of genetic correlations with field-based studies to determine whether the traits co-segregate in nature.

Trade-offs have been also identified within the immune response. For example, in *B. terrestis*, lines selected for increased resistance to trypanosome infection also had a higher investment in a phenoloxidase response coupled with a lower investment in AMP response (Wilfert *et al.*, 2007b). The Egyptian cotton leafworm, *Spodoptera littoralis*, demonstrated positive genetic correlations among haemocyte density, cuticular melanization, and phenoloxidase activity, but a negative genetic correlation between haemocyte density and lysozyme-like antibacterial activity (Cotter *et al.*, 2004). A different result is obtained from females of the mealworm beetle *Tenebrio molitor*, where cuticular melanization shows a negative genetic correlation with haemocytes and phenoloxidase, suggesting that the genetic architecture of these correlations can vary between species (Rolff *et al.*, 2005). These results demonstrate that increased investment in one component of the immune response can come at a cost to other immune functions, and indicate the potential for trade-offs within the immune response to place constraints on the evolution of global resistance.

Thus far, all resistance measures have been considered only in a single environment; however, the optimal immune strategy can be expected to vary based on environmental conditions (Lazzaro and Little, 2009). Selective pressures are heterogeneously distributed in the environment. Abiotic factors such as day length, temperature, and moisture vary between populations, affecting development time, metabolic flux, and other traits, and also altering the composition of pathogen communities and nutrient availability. Allelic variants in some genes respond differently to changes in the environment, termed genotype-by-environment interactions. If a genotype is particularly favoured in certain conditions, local adaptation to the proximate environment can occur. Temperate and tropical populations of *D. melanogaster* varied significantly in their resistance to the generalist fungal pathogen *Beauveria bassiana* (Tinsley *et al.*, 2006) and bacterial pathogen *Providencia rettgeri* (Lazzaro *et al.*, 2008). Considerable genotype-by-environment interaction was observed in resistance of *D. melanogaster* to *P. rettgeri* infection across multiple temperatures. Despite that observation, temperature populations were on average more resistant to *P. rettgeri* than the tropical one at lower temperatures, which potentially reflects adaptation to the local temperature. Spatial heterogeneity in the environment can lead to the maintenance of multiple resistance alleles if local adaptation is sufficiently strong to withstand erosion by migration and gene flow.

The magnitude, or even the existence, of genetic trade-offs can also vary between environments. In natural and laboratory settings, infestation by the mite *Macrocheles subbadius* negatively affects the fertility and body size of its host, *Drosophila nigrospiracula* (Luong and Polak, 2007). There is genetic variation for resistance to mites, which in this case is mediated by an avoidance behaviour. It has been demonstrated that, similar to *D. melanogaster* selected for parasitoid resistance, lines selected for mite resistance also suffer a cost in terms of decreased larval competitive ability. Manipulating the environment with high temperatures and increased larval density to create stressful conditions tends to increase costs of resistance. For instance, in previously considered examples from *D. melanogaster*, resistance to bacterial infection was correlated with low fecundity only in a food-limited environment (McKean *et al.*, 2008), and larval success of parasitoid-resistant larvae was compromised only under crowded conditions (Kraaijeveld and Godfray, 1997). In all of these cases, selection can act independently on the traits in a non-stressful environment but the traits are constrained to each other under resource-limited conditions. Genetic variation for different allocations of resources between resistance and fitness traits can be maintained by environmental heterogeneity since the optimal investment strategy will be context-dependent (Roff and Fairbairn, 2007). Selection on these variants will be inefficient because trade-offs will only be apparent in certain conditions.

The host immune response faces a special obstacle in evolving immunity: the immune system must respond to living organisms that are them-

selves free to evolve. Its pathogen 'environment' is capable of rapid evolution, often much more quickly than the host. Analogous to genotype-by-environment interactions, a genotype-by-genotype interaction occurs when the efficacy of a host resistance genotype is dependent on the genotype of the pathogen. Antagonistic pleiotropy can occur in this context if resistance to one pathogen genotype comes with susceptibility to another. The specificity of these interactions can allow for temporal fluctuations in host and parasite genotypes in a frequency-dependent manner. Such fluctuations are generally difficult to measure experimentally, but have been observed natural populations of the snail host *Potamopyrgus antipodarum* and trematode parasite *Microphallus* sp. as well as in the crustacean host *Daphnia magna* and bacterial parasite *Pasteuria ramosa* (Dybdahl and Lively, 1998; Decaestecker *et al.*, 2007). In both cases, resistant host genotypes are at an advantage when they are rare because their infective parasite genotypes are also rare, allowing resistant host genotypes to then to rise in frequency. This leads to a time-lagged increase in the infective parasite genotype, causing the host advantage to decline, subsequently reducing the frequency first of the host genotype and then the parasite genotype. This type of co-evolution is probably rare, occurring only when a parasite infects a narrow species range of hosts, allowing for specific, reciprocal adaptation, and when the parasite greatly reduces the fitness of the host such that selective pressure on resistance is high. In reality, many parasites are likely adapting to multiple host and impose only small reductions of fitness, placing more diffuse selective pressures on their hosts.

Environmental heterogeneity in pathogens and pathogen genotypes can lead to spatial adaptation to local pathogen pressures (Woolhouse *et al.*, 2002). Genotype-by-genotype interactions between hosts and pathogens allow for adaptation to proximate pathogen pressures. Experimental evolution has been used to demonstrate the potential for local adaptation. In an experiment where *P. ramosa* was serially passaged for several generations on *D. magna*, it evolved high levels of infectivity on the host used for passage and in some cases lost virulence on non-passaged hosts (Little *et al.*, 2006). This indicates that parasites can adapt to current hosts, perhaps at a cost of infecting alternate hosts, in only a few generations. Spatial variation in resistance can be detected by comparing the success of infection between host–parasite combinations that are either sympatric (local) or allopatric (foreign). Although most theoretical models predict that the parasite should be most successful in sympatric infections, in practice both parasite local adaptation and maladaption are observed (Woolhouse *et al.*, 2002). In *A. gambiae*, a locus that was found to control encapsulation response to the malaria parasite *Plasmodium falciparum* was strongest against allopatric infections (Niaré *et al.*, 2002). Another locus restricting infection intensity was strongest against sympatric infections. Despite the opposite directions of these responses, both findings demonstrate population variation in resistance. In some cases, host resistance and parasite virulence have been observed to covary. The parasitoid *Asobara tabida* has been reported to have the highest virulence in the Mediterranean and lower virulence in northern Europe (Kraaijeveld and Godfray, 1999). *D. melanogaster*, the host, was observed to have the highest resistance in the Mediterranean and southern Europe, and low resistance in northern Europe, evidence of adaptation to local parasitoid pressures.

Tremendous variation in immunocompetence exists in extant natural populations. Trade-offs within the immune response and between immunocompetence and other fitness components constrain the ability of natural selection to drive resistant genotypes to fixation. Variation in trade-offs is maintained in part by environmental variation, whereby the costs associated with a particular genotype are context-dependent. Genotype-by-environment interactions and local adaptation can potentially lead to the maintenance of multiple polymorphisms in heterogeneous environments. Furthermore, the pathogen 'environment' is itself evolving. These forces in combination oftentimes limit the evolution of a single globally resistant genotype.

13.5 Conclusion

Genes involved in the immune response show signals of rapid evolution, with the precise evolutionary mode varying among components of

the immune system. Extant populations harbour tremendous genetic and phenotypic variation in resistance, providing the substrate upon which selection acts. Examination of both evolutionarily ancient and current patterns has only rarely been performed. The most complete example is from the *ref(2)P* locus in *D. melanogaster*, which is polymorphic for the ability to permit or restrict Sigma virus infection. In natural populations, this locus shows evidence for elevated polymorphism, partial selective sweeps, and spatial heterogeneity in allele frequencies, all of which reflect an on-going battle between host and pathogen. These polymorphisms also often become fixed, driving long-term adaptive amino acid evolution. Other parts of the immune system could be equivalently studied, such as a polymorphic locus in the mosquito *A. gambiae* that confers resistance to malaria. In general, characterization of forces that facilitate or inhibit the spread of host resistance through populations, combined with genome-scale comparisons between species, will allow the linkage of short-term and long-term patterns to fully define the lability and constraint on adaptive evolution across the immune system.

Understanding the factors that influence the evolution of the immune response has important ramifications for diverse fields of study. Evaluation of the feasibility of applications such as the proposed engineering of transgenic disease-vector insects to control transmission and the use of pathogens to implement biological control of pest populations benefits from the most complete understanding possible of how resistance arises and propagates through natural populations. These are inherently evolutionary biological questions. The evolutionary dynamics of insect–pathogen interactions also has clinical importance in so far as insects can serve as model hosts for humans. Evolutionary inferences about how pathogens interact and interfere with different components of the immune system inform studies in molecular immunology. Advances in immunology, in turn, will test these predictions and identify new sets of genes and pathways in a wider range of organisms, further broadening the field of evolutionary genetics.

13.6 Acknowledgements

We thank Madeline Galac for helpful advice during the preparation of this manuscript and Jacob Crawford, Sarah Short, and Gerardo Marquez for feedback on the manuscript. We thank Tim Sackton for providing portions of the data illustrated in Figure 13.3. Work in the Lazzaro lab is funded by grants from the US National Science Foundation and National Institutes of Health.

