Insect Immunity: An Evolutionary Ecology Perspective

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Abstract

We review recent advances in our understanding of the mechanisms of insect immune defence, but do so in a framework defined by the ecological and

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evolutionary forces that shape insect immune defence. Recent advances in genetics and molecular biology have greatly expanded our understanding of the details of the immune mechanisms that enable insects to defend themselves against parasites and pathogens. However, these studies are primarily concerned with discovering and describing how resistance mechanisms work. They rarely address the question of why they are shaped the way they are. Partly because we know so much about the mechanisms that it is now becoming possible to ask such ultimate questions about insect immunity, and they are currently emerging from the developing field of 'ecological immunology'. In this review we first present an overview of insect immune mechanisms and their coordination before examining the key ecological/evolutionary issues associated with ecological immunity. Finally, we identify important areas for future study in insect immunity that we feel can now be approached because of the insight provided by combining mechanistic and ecological approaches.

1 Introduction

Insects are coelomate metazoans that have a dominant, open haemocoelic cavity in which the organs and tissue systems are suspended. This single pervasive and continuous body space is home to the last line of defence against pathogens and parasites: the insect's immune system. A single, relatively large, fluid-filled body cavity has several advantages (see Willmer, 1990 for a comprehensive analysis). It is relatively efficient at distributing nutrients from the gut and collecting waste products; it provides a discrete environment for the evolution of large, complex tissues and organs and consequently allows independent growth of the gonads and provides a hydrostatic skeleton. These, and other, features have profound influences on an organism's size, locomotion, life history and consequently ecology and evolution. Clearly, there are many aspects of insect biology that will influence and constrain the evolutionary paths that are available to this immensely successful taxon (see McGavin, 2001), but the consequences of, and constraints imposed by, an open haemocoel are core to understanding many aspects of the organisation and control of the insect immune system.

Convention dissects the insect immune system into cellular and humoral components, a division which probably reflects the historical unfolding of our understanding of the vertebrate system as well as the practically constrained approach to studying insect immunity. Most studies of insect immunity using this conceptual dichotomy acknowledge (usually implicitly) that the approach is convenient, rather than biologically meaningful. With the advent of, and advances in, genomics our understanding of the mechanistic basis of insect immunity has changed dramatically in the last few years. Coupled with these insights of the immune machinery of *Drosophila* and *Aedes* is the development

of an area of evolutionary biology that seeks to understand the basis for additive genetic variation in immune function. This microevolutionary perspective was initially focussed on sexual selection in vertebrates (Hamilton and Zuk, 1982; Folstad and Karter, 1992; Sheldon and Verhulst, 1996), but has more recently shifted its view to an ecological one that uses invertebrates as models (e.g. Schmid-Hempel, 2003; Schmid-Hempel and Ebert, 2003; Rolff and Siva-Jothy, 2003). The 'mechanistic' and 'evolutionary' approaches differ in several respects, but most importantly in how they deal with individual variation in immune traits and the kinds of pathogens they expose their model hosts to (see Hultmark, 2003). The mechanists necessarily remove individual variation from their systems because it is hard enough to isolate and identify mechanisms when the individuals are genetically similar. Consequently, what we know of immune mechanisms tends to come from genetically constrained models reared under ideal nutritional conditions in a relatively aseptic laboratory environment. This approach was, and is, a design necessity in addressing mechanistic questions. In contrast, evolutionary and ecological studies tend to use generalised assays of immune-function (for critiques see Siva-Jothy, 1995; Owens and Wilson, 1999; Ryder, 2003; Adamo, 2004) to address questions about the evolutionary maintenance of variation in immune systems, an approach that at best oversimplifies, and at worst ignores, the constraints imposed by and the meaning of quantitative measures of the underlying mechanisms. Clearly, the mechanistic and evolutionary/ecological approaches would, and have (see Kurtz *et al.*, 2002a), benefited from a formal synergism.