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CHAPTER 14

Ecological and evolutionary implications of specific immune responses

Ben M. Sadd and Paul Schmid-Hempel

14.1 Introduction

Specific immune responses will have implications for a number of ecological and evolutionary aspects of host–parasite relationships. Such immune specificity may be observed on two non-mutually exclusive levels. On one hand, genetically encoded responses against distinct parasite types can be discrete. Coupled with genetic diversity underlying these responses, this can lead to specific interactions between host and parasite genotypes. In addition, individual host experiences of parasites within their environment will shape immunity in response to the encountered circumstances. These primed levels of immunity can also show specificity. While vertebrates are well known for possessing specific immunity, and the associated mechanisms are well understood, specific immunity in invertebrates has been something of an enigma due to the lack of a clear mechanistic basis. Recently, studies in evolutionary ecology have shown that specific responses are widespread in invertebrates. In this chapter evidence for specific immune responses is presented, including both the cases where the specificity is observed in genetically determined host–parasite interactions and where specific responses are acquired depending on encounters with particular parasite types. Specific immune responses have implications for topics such as the evolution of virulence, the maintenance of diversity in natural populations, and the evolution and maintenance of sexual reproduction.

The study of ecological immunology has been motivated by a number of different questions that have emerged from various fields of enquiry. In particular, the field was strongly influenced by mathematically oriented population biologists interested in the dynamics of interacting host–parasite populations (Anderson, 1994a, 1994b; Anderson and May, 1979; May and Anderson, 1979) and the structure of host and parasite populations (Lythgoe, 2002). Regardless of how they come about, specific interactions between hosts and parasites are often assumed in these analyses. This population biology-centred view has focused the studies primarily on vertebrate host systems. Within these systems, the necessary specific immune responses have a clear and relatively well understood immunological basis in the highly specific adaptive immune system with a diverse repertoire of T and B cells (Lythgoe, 2002; Berding *et al.*, 1987). The interest in specific host–parasite interactions, and thus by implication in specific responses, has also been of considerable interest for the development of models to explain the maintenance of genetic diversity in host populations. If processes that maintain genetic diversity, such as sex and meiotic recombination, are driven by antagonistic co-evolution (the Red Queen hypothesis; Salathe *et al.*, 2008), then they will only be selected for if specific interactions of an appropriate kind do indeed exist (Box 14.1).

The second major influence on the developing field of ecological immunology came from

Box 14.1 Concepts and caveats

Immunity in the evolutionary ecology sense is an organism-level trait

Generally in this chapter, immunity and specific immunity are discussed in an evolutionary ecology sense. This sets them apart from any particular mechanism. Immunity here refers to any trait that leads to resistance and the prevention or reduction of parasite infection.

There is a spectrum of specificity and the level on which it occurs is important

Specificity is a quantitative measure, and discrimination of parasites by the immune system can take place on different levels of parasite relatedness. Specificity can be coarse-grained, for example between general classes of microbes, such as bacteria and fungi. This coarse specificity can be explained by differential effectiveness of pathways that are already relatively well understood (e.g. Toll and Imd). However, at the discriminative extreme of the specificity spectrum is the differentiation between strains of the same parasite species. Some of the examples given in this chapter suggest that the insect immune system can act on this level of specificity. This suggests that it is necessary to look beyond the currently well-studied mechanisms for the mechanistic basis of immune specificity in insects (see Chapter 5 in this volume). The evolutionary consequences of immune specificity will also depend on the level at which it acts. For example, selection for diversity of parasite genotypes will only occur if these genotypes are differentiated by the immune system.

Resistance is not the only means of coping with parasites

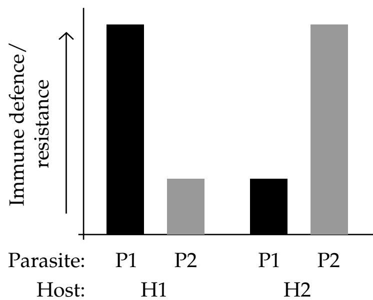
Resistance will limit infection intensity, whereas tolerance mechanisms will limit the fitness impact of a particular infection (Schneider and Ayres, 2008). Taken together, resistance and tolerance will determine the fitness loss that is incurred by an individual on parasite infection. It is plausible that there may be specific tolerance, just as there is specific resistance. The study in insects of these aspects of coping with parasite infection, and their consequences, is still in its infancy. As a result, this fascinating topic will not be discussed further in this chapter, but interested readers are directed to a relevant review by Schneider and Ayres (2008).

evolutionary and behavioural ecology. Hamilton and Zuk started this process with their influential paper on parasite-mediated sexual selection (Hamilton and Zuk, 1982). The study of sexual selection and the role of parasites has been increasingly affected by the search for evolutionary trade-offs between the investment into attracting mates (fertility selection/sexual selection) and other components of fitness (viability selection/natural selection). Similarly, the general framework of evolutionary life history theory is based on the existence of various trade-offs between different fitness components, such as reproduction and survival, the latter being very closely associated with immune defence. The idea that a less-than-perfect immune response and widespread susceptibility of hosts towards their parasites should be the result of unavoidable trade-offs has been a fruitful concept (Sheldon and Verhulst, 1996; Folstad and Karter, 1992), whose validity was documented early in the history of the field (Gustafsson *et al.*, 1994; Norris *et al.*, 1994; König and Schmid-Hempel, 1995). Further discussions on trade-offs involving the immune system can be found in Chapters 10, 11, and 12 in this volume. However, regardless of the usefulness of the trade-off concept, the element of specificity has not been a major consideration in trade-off discussions (see Schmid-Hempel and Ebert, 2003). Specificity has been touched on in terms of trade-offs within the immune system, for example between non-specific and specific defence (Moret, 2003; Mallon *et al.*, 2003), but has rarely been incorporated into any other framework in this branch of the field.

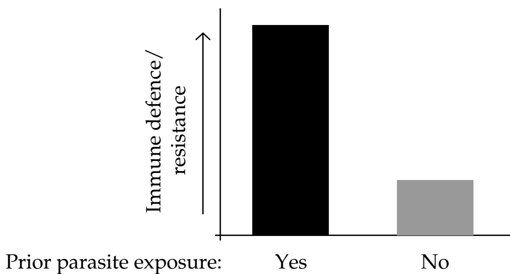
This chapter will approach the question of immune specificity from an evolutionary ecology perspective. For the sake of clarity, immune specificity will be addressed on two levels. First,

immune specificity will be considered in the light of evidence for specific interactions between hosts and parasites (Figure 14.1a). The importance of these specific interactions for questions concerning genetic diversity will then be discussed. The second level on which immune specificity will be addressed is in the context of immune priming (Figures 14.1b and 14.1c). It must be stressed that these two phenomena are almost certainly not mutually exclusive. For instance, the level of primed defences may be constrained by the innate defence capacity of an individual. Consequently, immune priming may play a role in the formation of specific interactions between hosts and parasites when re-infections are persistent or infections are chronic. Prior to concluding, the chapter will consider sociality, and in particular immune defence within social insects. Social insects have been important study organisms in elucidating the evolutionary ecology of immune defence. Furthermore, as they are particularly sensitive to issues relating to specific immune defence, empirical examples from the social insects are found throughout the chapter.

(a) Specific host–parasite interactions



(b) Immune priming (within a host type against a single parasite)



(c) Specific immune priming (within a host type)

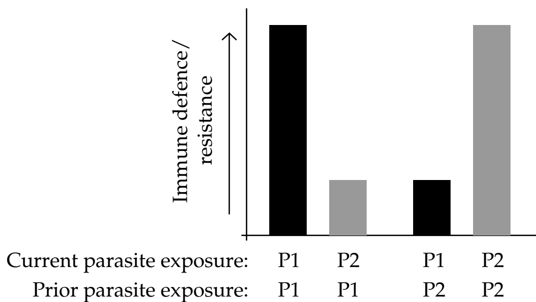


Figure 14.1 (a) Specific interactions. (b) Immune priming. (c) Specific immune priming.

14.2 Empirical evidence for specificity and specific interactions

For specific interactions (Figure 14.1a) to materialize within a host–parasite system, two conditions must be met. There must not only be polymorphisms between hosts in their immune defences, but the suite of responses effective against different parasite types should not entirely overlap. In other words, they should be specific to the parasite type. Whole-organism infection studies, and more recently genetic and molecular work, have shown that invertebrate hosts demonstrate such specificity. Using the snail *Biomphalaria glabrata* and its parasite *Schistosoma masoni*, host lines were separately selected for either resistance or susceptibility to two different parasite strains. While the selected lines responded as expected to the parasite strain they were exposed to, no concomitant change in resistance was found to the other parasite strain (Webster and Woolhouse, 1998). Similarly, quantitative trait loci (QTL) studies of the encapsulation of malaria parasites by mosquitoes has shown that a major QTL for the encapsulation of one parasite strain contributed little to the encapsulation of another (Carton *et al.*, 2005). It can be concluded that in these systems resistance is parasite-strain-specific, in that the suite of defences effective against one parasite type are not similarly effective against a second distinct parasite type. Relating to the idea that different immune defence components are specific against particular infections are more detailed mechanistic studies

of immune defence pathways, and their importance for combating pathogens. For instance, a plethora of work on *Drosophila* using mutants of certain immune system components has demonstrated specificity on a coarse level. Particular sets of recognition, signalling, and effector molecules are required for defence against certain classes of microbes but not others (Hultmark, 2003). For example, the two best-studied signalling pathways in *Drosophila* immunity, Toll and Imd, have been shown to be differentially required for an effective immune response depending on the microbe in question (Hultmark, 2003). Furthermore, post-genomic studies show different patterns of gene expression on exposure to distinct pathogens and parasites. When *Drosophila* received a fungal, protozoan, viral, or bacterial infection, 64% of the upregulated genes were specific to the infection type (Röxstrom-Lindquist *et al.*, 2004). Further evidence concerning the differential use and importance of immune system components, based on a coarse level of specificity between parasite types, can be found in Chapters 2 and 4. A mechanistic basis for a finer degree of immune specificity can be found in Chapter 5.

Note from the examples thus far that 'specificity' does not necessarily mean that resistance against one parasite type is traded-off with resistance to another, a pattern that would emerge as a negative co-variation between the two resistance components. While this trade-off could take place between two immune pathways or immune genes, an alternative is that alleles at a particular gene confer resistance or susceptibility depending on the parasite type. In this case, specific resistance against one parasite type will be antagonistic to resistance against another.

For many years, evolutionary ecology studies of host–parasite systems have strongly hinted that specificity exists within the immune defence of invertebrates. Several studies (Table 14.1) have demonstrated the existence of specific interactions between host and parasite types (see Figure 14.1a). Taking the evolutionary ecology perspective of immunity, as any host trait that influences the infection level of that individual (see Box 14.1), such host–parasite interactions would not manifest if the immunity of invertebrates were homogeneous and not specific. Instead, genotypic differences in hosts would relate to across-the-board resistance or susceptibility to parasites. This is not the case, and therefore the empirical evidence offers strong support for the existence of specific immune responses in invertebrates. This inference derived from macroscopic infection experiments is now being strongly corroborated by the discovery of specific immune mechanisms based on different principles than those found in vertebrates (see Chapter 5).

Table 14.1 Examples of specific interactions between invertebrate hosts and their parasites. The presence of such interactions hints strongly at the existence of specific immune responses.

Host	Parasites	Comments	Reference
Daphnia magna	Two bacterial and three microsporidia	Interaction between host clones and parasite species in measures of virulence, infectivity, and spore production.	Decaestecker *et al.* (2003)
	Pasteuria ramosa (bacterium)	Interactions between host clones and parasite clones with respect to virulence and infectivity.	Carius *et al.* (2001)
Bombus terrestris	*Crithidia bombi* (protozoan)	Interaction between host colony (relatedness between individuals = 0.75) and parasite isolate with respect to transmissible cells shed in the faeces.	Schmid-Hempel (2001)
		Interaction between host colony and parasite isolate with respect to infection intensity in the gut.	Mallon *et al.* (2003)
Caenorhabditis elegans	*Serratia marcescens* (bacterium)	Interaction between host and parasite strains in respect to virulence (expressed as survival).	Schulenburg and Ewbank (2004)
Anopheles gambiae	*Plasmodium falciparum* (protozoan)	Interaction between host family and parasite isolate with respect to the likelihood and intensity of infection.	Lambrechts *et al.* (2005)

Examples of specific host–parasite interactions have been found across the invertebrates, including insects, and for a variety of different parasite types. In an infection experiment with nine different clones of the host *Daphnia magna* and nine different isolates of the bacterial parasite, *Pasteuria ramosa*, the interaction term between host clone and parasite isolate was significant in determining resistance (Carius *et al.*, 2001). In other words, the influence of host clone on the outcome of infection was dependent on the parasite isolate it was infected with. No clone was completely susceptible or completely resistant to all parasite strains. The same was also true for the infection ability of parasite isolates. Similarly, when different isolates of the trypanosome *Crithidia bombi* were passed through a number of different colonies of its bumblebee host, *Bombus terrestris*, a clear association of different parasite isolates with a given host colony was found (Schmid-Hempel *et al.*, 1999). Furthermore, controlled infection experiments of host individuals with different parasite isolates yield results comparable to the *Daphnia* example above. Infection intensity is dependent on an interaction between the host origin and parasite isolate (Schmid-Hempel, 2001). A genetic screen of the naturally infecting *C. bombi* populations also supports this finding. This showed the population of the parasite to be highly structured and genetically diversified, a pattern that is expected to result from strong genotypic host–parasite interactions (Schmid-Hempel and Reber Funk, 2004). In light of the above results and those presented in Table 14.1, there is little doubt that many systems show specificity at the macroscopic level of infection and susceptibility.

14.2.1 Specific interactions and the maintenance of genetic polymorphism

The presence of specific immune responses and consequently specific host–parasite interactions not only has implications for the kind of immunological mechanisms operating in insects that might achieve such specificity, but also has many ramifications for a number of basic and important problems in ecology and population and evolutionary biology. Foremost among those is the expectation that the selective processes associated with specific interactions between hosts and parasites should lead to the maintenance of diversity, genetic and otherwise, in natural populations. This discussion was started by Haldane (1949) and has become a more intensively studied research topic over the last two decades. Theory has indeed shown that host–parasite co-evolution is likely to generate negative frequency-dependent selection that is able to maintain polymorphisms in host (or parasite) populations (Hamilton, 1982; Seger, 1988; Hamilton *et al.*, 1990). The topic is closely linked to the question of how sex and recombination can evolve and be maintained (Peters and Lively, 1999; Salathe *et al.*, 2008). One prominent hypothesis for the widespread occurrence of sex and recombination, the Red Queen hypothesis, requires antagonistic co-evolution between species resulting in genotype-frequency-dependent selection. The idea is that sex and recombination might allow hosts to escape parasites by creating rare offspring genotypes (Salathe *et al.*, 2008). Genetically encoded host–parasite interactions are a requisite to achieve the necessary co-evolutionary dynamics. As discussed above, these interactions are most likely present due to the existence of immune responses specific to parasite types. However, in all these discussions relating to genetic diversity and co-evolution, the level of specificity is key (see Box 14.1).

The exact way in which specific responses influence dynamics and evolution in host–parasite systems will greatly depend on the genetic architecture that underlies these traits. Work on this area has focused mainly on organisms and interactions that are of economic or medical importance, but general patterns have begun to emerge. Resistance seems to be achieved by one or a few major effect loci, including where specificity has been considered (Carton *et al.*, 2005). In a meta-analysis of published QTL studies it has been found that between one and six (mean 2.47) main effect loci are responsible for resistance to parasites in animal hosts (Wilfert and Schmid-Hempel, 2008). Relevant for theories linking host–parasite interactions with the evolution and maintenance of sex and recombination (Salathe *et al.*, 2008), it seems that epistatic interactions between loci contribute

substantially to the observed variance (Wilfert and Schmid-Hempel, 2008). For example, for the Red Queen hypothesis to work epistasis between parasite-resistance loci is necessary (Salathe *et al.*, 2008).

14.2.1.1 Empirical evidence for genetic diversity versus parasitism

That specific responses exist, and genetically distinct individuals show—depending on their genotypes—differential immune responses will mean that the resistance of a population will cover a greater range of parasite types than the resistance of a single individual within that population. Clearly, as genetic diversity increases the discrepancy increases. The same principle of genetic diversity will apply to diploid individuals, and individuals heterozygous at loci related to specific responses will cover a greater range of parasite types than those that are homozygous. It has been demonstrated that in vertebrates individual heterozygosity at resistance-related loci, but not neutral loci, is negatively correlated with parasite burden (Luikart *et al.*, 2008). For the major histocompatibility complex in vertebrates, a relationship between locus allelic diversity and parasite load was found, showing a minimum parasite load at an intermediate rather than maximum number of alleles (Madsen and Ujvari, 2006; Kurtz *et al.*, 2004). However, limited data exist for insects or other invertebrates. In *Drosophila* bacterial infections were shown to induce more damage in more inbred individuals (Spielman *et al.*, 2004), but no corresponding effect was found for the immune response in bumblebees (Gerloff *et al.*, 2003), for example. This is an interesting area of research that deserves further attention in insects. However, it also requires a more in-depth understanding of the host genetics behind specific host–parasite interactions.

The relevance of group diversity will be particularly pertinent for those animals living socially, such as the social insects. Indeed a variety of studies in social insects have shown the benefit of diversity when it comes to defence against parasites and pathogens (Table 14.2). It is the very fact that responses are specific that increasing genetic diversity has such an effect. However, it has also been suggested that diversity and higher heterozygosity may increase the efficiency of social antiparasite behaviour and in this way reduce infection levels, rather than having implications for individual immune defence (Calleri *et al.*, 2006). Yet, it is hard to imagine that individual immunity does not play any role at all in generating the observations documented in Table 14.2. As such, the interplay of host diversity, specific responses, and parasites will have a number of consequences in social groups. For example, these factors are likely to have played a role in the evolution of particular mating strategies in social insects (Sherman *et al.*, 1988; Schmid-Hempel and Crozier, 1999; Brown and Schmid-Hempel, 2003). Polyandry, mating with multiple males, has been proposed to have benefits in that it will increase genetic variation in offspring, thus reducing parasite infection (Brown and Schmid-Hempel, 2003).

The focus here has been the maintenance and benefits of genetic diversity of hosts. However, parasite genetic diversity is also likely to be influenced by the presence of genetically encoded specific responses that discriminate between parasite strains. Host selection of parasite genotypes will maintain diversity when specific responses occur and host populations or communities are made up of individuals that differ in these responses (Hitchman *et al.*, 2007).

14.3 Immune priming as a specific response to the current environment

The observation of specific immune responses, specific interactions between host and parasite types, and the rapidly accumulating knowledge on the molecular mechanisms of the insect immune system (Chapters 2, 4, and 5 in this volume; Du Pasquier, 2005; Dong *et al.*, 2006) collectively suggest that the insect immune system is much more sophisticated in its performance than was traditionally believed. Two additional responses are particularly interesting in the context of specific responses. These are immune priming across generations (trans-generational immune priming) and immune priming within an individual. These two phenomena are based on immune priming; that is, the capacity of generating an improved immune response depending on previous experience with the environment (Figure 14.1b and 14.1c).