One core aim of this review is to examine insect immunity from a perspective that is integrated with ecology and evolution in the belief that synergism will offer insight. In reviewing the mechanistic components of the insect immune system, we have moved away from the humoral/cellular dichotomy and instead organised defence mechanisms from the viewpoint of how individuals are organised (by selection) to defend themselves: We structure this review by examining how individuals avoid the negative effects of pathogens and parasites. The first line of defence is behavioural avoidance, the second is boundary defence. Immunity is the last line of defence, and represents a collective 'emergency service' that the organism calls on when the standing precautions and defences fail. This scheme is developed from Schmid-Hempel and Ebert's (2003) 'defence components model' in which they seek to (and succeed in) reconciling two disparate evolutionary approaches to understanding how hosts and parasites coevolve (Fig. 1).

Such a structure is biologically relevant because clearly individual organisms are the units of selection on which pathogens and parasites act: change in immune genetics is only one response to that complex selection pressure. It is important to bear in mind that insect immune systems are also under selection from sources other than pathogens and parasites since certain components and systems have additional functions (e.g. phenoloxidase (PO)). We do not

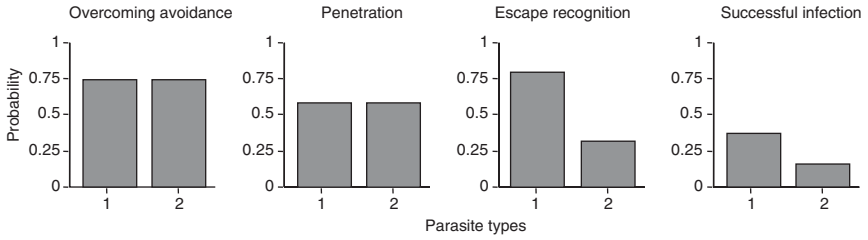

FIG. 1 The defence component model as proposed by Schmid-Hempel and Ebert (2003). It shows a hypothetical host–parasite/pathogen interaction with three consecutive steps from encounter to successful infection. The probabilities on the *y*-axis are the probabilities of the parasite overcoming host defence at each step. For the sake of simplicity, a multiplicative model is assumed, whereby the probabilities of all three steps are multiplied to estimate the probability of successful infection. The most specific components of the system determine the overall outcome of the infection, and hence the specificity to the parasite (geno-) types.

deal with the biochemical, genetic or structural details of immune system components in this review since these topics are covered by a host of recent reviews, which are cited in the relevant sections.

2 Defence via behaviour

Behavioural mechanisms directed against the parasites in an organism's environment is necessarily the first line of defence (e.g. Hart, 1997). Behavioural mechanisms for removing parasites are well known and range from simple, individual-based behaviours like dust-bathing in birds, through more complex interactions that often serve important social functions, such as grooming in primate groups. Such behaviours have even evolved into the complex interspecific interactions that exist on tropical reef cleaning stations where one species makes a living by removing ectoparasites from other species (e.g. Cote and Molloy, 2003).

In insects defence behaviours directed at pathogens and parasites tend to be less well studied, but there are several good examples of how ecology and behaviour are used to reduce the risk, and effects, of parasitism. Several insects use acoustic signals to attract mates and certain sarcophagid and tachinid fly parasitoids use these cues to locate their singing cricket hosts (see Cade and Wyatt, 1984; Cade, 1991). A male cricket has a range of singing options with two extremes: sing loudly and attract females and parasitoids rapidly, or do not sing and do not reproduce but avoid parasitoids. In nature, male crickets utilise one of the two tactics. The high-risk, high-gain tactic where they sing and mate with as many attracted females as possible before the parasitoid strikes, or the alternative, low-risk, low-gain tactic where they remain silent and loiter near a singing male. This tactic has a much reduced risk of parasitism, but provides an