Table 14.2 Examples from the social insects showing the benefits of group diversity against parasites. This benefit is, at least in part, likely to be a consequence of specific responses.

Host	Parasite	Method used to achieve variation in diversity	Influence of diversity	Reference
Formica selysi	*Metarizhium anisopliae* (fungi)	Monogynous versus polygynous colonies	Survival following exposure	Reber *et al.* (2008)
		Mixing of individuals from separate colonies	Survival following exposure	
Apis mellifera	*Paenibacillus larvae* (bacterium)	Artificial insemination with one or 10 males	Intensity of infection	Seeley and Tarpy (2007)
	Natural parasites in the field			Tarpy and Seeley (2006)
	Ascosphaera apis (fungus)		Variation in disease prevalence	Tarpy (2003)
Acromyrmex echinatior	*Metarizhium anisopliae* (fungus)	Serial passage through groups of individuals from one or three patrilines	Evolution of parasite virulence	Hughes and Boomsma (2006)
		Serial passage through full-sibs, half-sibs, unrelated conspecifics, and congenerics	Probability of parasite extinction	
		Grouping of individuals from one or three different patrilines	Survival following exposure	Hughes and Boomsma (2004)
Various	Various	Cross-species comparative analysis of colony relatedness	Parasite richness	Schmid-Hempel and Crozier (1999)
Bombus terrestris	*Crithidia bombi* (protozoan)	Keeping workers with kin or non-kin	Parasite prevalence	Shykoff and Schmid-Hempel (1991)
	Natural parasites in the field	Transplantation of brood between colonies	Parasite prevalence, infection intensity, and species richness	Liersch and Schmid-Hempel (1998)
		Artificial insemination with sperm of four unrelated males or four brothers		Baer and Schmid-Hempel (1999)

14.3.1 Trans-generational immune priming

Vertebrate mothers pass antibodies to their offspring via milk (Hanson, 1998) or the placenta in mammals, and through yolk in birds and fish (Grindstaff *et al.*, 2003). In these cases, the mechanism of immunity is clear. Functionally homologous phenomena have been observed in insects, but the mechanisms have yet to be uncovered.

In a study on bumblebees, it was shown that there are higher induced levels of antibacterial activity in offspring from mothers who had received a bacterially based immune challenge, prior to colony founding and subsequent egg laying (Sadd *et al.*, 2005). Cross-fostering experiments confirmed this result and showed that the cue for the trans-generational effect is passed through the egg and persists into the adulthood of the offspring (Sadd and Schmid-Hempel, 2007). The phenomenon of trans-generational immunity has also been demonstrated in mealworm beetles (Moret, 2006). While none of these studies have dealt with the issue of specificity, a study in the crustacean *Daphnia* demonstrated maternal transfer of specific resistance. The resistance transferred from mother to offspring was highly specific, differentiating on the level of bacterial strains (Little *et al.*, 2003). One proposed molecular basis for creating immune receptor diversity, *Dscam* (see Chapter 5), seems conserved between *Daphnia* and insects (Brites *et al.*, 2008). Given this, we may well expect trans-generational immune priming of a similar level of specificity in insects.

In trans-generational immunity, both the mother and her environment influence the phenotype of

the offspring. This will be a particular advantage when the environment experienced by the mother, in this case the prevailing parasite and pathogen community, is closely related to that encountered by her offspring. Such defence-associated trans-generational effects will enable offspring to reap the benefits of defence when required, avoid the costs of investment when it is not needed, and avoid the potential lags that are often involved in induced defences (Agrawal *et al.*, 1999). While unlikely to be directly relevant for the issue of specificity, offspring immunity and resistance in invertebrates may also be influenced by maternal environmental cues, outside of those related to immunological experience. For example, mother *Daphnia* subjected to an environment with poor food during reproduction produce offspring that are more resistant to a bacterial pathogen than offspring of mothers reproducing in a high-food environment (Mitchell and Read, 2005).

14.3.2 Individual immune priming (immune memory)

In vertebrates, the development of B and T cells into memory cells provides a molecular mechanism for the formation of an immune memory. No such cells exist in invertebrates such as insects, and therefore it has long been controversial whether something functionally akin to immune memory can exist in this taxon. However, it has been known for some time that in moths 'immunity' is transferable. Injecting the haemolymph of challenged individuals into a test individual led to an improved resistance of the test individual when it was challenged (Wagner, 1961). Similarly, in a series of studies with cockroaches results hinted that immunization of these animals was at least plausible (Rheins and Karp, 1985; Faulhaber and Karp, 1992). The shortcomings of these experiments, and the failure to find appropriate molecular mechanisms have lead to the dogma that invertebrates cannot possess an immune phenomenon that is functionally analogous to immune memory (Klein, 1997). While it is unlikely that this dogma will go away overnight, evidence has started to accumulate that invertebrates do possess a functional equivalent of immune memory. That is, a lasting improved response after an initial exposure is here referred to as immune priming.

In crickets lifetime upregulation of immune components following a nymphal immune challenge has been demonstrated (Jacot *et al.*, 2005). Furthermore, it has been demonstrated in insects that immune priming increases the probability of survival against subsequent exposures of fungi (Rosengaus *et al.*, 1999b; Moret and Siva-Jothy, 2003) and bacteria (Rosengaus *et al.*, 1999b). In shrimps, the effects of a commonly occurring viral infection are reduced following an earlier priming with a controlled dose of the virus (Wu *et al.*, 2002), and this effect can be replicated when only using a particular viral envelope protein (Witteveldt *et al.*, 2004). These studies do not show specificity, nor aim to investigate it; however; functional studies showing protective immune priming and specificity in tandem are beginning to emerge. Strain-specific immune priming has been shown for crustaceans infected by cestodes (Kurtz and Franz, 2003), but some of the best evidence for lasting specific immune priming in invertebrates comes from a well-controlled study of antibacterial immunity and resistance in the bumblebee. Workers of *B. terrestris* were challenged with sublethal doses of different bacteria before being exposed to a lethal dose 8 or 22 days later. In these tests, worker bees exposed to the same bacterium had a greater probability of survival and a higher proportion of them cleared the bacteria from their haemocoel compared to those exposed to a different bacterium (Sadd and Schmid-Hempel, 2006) (Figure 14.2). This study strongly suggests that insects do have a functional equivalent of immune memory even though the mechanisms are not yet known (Arala-Chaves and Sequeira, 2000; Kurtz, 2004).

Clearly, the level of specificity associated with immune priming will depend on the exact immune mechanisms that are involved. Initial studies suggest that a wide spectrum, through cross-reactive, coarsely specific, and highly specific immune priming, is present in invertebrates. This level or even the existence of immune priming may also depend on the pathogens involved. Specific immune priming in *Drosophila* was found for the bacteria *Streptococcus pneumoniae*, but not for

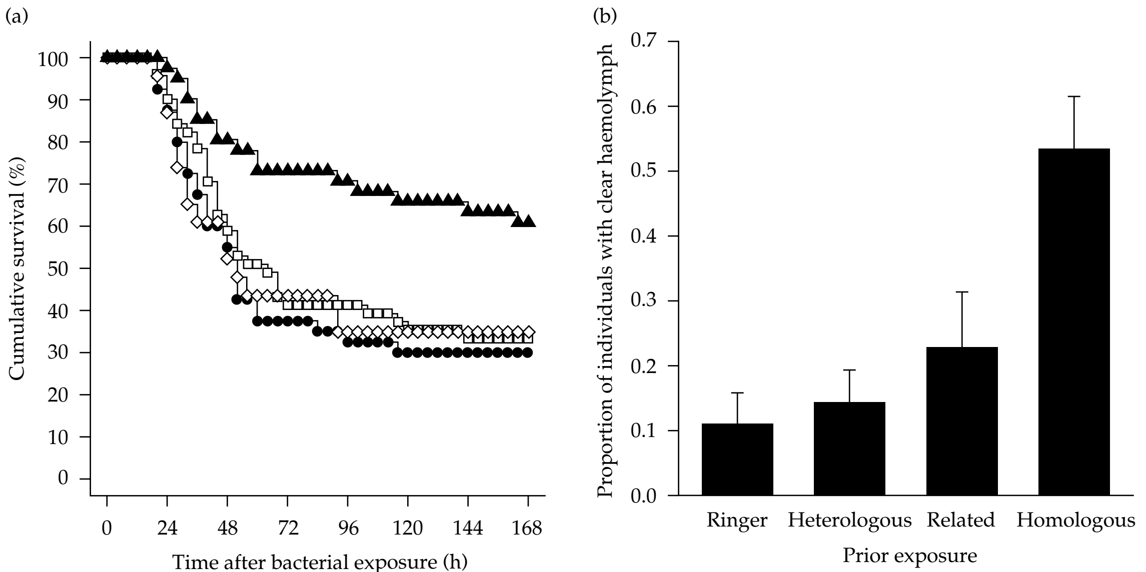


Figure 14.2 Demonstration of specific immune priming in insects. (a) Bumblebee workers exposed to a clearable dose of bacteria had an increased probability of survival when challenged with a lethal dose of the same bacteria (homologous ▲) 22 days later, over and above that of animals challenged with a different bacteria (heterologous □ and related heterologous ◇) and those that had only previously received a control Ringer injection (●). (b) This specificity is also reflected in the proportion of individuals that have cleared their haemocoel of bacteria 24 h after a second challenge. Redrawn after Sadd and Schmid-Hempel (2006).

Salmonella typhimurium, *Listeria monocytogenes*, and *Mycobacterium marinum* (Pham *et al.*, 2007). In the beetle *Tribolium castaneum*, results suggestive of strain-specific immune priming against *Bacillus thuringiensis* were found, but no such results could be found for defence against *Escherichia coli* (Roth *et al.*, 2009).

14.3.3 The consequences of immune priming within and across generations

The demonstration in insects of specific immune priming and trans-generational immunity suggest that while they rely on different mechanisms, the immune systems of both vertebrates and invertebrates have been selected by parasites and pathogens to perform similar functions. It is likely that a particular level of spatial and temporal variation in exposure to parasite types would give a benefit of immunity that is acquired depending on prior experiences. For immune priming to be beneficial variation in the exposure to parasite types should be rapid enough to stay ahead of changes in innate defence levels, but slow enough that secondary exposure to a distinct parasite type is likely once it has already been encountered.

The existence of immune priming both within individuals and across generations will have implications beyond that of understanding the evolution of immune system function. Once they have evolved in hosts, these phenomena of immune priming would have a set of consequences for the further evolution of hosts and parasites, and the co-evolutionary interactions between the two. While this area has received little attention, it is possible to make some intuitive predictions. The postulated examples and potential outcomes given below are generalizations and are by no means exhaustive. However, their aim is to demonstrate that specific immune priming will have far-reaching consequences for the evolution of host and parasite traits, and their co-evolution.

Immune priming in a basic sense can be seen as an acquired element of host resistance. This acquired resistance will only be present in a host individual if it, or its mother, previously

encountered a particular parasite type. This is in contrast to the genetically encoded innate levels of resistance that can be found even in immunologically naïve individuals. It can reasonably be assumed that the presence of these two immune strategies will have certain impacts on the effectiveness of the other, and thus on each other's evolution. Logically, acquired resistance will have no value in a system where the innate defence of individuals does not allow them to overcome an initial infection. However, it is more plausible that immune priming will be found in a host population that exhibits a spectrum of genetically encoded defences from susceptible through to resistant. Where this is the case, and not all susceptible individuals are removed from the population on an initial exposure, immune priming may maintain more innately susceptible genotypes within the population. This will be the result of immune priming turning susceptible phenotypes into resistant phenotypes. As a consequence this would probably slow down the evolution of genetically based resistance against parasites. This has been considered for the case of vertebrates, where modelling the evolutionary dynamics of host resistance traits showed that while acquired resistance has benefits, it decreases the rate of evolution in innate resistance traits (Harding *et al.*, 2005). However, to understand how the evolution of immune priming and innate resistance traits are linked in insects, it will be necessary to understand more about their mechanistic basis. It is possible that they employ similar pathways, and as such, levels of acquired resistance mediated by immune priming will be tied to innate levels.

Virulence evolution is a topic that has received a great deal of attention and controversy in evolutionary biology (Bull, 1994; Frank, 1996). If immune priming in insects affects the fitness of parasites, then it must have had implications for the evolution of parasite virulence. At a basic level, it can be imagined that heightened immune capabilities of a primed host will select for faster and more efficient immune evasion strategies in parasites; immune evasion strategies that may contribute considerably to virulence (Frank and Schmid-Hempel, 2008). Borrowing results derived from models based on vertebrate immunity and vaccination, it can be suggested that immune priming will enable the co-existence of inferior parasite types by mediating competition (Wodarz, 2003), and promote the evolution of faster-replicating, more virulent strains (Fenton *et al.*, 2006; André and Gandon, 2006). This latter consequence has been confirmed empirically in a mouse/malaria system that investigated the effect of immune selection via vaccination. Parasite lines transferred through mice with acquired resistance (via immunization) were found to be more virulent than lines transferred through naïve mice (Mackinnon and Read, 2004).

In the prior two paragraphs we have considered how host and parasite traits may evolve in response to the presence of immune priming in the system. However, it is clear that it is not possible to separate the two protagonists, and what we observe in nature will be ongoing co-evolutionary dynamics. Such dynamics between hosts and parasites are important for a number of evolutionary theories. As touched on above in a different regard, these include the Red Queen hypothesis that invokes the role of parasites in the maintenance of sexual reproduction and recombination (Peters and Lively, 1999; Salathe *et al.*, 2008). This hypothesis is based on the existence of oscillations in the frequency of host and parasite genotypes. These oscillations are produced by negative frequency-dependent selection, where rare genotypes are at a fitness advantage. Based principally on the idea that more susceptible host genotypes will be maintained in a population with immune priming, it has been suggested that immune priming will dampen the amplitude of co-evolutionary oscillations (Little and Kraaijeveld, 2004). While this may have complications for theories that depend on co-evolutionary dynamics, the issue clearly needs to be given greater consideration both theoretically and empirically. It is possible that, as in vertebrates (Borghans *et al.*, 2004), immune priming is achieved by highly polymorphic loci, and as such will add more material for co-evolution to act upon.

14.4 Immunity and sociality in insects

An important and fascinating group of insects are the social insects, essentially the ants, bees,

wasps, and termites. Throughout this chapter, many examples pointing to insect immunity having specificity and greater functional complexity than previously assumed come from social insects. Social insects will also be particularly sensitive to issues relating to the existence of specific immune responses (e.g. Table 14.2). Therefore, time will be taken here to briefly outline further related aspects of the evolution of social insects and their immune defences.

Social insects invest heavily in brood care; in fact, co-operative brood care is a defining element of sociality in insects (and elsewhere). It is therefore fitting that the first demonstration in insects of the transfer of immune priming to offspring should have been in a social insect, the European bumblebee *B. terrestris* (Sadd *et al.*, 2005). In a social insect colony there are overlapping generations, and both temporal and spatial stability of the environment. In such a setting, there is a clear benefit if a mother can influence her offspring's immunity in line with the pathogen environment that she has herself encountered. Additionally, specific immune priming within individuals, as has also been shown in *B. terrestris* (Sadd and Schmid-Hempel, 2006), will be beneficial where the probability of individual re-exposure is high.

14.4.1 Social immunity

Given the lifestyle of social insects they are particularly vulnerable to the threat posed by parasites. Colonies are made up of many highly related individuals that live in close proximity in a comparatively stable environment. The larger the colony the greater the probability that it is exposed to parasites given increased numbers of foragers (Naug and Camazine, 2002). Once exposure takes place, the high density of related individuals is likely to facilitate the spread of infection (Gadagkar, 1992; Schmid-Hempel, 1998; Pie *et al.*, 2004). As discussed earlier, diversity and relatedness of individuals within a group can influence parasitism in a number of ways (Table 14.2). Fitting with predictions concerning increased sociality and parasitism, it has been shown that in bees antibacterial activity correlates positively with the level of sociality (Stow *et al.*, 2007).

Outside of individual-based immune defence, social insects have additional possibilities to protect themselves through their social living and co-operation (Cremer *et al.*, 2007). Indeed, in *Acromyrmex* leaf-cutting ants these social immune traits are effective to such a degree that life in a group gives a net benefit on exposure to parasites, despite the increased risks (Hughes *et al.*, 2002). Following the sequencing of the first social-insect genome, that of *Apis mellifera*, it was even suggested that the apparent paucity of immune genes, relative to the numbers found in other insects, might have been due to the presence of these social defences (Evans *et al.*, 2006). Colony structure and division of labour between the social individuals may act as a defence (Schmid-Hempel and Schmid-Hempel, 1993). More active defences may also be employed, for example increased allo-grooming to remove fungal spores from nest mates (Rosengaus *et al.*, 1998) and alarm signals leading to avoidance of pathogen-exposed individuals (Rosengaus *et al.*, 1999a). These social immune traits on the colony level may also mimic individual responses to infection. Fever, the raising of the body's temperature in an attempt to counter a perceived parasite or pathogen threat, is a well-understood response against infection in individual organisms. In honey bee colonies exposed to the fungal pathogen that causes chalkbrood disease in larvae, *Ascosphaera apis*, nursing adult workers were found to raise the nest temperature (Starks *et al.*, 2000). Against a heat-sensitive pathogen such as *A. apis*, this colony-level response can been seen as analogous to fever within an individual.

Additionally, social immune priming may occur. Immunity of a naïve individual within a social colony is induced not only on contact with the infectious agent directly, but also on contact with infected nestmates. Social immune priming of this kind has been demonstrated for fungal resistance in termites (Traniello *et al.*, 2002) and for immune traits in bumblebees (Moret and Schmid-Hempel, 2001).

All the aforementioned defences of social insects are collectively referred to as social immunity (Cremer *et al.*, 2007). While it is yet to be investigated, it is reasonable to expect that they may also show some level of specificity.

14.4.2 Immunity and social evolution

Specific immune responses and some of the immune related phenomena that have been discussed earlier in this chapter will not only be beneficial in social insect colonies, but may also feedback on the evolution and maintenance of sociality. The costs and benefits of social behaviour were formulated into a rule by Hamilton (Hamilton, 1964a, 1964b). The terms factored into this rule could well be altered when taking into account specific immunity, immune priming, and parasites. A particular case that is likely to have a strong influence on the costs and benefits of sociality is the tailoring of immune responses to the prevailing environment, as is the case in trans-generational immune priming (Sadd *et al.*, 2005; Sadd and Schmid-Hempel, 2007). If parasite pressure is heterogeneous across an environment, the immune capabilities bestowed on offspring will have been customized to the local environment, but will not be globally optimal. Due to this fact, dispersal away from the local parasite environment will be costly, as levels of defence will not match those that are required. This will mean there is an advantage of 'stay-at-home' behaviour, a prerequisite for the formation of social groups, such as those seen in social insects.