opportunity to sequester females that are attracted to the singing male. A simple, and probably common, behavioural mechanism for avoiding parasites is to disperse away from aggregations or populations that are likely to have a prevalence of parasites (e.g. Bischoff, 2003; and see Kurtz *et al.*, 2002b for the way immune systems respond to such behaviour). Another simple, but very effective, behavioural weapon in a host's battle with parasites is thermoregulatory behaviour. The thermal optimum of an insect host will often not be the same as the thermal optimum of a parasite and selection has favoured behavioural thermoregulation that elevates the host's core temperature to disadvantage the parasite (see Thomas and Blandford, 2003). This 'behavioural fever' has been shown to be quite subtle, often being directed at specific pathogens (e.g. Adamo, 1998).

Behavioural avoidance of parasites and pathogens is an understudied area but there is good reason to suppose it is an effective response against selection from predictable, relatively high-cost parasites or pathogen insults. Since many insect parasites enter with the host's meal, it is likely that foraging behaviour will be under selection to reduce parasite exposure (while selection on parasites probably favours entry with food since resource acquisition by hosts cannot be compromised). Theory suggests that selection for parasite avoidance may even promote the evolution of eusocial behaviour (O'Donnell, 1997).

It is beyond the scope of this review to address behavioural avoidance in detail, but we will reconsider this aspect in Section 7 when we reexamine the defence components model of Schmid-Hempel and Ebert (2003) (see Fig. 1) in light of this review.

3 The insect immune system – boundary defence

The second line in an insect host's defence against pathogens and parasites is the outer body covering. This consists predominantly of a toughened cuticle forming a protective integument over the insect's external surface. Even in the midgut, the one place where the insect's external surface is formed by a relatively delicate epithelium, there is still a protective cuticular membrane (the peritrophic membrane) forming a static defence against the outside world (Peters, 1992). Although the integument forms a formidable barrier to the outside world, there are potential weak points in the intact external surface that parasites and pathogens might be expected to target (see Fig. 2). Moreover, once the largely physical barrier of the cuticle is breached, the epidermis is the next line of defence. The epithelium is likely to be a rather ineffective physical barrier, and appears to be the site of expression of a number of key immune effector systems. It is an oversimplification to regard the interface between an individual insect and the outside world as an inert barrier; in reality, 'boundary defence' is a combination of inert physical barriers that have a limited immune capacity.

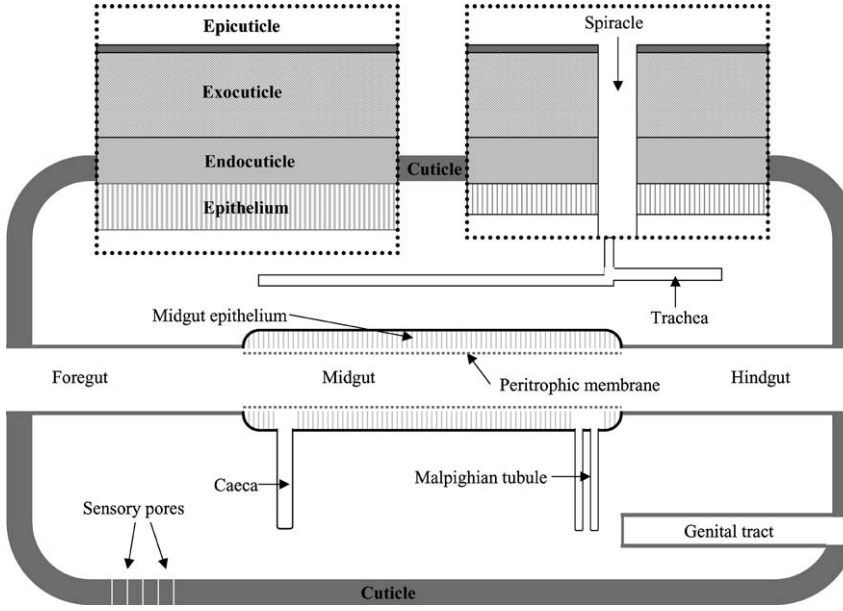

FIG. 2 A schematic figure of the insect's barrier defences, showing the regions where invasion is likely. The dotted boxes reveal detail within the integument.