14.5 Conclusion

It is almost certain that specific immunity plays a crucial role in natural systems, and it would be an important advance to understand specific immunity in insects to a level that allows the development of field-based assays. While it is clear that the presence of specific responses adds an extra dimension to studies of ecological immunology in insects, caution must be exercised when considering simple measures of immunity in the field. Standard assays of immunity in insects tend to consider relatively general effector systems, and these are usually measured against standardized microbes or immune elicitors. While measuring important aspects of the insect's immune defence, these assays overlook specificity, and may therefore not give an appropriate or full picture of the defence capabilities of a certain individual. This incomplete picture of resistance, together with the potential importance of tolerance (see Box 14.1), may explain results that fail to find clear correlations between measured immune parameters and levels of infection or survival on exposure to parasites and pathogens (Adamo, 2004; Mucklow *et al.*, 2004).

The presence of specific immune responses in insects has the potential to open up avenues in the study of more general questions in evolutionary ecology research. It is clear that specific interactions between a host's immune system and its parasites will have profound implications for many ecological and evolutionary processes. A selection of the potential consequences has been discussed in this chapter, but this list is by no means exhaustive. The presence of specific immune responses in insects, while essentially mechanistically distinct from those occurring in vertebrates, offers the opportunity to further experimentally investigate this phenomenon. The potential for experimental manipulation of systems used in the study of insect immunity and host–parasite interactions will enable the true nature of specific immune defence and its ecological and evolutionary implications to be uncovered.

Integration of the whole-organism approach—addressed prominently in this chapter—with more mechanistic studies is vital for further understanding of the importance of specific immune responses in insects. However, functional assays that allow organisms themselves to show what the immune system is capable of have proven to be a valuable tool in gaining insights into the existence of specific responses in insect immunity. These insights have demonstrated that the conclusion that the insect immune system is devoid of adaptive immune reactions and only capable of coarse specificity (Hoffmann, 2003) is incomplete and unjustified.

14.6 Acknowledgements

We would like to thank David Schneider and an anonymous referee for comments that helped improve this chapter.

14.7 References

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CHAPTER 15

Reproductive immunity

Michael T. Siva-Jothy

15.1 Introduction

One of the most predictable episodes of a female insect's life is the timing of mate-encounter and mating. In this chapter I propose that females are often subjected to predictable wounding during mating and that this wounding provides opportunity for environmental microbes to enter the female's haemocoel, thereby presenting immunological costs. I argue that this combination of factors is likely to lead to reproduction being a period of heightened immunological activity that has resulted in specific immune defence mechanisms and management systems that function to minimize costs while maximizing immunological efficacy. If true, these phenomena may provide valuable insights into how organisms with mechanistically simple immune systems protect themselves against a complex pathogenic world, and may also provide logistic opportunities to better study immunity in the wild.

Parasites and pathogens are ubiquitous and major agents of selection, affecting host fitness by reducing longevity and/or reproductive output. Even if they do not kill their hosts, they often make them more susceptible to other forms of death. An organism's main form of defence against them is the immune system and, throughout this review, I use the concept of immunity developed by the field of ecological immunity (e.g. Rolff and Siva-Jothy, 2003; Siva-Jothy *et al.*, 2005). Immune defence as envisaged by ecological immunity is broader than the physiological mechanisms examined by 'classical' immunologists: it encompasses all mechanisms that an organism uses to protect itself and so includes behavioural and anatomical traits, as well as the complex suite of systemic physiological mechanisms. In this chapter I consider the male–female interactions during reproductive encounters that are likely to affect overlooked patterns of female investment in immune function. Surprisingly little is known about how reproduction and immunity interact (but see Lawniczak *et al.*, 2007), despite (a) the core position of this trade-off in evolutionary and ecological thinking, (b) its proposed role in sexual selection (e.g. Hamilton and Zuk, 1982; Folstad and Karter, 1992), and (c) the fact that immune genes are known to be activated by mating (Lawniczak and Begun, 2004; McGraw *et al.*, 2004). I will concentrate on intersexual interactions and will not consider obligate sexually transmitted diseases (see review by Knell and Webberley, 2004). Rather I consider male traits that function to enhance male mating success but simultaneously increase the likelihood of wounding and therefore microbial transmission to females. The consequence is an increased exposure to 'opportunistic' microbes (e.g. bacteria, fungi, and fungal spores) that are present on the insect cuticle and in the immediate environment (see Figure 15.1). If females are wounded during mate encounters (which I propose is frequent and predictable across insect taxa), because of incidental damage caused by competition between males, courtship, copulation, and/or copulatory guarding then selection will favour the evolution of immune-management systems that offset these costs and operate in anticipation of these costs, or when they are incurred. My purpose is to stimulate research into this potentially rich but overlooked immunological episode of an insect's life.

It is axiomatic that hosts encounter their parasites unpredictably. However, recent work on insects has uncovered some subtle immune regulation

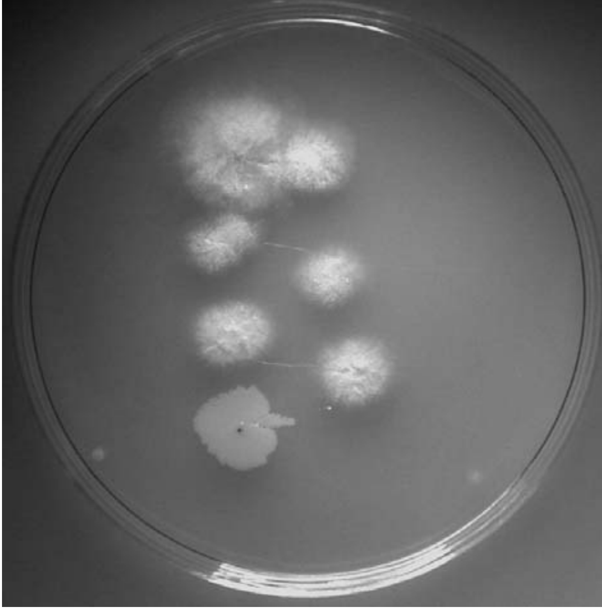

Figure 15.1 An illustration of the natural microflora on the aedeagus of an insect (a bedbug, *Cimex lectularius*). The ablated structure was removed and held in sterile forceps and drawn in a zig-zag pattern across the sterile agar plate. The aedeagus is visible as the dark central dot in the bacterial colony at the bottom of the plate.

where hosts monitor their current level/likelihood of infection and adjust investment either in their offspring (e.g. Moret, 2006) or in later ontogenetic stages (e.g. Wilson and Reeson, 1998), suggesting that they are capable of 'predicting' periods of increased likelihood of infection. Alongside these phenomena are studies showing that insect immune genes are under circadian control (e.g. Lee and Edery, 2008). Whereas there are several interpretations for the functional significance of such oscillations, one simple explanation is that these cycles occur because there are diurnal changes in the likelihood of infection; that is, they function, at least in part, to balance costs and effectiveness in the context of the likelihood of infection. Insects may be very good at anticipating immune insult and modulating their immune-effector systems to optimize this balance.

I propose that insects may show well-developed abilities to 'anticipate' immune insult in relation to their environmental conditions/life-history stage and adjust investment in immunity accordingly. One aspect of all female insects' life cycle that is both predictable and, I suggest, associated with a high risk of infection is mating and its associated behavioural, anatomical, and physiological interactions. These encounters tend to be highly predictable for females because it is usually females that determine the temporal occurrence of mating. Insect mate-encounter sites are usually spatially predictable and males are often selected to concentrate their efforts defending and/or competing for resources that attract females, resulting in a highly male-biased operational sex ratio (the ratio of reproductively active males to reproductively active females) at these sites (e.g. Thornhill and Alcock, 1983). Females can therefore avoid exposing themselves to males until they need to. However, a consequence of the male-biased operational sex ratio at these encounter sites is that females are then exposed to male traits that function to secure matings with females, and physically or physiologically 'subdue' them, often at a cost to their fitness. It is important to note that males will be subjected to similar selection, especially in species where fighting, and therefore wounding, is likely to lead to infection.

15.2 Mating and conflict

The notion that males and females of the same species may have different reproductive interests lies at the heart sexual selection theory (Bateman, 1948), and has recently been formalized under

the heading of sexual conflict. Sexual conflict theory (Parker, 1979) was born out of Trivers' (1972) realization that the sexes invest differentially in parental effort and gametes. Today it is a flourishing field of enquiry providing insight into the nature of evolution and reproductive interactions (Arnvist and Rowe, 2005). The most obvious form of sexual conflict arises because males tend to have higher mating-frequency optima than females, sparking an evolutionary interaction between the sexes where males express traits that enable them to secure matings at rates that exceed the female optima. These, usually agonistic, traits are readily observable in a range of insect taxa (e.g. Thornhill and Alcock, 1983; Rowe *et al*., 1994). For example, the competitive struggles between male dungflies to mount and defend females often causes physical damage to the female (Hammer, 1941). Likewise several males of the digger-bee *Centris pallida* will converge on, and struggle to grasp newly emerged females (Thornhill and Alcock, 1983). In some species of insect the males are so tenacious they cut the female in two while exerting an ever-tighter grip (Hölldobler, 1976), or bite through cuticular structures to maintain a better hold (Sivinski, 1981). Many male insects show specialized clasping organs which function to maintain a grip on either the female's external genitalia or delicate structures such as wings or eyes (e.g. MacKerras, 1970; Colless and McAlpine, 1970; Gerber *et al*., 1972; Thornhill, 1984). Some chironimid males have large, powerful, sclerotized grasping structures on their genitalia (Wirth and Sublette, 1970), and all odonate males grasp the female's head or prothorax prior to mating with special appendages on the end of the abdomen (Corbet, 1999). In two groups of odonates these male appendages tear the cuticle covering the compound eyes (Dunkle, 1984), resulting in reduced foraging efficiency and repair/immune costs (see Figure 15.2). As well as these manifestations of competition, males are also likely to damage females when selection drives males to mate with freshly eclosed females, that may be susceptible to physical damage. Male *Drosophila*