3.1 THE CUTICLE

It is an axiom in entomology that insect cuticle is the key to understanding the phenomenal success of this taxon. The cuticle is a tough, flexible and waterproof barrier against the outside world and is formed by the basal epidermal cells (Neville, 1975). The outer surface of insect cuticle probably harbours a diverse and abundant microbial community (see Brey *et al.*, 1986) even in the most aseptic habitats. However, given the propensity of many insects to live in microbial-rich environments, it seems likely that opportunistic infections will accompany each integumental breach. Very little data currently exist to indicate the frequency of such wounding events in natural populations: an irony given that the launch pad for host–pathogen studies, and insect immunity in particular, was Pasteur's seminal study (see Brey and Hultmark, 1997) showing that frequent cuticular wounding in *Bombyx mori* was responsible for the transmission of the silkworm plague.

The main features of cuticle that make it a good barrier are its thickness and its strength. The latter is achieved by cross-linking proteins in the exocuticle via melanisation and sclerotisation (Neville, 1975), processes that share their core enzymes with the immune system (see Section 4.4.1).

The first part of the cuticle that a potential pathogen/parasite encounters is the outer, complex, but usually very thin, epicuticle. This layer is unlikely to

provide physical protection (with the possible exception of scale insects (Homoptera: Coccoidea) where it often consists of thick, complex stacks of wax) but may harbour some, as yet unquantified, antimicrobial activity.

The procuticle (combined exocuticle and endocuticle) forms the next barrier, mainly because of its thickness and architecture. The only entomopathogens, which invade directly through the exoskeleton are fungi (Charnley and St. Leger, 1991) and studies of their invasion mechanics suggest several aspects of cuticular architecture are important in resistance. Perhaps the best studied fungus in this respect is *Metarhizium anisopliae*, which invades by combining physical and enzymatic processes. Hajek and St. Leger (1994) suggest, and David (1967) supports, the notion that resistance to fungal entomopathogens resides mainly in cuticular thickness, the degree of cuticular cross-linking within the cuticular laminae (i.e. cuticular strength) and the degree of sclerotisation in the cuticle. Moreover, pore-canals (narrow trans-cuticular ducts that transport material to the epicuticular surface (Neville, 1975)) may also afford a path-of-low-resistance for the diffusion of enzymes released by the invading fungi (Zacharuk, 1970).

As well as its obvious physical characteristics cuticle also provides an active biochemical barrier. Brey *et al.* (1993) showed that bacterial challenge to abraded cuticle resulted in antimicrobial activity in the vicinity of the abrasion. PO activity (an immune effector system responsible for producing melanin, see Section 4.4.1) has also been detected in insect cuticle (Ashida and Brey, 1995), although, whether it is there in a structural context, or to afford defence is unclear. Regardless of why it is in the cuticle, the activity of this enzyme is directed towards pathogens in the cuticle: fungal germ tubes are melanised as they pass through procuticle before they entered the haemocoel (Golkar *et al.*, 1993).

Schal *et al.* (1998) have shown that there is an active association between the haemolymph and the cuticle: compounds in the haemolymph, probably residing in oenocytes, are readily transported to the outer epicuticular surface. Although these compounds were not immunologically active, it seems likely that the existence of such transport mechanisms means immunologically active compounds could also be easily transported to the surface of the cuticle (although no evidence exists for such a phenomenon at the time of writing).

The remarkable physical properties of cuticle combined with its ability to respond biochemically to pathogens means that it is an extremely effective barrier to infection, preventing or slowing down pathogen invasion (St. Leger, 1991).