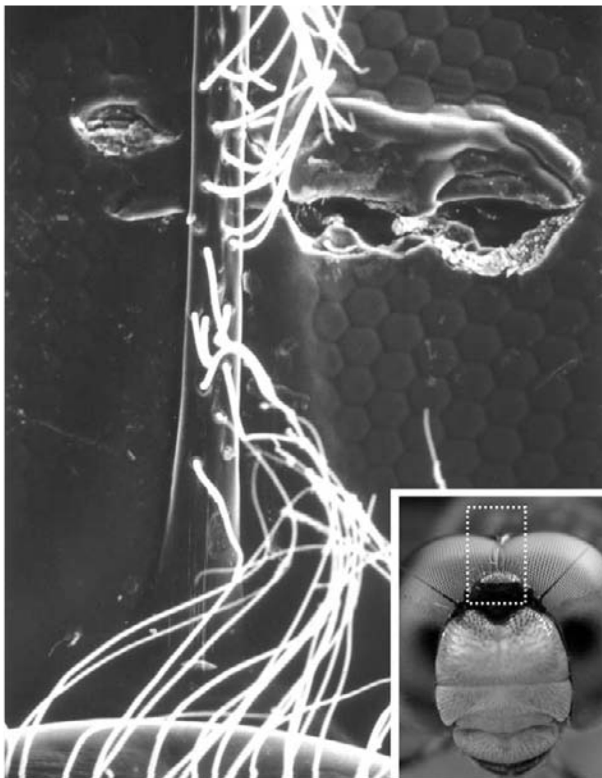

Figure 15.2 Damage to the vertex of the female's compound eyes caused by the male's abdominal claspers during pre-mating tandem formation in a dragonfly. The contact surface of the two disc-shaped projections can clearly be seen in the torn cuticle, as can the surface architecture of the ommatidia. The tear is approximately 1 mm across. The dotted rectangle in the inset photograph shows the region represented in the scanning electron micrograph.

melanogaster and *Drosophila simulans* mate with soft and vulnerable freshly eclosed adult females (Markow, 2000), and pupal mating is known in mosquitos (Slooten and Lambert, 1984) and heliconids (Deinert *et al.*, 1994). Each of these, and many other intersexual interactions during mating, are likely to cause external damage to females.

Sometimes mating is extremely prolonged in insects: up to 1 week in the Brimstone butterfly (Labitte, 1919). The male probably prolongs genital contact to prevent the take-over of mating by competitors (e.g. Parker, 1970). Although many insects maintain strong genital contact during copula, and often for prolonged periods, there are almost no studies that examine the effect these extended and mechanically powerful couplings have on the integrity of the cuticle in the female genital tract, especially in the propensity of males to wound the female.

15.3 Genital-tract wounding

As well as the range of scenarios in which the female's external body structures can become damaged, recent studies have revealed that males can, and do, damage female genitalia with specialized structures on the aedeagus. There has been an ongoing debate about whether such genital wounding is adaptive (i.e. males gain a direct benefit by wounding the female, e.g. delaying re-mating), or a by-product of competition between males (e.g. Merritt, 1989; Civetta and Clark, 2000; Morrow *et al.*, 2003). Regardless of the ultimate reason(s) for these 'harmful' male traits, the proximate consequence is the wounding of the female's genital tract (e.g. Crudgington and Siva-Jothy, 2000; Blanckenhorn *et al.*, 2002; Kamimura, 2007). That this has negative fitness consequences for the female has been demonstrated in the bean weevil *Callosobruchus maculates* (Crudgington and Siva-Jothy, 2000) and in the bedbug *Cimex lectularius* (Morrow and Arnqvist, 2003; Reinhardt *et al.*, 2003). Genital wounding has been regarded as a restricted phenomenon, but it is likely to be more widespread than previously thought. Recent work by Kamimura (2007) on the *Drosophila bipectinatta* complex has revealed that the males of 10 out of the 13 studied species wounded the female genitalia during mating. His work showed that this phenomenon also occurred in male *D. melanogaster* (Figure 15.3) and had been overlooked despite this insect's central role in identifying mechanisms of sexual conflict (e.g. Chapman *et al.*, 1995). Genital wounding has probably been overlooked in nature because of the lack of a conceptual framework: it is doubtless no coincidence that the recent explosion of interest in sexual conflict has coincided with the identification of this reproductive phenomenon.

Male insects have a wide range of melanized spines and bristles on their aedeagi that are interpreted as having a range of functions including the maintainance of genital contact. They may also generate copulatory wounding and/or traumatic insemination. Why would males 'want' to wound the female's genital tract? One proximate reason is that if they transfer physiologically active compounds that alter the female's receptivity or fecundity (e.g. Chapman *et al.*, 1995) these have to diffuse across the female's genital-tract cuticle. The potency of these compound(s) could be enhanced by ensuring they get directly into the haemocoel by breaching the cuticle. This may well have been the first step in the path that led to full-blown traumatic insemination. However, such genital-tract damage leaves the door open to costs to the female via infection from opportunistic pathogens and requires the female to repair the wounds. Activation of the female's immune-effector systems and wound repair will have to be completed at the expense of investment in other traits and so are likely to reduce female fitness. In this scenario the harmful male trait evolves because of the advantage it confers on males. Rönn *et al.*'s (2007) comparison of bean weevil species shows that females may respond to this form of selection by evolving thicker genital-tract cuticle, thereby reducing the likelihood of damage and subsequent physiological immune activation.

15.4 Female genital-tract integrity and immunity

The insect's cuticle is a major barrier to pathogens (Neville, 1998; Siva-Jothy *et al.*, 2005), and once it is breached the insect's constitutive effector systems work very rapidly to clear the infection (see

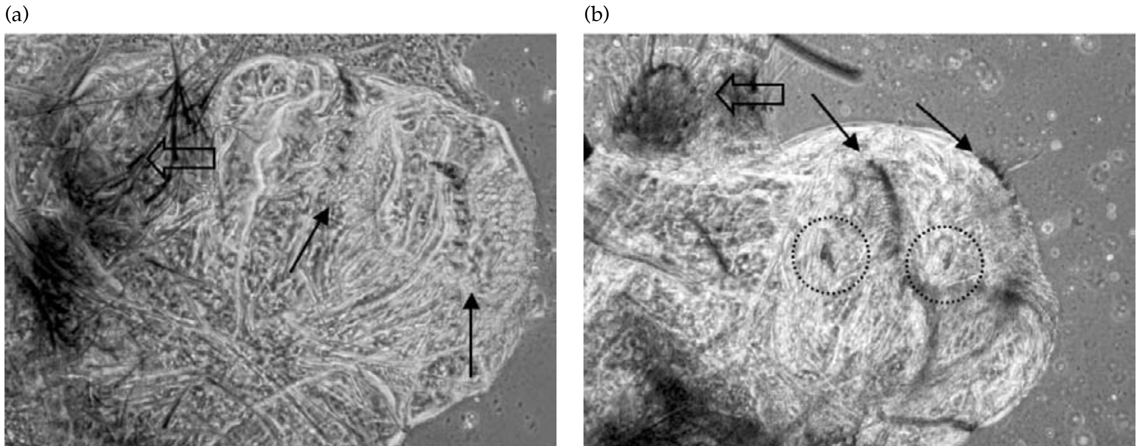

Figure 15.3 Phase-contrast micrographs of the genital damage caused during mating in *D. melanogaster* (see Kamimura, 2007). (a) Virgin female's genital tract. (b) Single-mated female's genital tract showing the melanized wounds (broken circles) caused by the male's intromittent organ. The solid black arrows indicate the oviscapt and the open black arrows indicate the analia. Figures reproduced by permission of Y. Kamimura.

Haine *et al.*, 2008). Although the insect cuticle is an unfavourable environment for microbes (Steinhaus, 1947; Brooks, 1963), it does not appear to be sterile (e.g. Rivault *et al.*, 1993; Sukontason *et al.*, 2000) and at least one study has shown that male genitalia harbour a range of microbes that are potential pathogens (Reinhardt *et al.*, 2005) (see Figure 15.1). Any breach in the female's genital tract therefore affords surface microbes rapid access to the female's haemocoel. Not surprisingly female insects have been shown to respond to potentially damaging male genital traits by evolving thicker cuticle (Rönn *et al.*, 2007) but it is likely that this response is constrained by the need to process and eject eggs: the genital-tract cuticle can be only so thick and tough. Females are therefore likely to have to rely on physiological defence and several studies have shown that female *Drosophila* express antimicrobial peptides in their genital-tract epithelium (Charlet *et al.*, 1996; Ferrandon *et al.*, 1998; Tzou *et al.*, 2000). Moreover, these genital-tract peptides are regulated by a different mechanism to systemic antimicrobial peptide expression, suggesting their production in the genital tract is linked to reproduction (Ryu *et al.*, 2004). It is possible that these antimicrobial peptides have functions other than/additional to immune defence of the genital tract; for example, they may function to protect the eggs after they have been laid (Marchini *et al.*, 1997). Studies of genital-tract wounding have also revealed efficient melanization responses in the damaged regions (Crudgington and Siva-Jothy, 2000; Kamimura, 2007; Reinhardt *et al.*, 2007), although these are likely to be a function of the haemolymph, rather than the genital-tract epithelium. The melanized wounds identified in these studies (see Figure 15.3) are small and localized, suggesting that the breach of the genital-tract cuticle may be sealed rapidly by haemolymph clotting mechanisms (see Theopold *et al.*, 2002; Haine *et al.*, 2006). It is interesting to note that in traumatically inseminating insects, where males always wound females during mating, the female has evolved an immune organ underlying the region where the male causes damage (Usinger, 1966; Reinhardt *et al.*, 2003). The localization of immune-effector systems in regions subject to predictable damage may therefore be a widespread, but overlooked, phenomenon.

In summary so far, males possess a suite of traits that can cause damage to females during mate encounter. The cost of this damage to females is cuticular wound repair as well as the potential associated immune costs in dealing with opportunistic

pathogens that gain entry to the haemocoel. I suggest that reproduction is a period in a female insect's life when immune insult is predictable in time (i.e. driven by temporal decisions by the female to mate) and in space (i.e. the consequence is localized wounding of the genital tract and other regions subjected to male gripping/restraint). Moreover, the fact that (a) reproductive events are usually under the temporal control of the female and (b) insects have revealed a sophisticated ability to anticipate immune insult suggests that female insects may be under selection to modulate their immunity to offset the costs of damage associated with mating. Such 'reproductive' immune anticipation remain to be demonstrated.

15.5 The bedbug and traumatic insemination

Male bedbugs breach the female's cuticle during copulation and inseminate directly into the female's haemolymph (Usinger, 1966), a mode of insemination that has been shown to be potentially very costly to females (Stutt and Siva-Jothy, 2001). Since the male inserts his aedeagus directly through the female's abdominal cuticle and inseminates into the haemocoel, rather than use the genital tract (Carayon, 1966), any surface microbes (Reinhardt *et al.*, 2005) will be introduced directly into the body cavity. Moreover, this will occur with the simultaneous introduction of a large number of sperm, another form of non-self as far as the female's immune system is concerned. Reinhardt *et al.* (2003) experimentally revealed that the septic consequences of traumatic insemination formed a large part of the cost base identified by Stutt and Siva-Jothy (2001). Interestingly, male bedbugs direct their mating efforts at females only if they have recently fed (Siva-Jothy, 2006), probably because females cannot prevent mating when engorged with blood (Reinhardt *et al.*, 2009). Nonetheless, mating is so frequent and tightly linked to feeding (Reinhardt and Siva-Jothy, 2007) that mating should be readily predictable when the female leaves her refugium to feed. As might be expected with such a potentially harmful male mating tactic the females have responded in a unique way. Female bedbugs have evolved a discrete immune organ that lies under the cuticle at the place where the male traumatically inseminates (Carayon, 1966). This organ is full of haemocytes (Klein and Kallenborn, 2000) and shows humoral immune activity (M.T. Siva-Jothy, personal observations) that combine to defend the female from the microbes that are introduced during traumatic insemination (Reinhardt *et al.*, 2003). Whereas the evolution of a specialized reproductive immune organ is likely to be restricted to this reproductively unique taxon, its presence and function testifies to the immunological adaptations that can arise as a result of males breaching the female's cuticle during prolonged periods of close contact in insects. Given this predictability, and the fact that mating results in the introduction of microbial pathogens, it is likely that females may benefit from anticipating mating by upregulating antimicrobial effector systems prior to feeding, and the inevitable mating-induced immune insult that follows. Preliminary results suggests such immune anticipation of mating occurs in *C. lectularius* (M.T. Siva-Jothy *et al.*, unpublished results). I predict that similar anticipatory upregulation of induced effector systems will occur in other insect taxa where mating is associated with immunological costs to the female.

15.6 Other issues

15.6.1 Sperm as non-self

Female vertebrates are faced with an immunological dilemma upon receiving their mate's gametes: sperm are non-self. Whereas this is also true for female insects, they (a) do not possess the sophisticated allograft-recognition mechanisms of vertebrates and (b) usually maintain sperm in a cuticle-lined structure(s). Consequently, there are unlikely to be any direct immunological consequences of having to deal with allogenic cells. However, cimicid females *are* faced with this situation since sperm are injected directly into the haemocoel and must swim through the haemolymph to reach the ovaries (Usinger, 1966). The females of *C. lectularius*, and other cimicids, are known to use their haemocytes to phagocytose sperm (Carayon, 1966), although it is far from clear whether this is an immunological response to the detection of 'non-self', the removal

of dead or dying cells from the haemocoel or, as suggested by Eberhard (1996), a mechanism to select the sperm of desirable males.

Almost no work has been done to examine the immune consequences of storing and maintaining non-self cells in insects (or, for that matter, in vertebrates) despite the fact that female insects often do this for prolonged periods. In most insects this is unlikely to be problematic since there is a cuticular haemocoel/gential-tract barrier and insects appear to have weakly developed allograft-recognition systems (Chapman, 1998). However, a recent study of vertebrate antimicrobial peptides revealed that these compounds can immobilize sperm in the female genital tract (Reddy *et al.*, 2004). I am not aware of any studies that examine the interplay between insect immune effector compounds and sperm function/survival in the female's genital tract, but suspect that the wealth of female genital-tract accessory glands in insects will reveal some interesting immunological phenomena in relation to the female's interaction with sperm.

15.6.2 Physiological consequences of mating on immunity

Recent studies have shown that mating induces downregulation of immune function in males and females (e.g. Siva-Jothy *et al.*, 1998; McKeen and Nunney, 2001), which does not appear to be caused by energetic demands. Whereas it is possible that the effects in McKeen and Nunney's (2001) study were linked to the copulatory wounding processes identified by Kamimura (2007), that seems unlikely in Siva-Jothy *et al.*'s (1998) study (however, the aedeagi of odonates are known to bear recurved spines that function in sperm competition (Corbet, 1999) and might therefore damage the female's genital tract). A physiological candidate for generating/mediating the observed reduction in immune function is juvenile hormone (e.g. Zera and Harshman, 2001) and Rolff and Siva-Jothy (2002) revealed that the endogenous release of this hormone stimulated by mating was responsible for reducing immune function after mating. Whereas immune and juvenile hormone function are conserved across insects, it is likely that in species where post-mating immune insult has serious costs, mechanisms will exist to minimize the effects of mating-induced, juvenile hormone-mediated, immune suppression; for example, Shoemaker *et al.* (2006) showed that cricket immune function was elevated by mating.

15.6.3 Male compounds that may affect female immunity

Male insects are known to transfer compounds in their seminal fluids that affect female receptivity (e.g. Thornhill and Alcock, 1983), oviposition rates, and longevity (e.g. Chapman *et al.*, 1995). Given that these physiologically active compounds affect a complex range of female traits it is likely that they may coincidently, or deliberately, enhance/reduce female immune function. For example, it has been established that male *Drosophila* transfer three different antimicrobial peptides in their seminal fluid (Lung *et al.*, 2001) as well as transfer compounds that activate phenoloxidase in the female's genital tract (Asada and Kitagawa, 1988). These male-transferred compounds may function primarily to protect and/or enhance the competitiveness of the male's ejaculate, but may have the correlated effect of protecting the female and/or reducing her immune costs as well. We know almost nothing about the immunological function of insect seminal fluid and/or why males transfer these compounds.

15.7 Conclusion

In this short survey of the relationship between reproduction and immunity in female insects I have made a case that this is a potentially rich area for uncovering novel immunological phenomena. This is because immune insult is relatively predictable, and to some extent controlled by females. Because of this predictability, and the frequency of mating-associated immune phenomena, examination of this aspect of immunology provides a tractable opportunity to study immunity in field systems and thereby better understand the ecological context of immune function.

Male genitalia evolve rapidly and divergently (see Eberhard, 1985) and despite a broad understanding of the selection pressures that might drive this evolution (e.g. Parker, 1970; Eberhard,

1985; Reinhardt, 2009) the functional interactions between male and female genitalia during mating remains relatively obscure. Sclerotized spines and bristles on the male's aedeagus have the potential to damage the female's genital tract and, despite the increasing number of examples of this phenomenon, we still know almost nothing about the immunological consequences of mating-induced damage. Given that females have responded to damaging male genital traits by evolving a novel immune organ in the extreme example of the bedbug (Morrow and Arnqvist, 2003; Reinhardt *et al.*, 2003), it is likely that more subtle immunological responses are waiting to be discovered in taxa where males do less obvious harm to their mates.

Although this chapter has concentrated on the immunological consequences of damage imposed on females by males, it is likely that in species where males damage each other in intrasexual fights males may also display specific immunological adaptations to cope. Despite a large literature that (a) documents fighting and (b) examines the underlying sexual selection in insect taxa (e.g. Thornhill and Alcock, 1983) almost no studies have examined how males respond to wounding before, during, or after fighting.

In conclusion, I propose that wounding associated with mating and mate encounter is predictable in space and time, especially in females. The resulting immunological phenomena are likely to be be linked to patterns in the insect's behaviour, reproductive anatomy, cuticular microbial flora, and life-history investment. The study of these phenomena therefore offers the opportunity to disentangle the important relationship(s) between ecology and immunity.

15.8 Acknowledgements

Many thanks to K. Reinhardt, J. Rolff, and S. Reynolds for their patience and constructive feedback on the manuscript and to Y. Kamimura for granting permission to use his unpublished micrographs.

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