The one external surface of the insect which has no immediate cuticular covering is the midgut. Here, the insect must balance the need to absorb nutrients across an extended surface in an efficient manner with the need to defend itself from non-self (with which the midgut is replete). Here, again insects rely on cuticle. As food passes into the midgut it is sleeved with a chitinous, porous 'peritrophic' membrane, which is permeable to nutrients and

enzymes, but affords the delicate midgut epithelium some physical protection from damage as well as protection from invaders (Richards and Richards, 1977; Peters, 1992).

3.2 THE INTEGUMENTAL EPITHELIUM

Once the cuticular barrier is breached, the pathogen either encounters the underlying cuticular epithelium or the haemolymph. The cuticular epithelium associated with the external integument appears to become immunologically active mainly upon wounding (Brey *et al.*, 1993; Meister *et al.*, 1994).

Recent studies of gene expression in epithelia associated with the respiratory, digestive and reproductive systems reveal much of it to be immunologically active (see Tzou *et al.*, 2000). Whether this indicates constitutive immune function in these tissues, or is indicative of persistent challenge by opportunistic pathogens via these physically relatively weak lines of defence is unclear. What is clear is that the epithelium underlying the insect's external surface is capable of immunological activity. Because of its spatial situation it seems likely that this tissue boundary between the physical defences and the haemolymph not only plays a part in barrier defence, but is also likely to release 'early-warning' signals that recruit and activate haemolymph-based effector systems.

3.3 SENSILLA

Insect contact chemosensilla have an opening in the cuticle at their tip that provides access to the sensillum lumen, wherein lie the sensory dendrites, while olfactory sensilla are covered with abundant small pores. These openings are typically 0.2 μm in largest diameter (Chapman, 1998) and so represent potential entry points for small microorganisms. However, little evidence exists to indicate that active chemical-mediated immunity occurs at these sites. Semi-porous barriers are, however, placed in these openings. Viscous fluids and fibrillar cuticular plugs (Shields, 1994) erected across these access points presumably exclude microorganisms, while allowing the passage of the important biochemicals. The internal lumen of chemosensilla is in turn isolated from the haemocoel by a barrier of specialised epidermal cells (Chapman, 1998), providing a further impediment to invasion.

3.4 THE DIGESTIVE SYSTEM

The digestive system probably offers most invasion opportunities for pathogens since it is the least well-defended region physically, and is constantly exposed to non-self (i.e. food). The need to digest food (often with the aid of symbiotic gut microbes that need a favourable environment) and efficiently absorb nutrients (achieved across the cuticle-free midgut) results in the least

well-defended region of the insect's body in terms of barriers. The foregut and hindgut are physically protected to some extent, being lined with a relatively thin layer of cuticle (Chapman, 1998). The midgut, and its associated structures, present an unprotected epithelial surface to the outside world, often providing parasites with specific points of entry into the host (e.g. Han *et al.*, 2000). Physical protection in this vulnerable region is afforded by the delicate cuticular peritrophic membrane. The cuticle lining in the foregut provides more than just a passive inert barrier; however, it is sloughed off when pathogenic bacteria attached to it (Binnington, 1993), a reaction that presumably denies the pathogens a foothold and results in passage of the sloughed off material into the chemically hostile midgut.

The midgut epithelium is an immunologically active tissue that produces a host of defence peptides, including defensins, Gram-negative binding protein, chitinase-like protein, serine proteases and lectin-like protein (Lehane *et al.*, 1997; Barillas-Mury *et al.*, 2000; Tzou *et al.*, 2000) as well as NO (e.g. Hao *et al.*, 2003) and PO (Wilson *et al.*, 2001). There is also strong evidence that lysozyme-like activity occurs through the midgut and the caecae (Daffre *et al.*, 1994) but it is unclear whether this activity functions in digestion, or provides protection against bacteria. We know that the caecae are immunologically active in *Drosophila*, where they produce diptericin (Tzou *et al.*, 2000).

Another tissue that is unprotected by cuticle and is specifically targeted by parasites (e.g. Fries *et al.*, 2001; Weiser and Zizka, 2004) as well as opportunistic infections (e.g. Franco *et al.*, 1997) are the malpighian tubules. These structures are immunologically active (Sagisaka *et al.*, 2001; Bao *et al.*, 2003) and produce a range of antimicrobial peptides in *Drosophila* (Tzou *et al.*, 2000).

3.5 THE SPIRACLES AND RESPIRATORY SYSTEM

An important potential site of entry into an insect host is the spiracles and tracheal system. As well as entomopathic nematodes that invade through the spiracles (e.g. de Doucet *et al.*, 1998) and/or live in the tracheal system (e.g. Aikawa and Togashi, 2000), this route is likely to be used by opportunistic bacteria and fungal spores. Despite the potential ease of entry via this route there is very little information about how insects avoid infection through it apart from studies showing that the epithelium associated with the trachea is immunologically active (Tzou *et al.*, 2000), and that there are intimate spatial relationships between haemocytes and trachea (Wigglesworth, 1965) suggestive of a defensive role.

3.6 REPRODUCTIVE TRACT

Much of the reproductive tract that comes into frequent contact with the outside world has a cuticular lining: the female's genital tract and sperm storage organs are lined with cuticle, which stops at the junction with the oviducts

(Chapman, 1998). Because copulation and insemination provide pathogens with an opportunity for horizontal transmission one would expect the reproductive tract to be immunologically active. Studies of *Drosophila* have shown gender-specific expression of antimicrobial peptides in the reproductive tract epithelium (Tzou *et al.*, 2000) and it appears that males also incorporate antibacterial peptides in their seminal fluid (Lung *et al.*, 2001), presumably to afford their genetic investment some protection while it is in storage in the female's spermatheca. Recent observations of the interactions between males and females during mating suggest that males may deliberately wound female genitalia in order to delay female remating, and thereby enhance the wounding male's fertilisation success (see Crudgington and Siva-Jothy, 2000). The damage caused to the cuticular lining of the female's genital tract is relatively extensive and stimulates a wound-healing response culminating in the production of melanic plugs (see Crudgington and Siva-Jothy, 2000). Of interest in this respect is the hemipteran family, the Cimicidae, or bed bugs. Males of this taxon utilise traumatic insemination and introduce their intromittent organ through the female's abdomen wall and inseminate into her haemolymph (Carayon, 1966). These insects live in unsanitary conditions and recent studies have shown that females pay a large fitness cost associated with the introduction of bacteria during traumatic insemination (Morrow and Arnqvist, 2003; Reinhardt *et al.*, 2003). This selection pressure appears to be so strong that female cimicids have evolved a unique immune-organ (the mesospermalege) in the region where males pierce and inseminate them (see Reinhardt *et al.*, 2003).

4 The insect immune system – haemocoelic defence

Once an invader has breached the barrier defences, the insect has to produce a rapid and effective response that localises and neutralises the reproductive capacity of the pathogen or the growth potential of the parasite. This is best achieved by killing the invader (but see Sasaki and Godfray (1999) and Boots and Bowers (2004) for models that predict that hosts should produce no immune response to pathogens under certain, ecologically realistic, conditions). Insects rely solely on innate immune effector systems. Boman (1998) described these processes as 'insect germ-line encoded anti-infection responses.' This distinguishes these innate responses from the sophisticated immunity of vertebrates afforded them by the immunoglobulin gene superfamily. Insect immune systems have no specific immunoglobulin-based memory and are traditionally viewed as being relatively indiscriminatory when confronted with subtly different types of non-self. However, recent work on Crustacea suggests that invertebrate innate systems are capable of some remarkably specific immunological phenomena (Kurtz and Franz, 2003; Little *et al.*, 2003) (see Sections 6.1 and 6.2). 'Simple' is often misinterpreted as evolutionarily

inferior to 'complex,' a viewpoint that falsely equates phylogenetic basality with functional inferiority. Insects are the most successful class of organism on the planet (see, for example, Gullan and Cranston, 2000) and their 'simple' immune system plays an important role in that success.

The open haemocoel (cf. the closed circulatory networks of higher vertebrates) provides some advantages in terms of the function of the immune system. For example, mediators, effector systems and haemocytes can be more rapidly disseminated and organised. However, the open architecture also presents a problem when the insect is faced with systemic immune insult. An open body cavity facilitates rapid movement of infective agents through the host. Consequently, selection should favour the evolution of effector systems that rapidly and efficiently localise and neutralise invaders. One could argue that these needs render any acquired, or acquired-like, immune response pointless, since such responses are also characterised by their relatively slow response time.

The following section is organised, where possible, according to the chain of temporal and organisational events that follow a systemic immune insult (i.e. a breach in the barrier defences).

4.1 CLOTTING AND WOUND CLOSURE

A septic wound presents a series of major physiological problems that must be dealt with rapidly. The major priority, particularly in holometabolous larvae where the haemolymph is under pressure, will be to plug the wound. This will prevent excessive blood loss and close the invasion route behind the outflowing haemolymph (which will, to some extent, flush out invaders in a hostile medium). The main reaction to blood loss is clotting, which also functions to immobilise, localise and begin neutralising pathogens that have entered via the wound. Clotting has been extensively studied in Crustacea (see Theopold *et al.*, 2004 for review) where the reaction is triggered by pathogen-associated motifs (the so-called PAMPs – pathogen-associated molecular patterns) like lipopolysaccharide, peptidoglycans and β-1,3 glucan. Non-biotic stimuli for clotting probably also exist since clotting has non-defence roles as well (i.e. wound closure).

The first physical changes that occur during clotting are an increased viscosity of the haemolymph and the inclusion of insoluble, glycosylated 'sticky' fibres which, in *Drosophila*, contain several clotting proteins including hemolectin and tiggrin (Scherfer *et al.*, 2004). The production of these fibres, which adhere to each other and form a sticky net, begins to seal the wound, trap microbes and trap haemocytes (Gregoire, 1974), some of which are responsible for secreting the material that forms the fibres (e.g. Goto *et al.*, 2003). Haemocytes are also attracted to/remain in the vicinity of the wound because the damaged epithelial cells near the wound release hemokinin (Cherbas, 1973), a compound that aids cell aggregation. PO usually becomes activated during

wound closure, particularly in the later stages once the 'soft clot' is established. Although this enzyme cascade is probably not involved directly in coagulation (Scherfer *et al.*, 2004 but see also Li *et al.*, 2002 and Cerenius and Söderhäll, 2004 for an alternative perspective), it will kill invaders as well as melanise the material that constitutes the clot (Rämet *et al.*, 2002a) thereby reestablishing an impermeable physical barrier.

4.2 SELF/NON-SELF RECOGNITION

The insect immune system recognises a range of non-self motifs, from well-characterised pathogen cell surface molecules including peptidoglycans, β-1,3 glucans, lipopolysaccharides and other sugar moieties (Theopold *et al.*, 1999), collectively referred to as PAMPs.

Insect hosts need to avoid reacting to self in the absence of immune challenge but, upon septic insult, must target non-self, and sometimes specific components of self, in order to neutralise the insult. For example, the haemocytes that encapsulate and isolate larger immune insults die (apoptose) and are melanised by PO, probably in the same way haemocytes in the vicinity of cuticular wounds die and are melanised during wound repair. Such reactions are probably mediated by signals of 'altered self' (e.g. Franc *et al.*, 1999). The insect system's ability to separate different types of non-self from each other will be relatively restricted (cf. vertebrates) because they lack immune-functional immunoglobulin superfamily proteins. Despite this, however, recent studies of invertebrate immunity in an ecological context show that invertebrate innate systems are capable of some remarkable feats of recognition (Kurtz and Franz, 2003; Little *et al.*, 2003) (see Section 6.1). Exactly how this discriminatory capacity arises is currently far from clear.

The organs and tissues in the haemocoel (with the exception of haemocytes) are covered by the basal lamina, or basement membrane. It is believed that the basal lamina is produced by haemocytes (Wigglesworth, 1956, Ball *et al.*, 1987) because (a) there is an intimate association between haemocytes and the basal lamina during morphogenesis and rebuilding (Wigglesworth, 1973; Nardi and Miklasz, 1989; Nardi *et al.*, 2001, 2003), (b) haemocytes are recruited to areas of basal lamina disruption during wounding (e.g. Lackie, 1988) and (c) haemocytes and basal lamina share immunogenic epitopes (Chain *et al.*, 1992). One important function of the basal lamina in the context of self/non-self recognition may be to provide a uniform background signal of 'self' within the haemocoel, against which any non-self signal becomes more conspicuous. This notion is supported by the observation that termination of the encapsulation response (i.e. a coordinated haemocytic response to large non-self (see Section 4.6)) occurs when a basement membrane-like layer appears on the outside of the encapsulating, dead and melanised haemocytes (e.g. Liu *et al.*, 1998; Pech and Strand, 2000).

Against this background of self, insects appear to distinguish non-self by relying largely on a host of pattern recognition peptides (see Janeway, 1989) that usually identify PAMPs. We concentrate our overview on two relatively well-studied groups of these pattern recognition peptides: the peptidoglycan-recognition proteins and the Gram-negative binding peptides.

Peptidoglycan-recognition proteins are relatively conserved molecules that bind to peptidoglycans (a conserved, essential and unique component of the microbial surface) and thereby sense an infection (Dziarski, 2004). *Drosophila* has 12 peptidoglycan-recognition protein genes (Werner *et al.*, 2003), not all the products of which function in alerting the immune system to the presence of invaders (see Mellroth *et al.*, 2003). Insect peptidoglycan-recognition proteins have four main identified functions in terms of the immune effector systems they activate when bound to bacteria. (1) They activate the prophenoloxidase cascade (Yoshida *et al.*, 1996; Kang *et al.*, 1998; Takehana *et al.*, 2002) by activating serine proteases. (2) They stimulate antimicrobial peptide production via the *Toll* and *Imd* pathways (see Gottar *et al.*, 2002; Royet, 2004). (3) They appear to activate phagocytosis in some haemocytes (Rämet *et al.*, 2002b), and (4) some peptidoglycan-recognition proteins seem to function to remove, or 'clean up', excess peptidoglycans in the haemocoel (Mellroth *et al.*, 2003). Some peptidoglycan-recognition proteins are transmembrane proteins, the best studied of which is PGRP–LC. Mutants of PGRP–LC fail to respond to G^-, but not G^+ bacteria (Choe *et al.*, 2002; Rämet *et al.*, 2002b). These phenomena are curious for two reasons. First, the peptidoglycans in G^- bacteria are concealed beneath the outer cell wall (e.g. Doyle and Dziarski, 2001). Second, G^- bacteria have a lipopolysaccharide-rich outer coating (G^+ bacteria have no lipopolysaccharide). Since lipopolysaccharide is highly immunogenic, it is counter-intuitive that part of the recognition system that distinguishes G^- from G^+ bacteria operates by detecting the concealed PAMP (see Leulier *et al.*, 2003). The response, in *Drosophila*, of detecting G^- bacteria with PGRP–LC is the production and secretion of the potent antibacterial peptide diptericin, a member of the gloverin family of antimicrobial peptides (Bulet *et al.*, 1999). Another well-characterised peptidoglycan-recognition protein is PGRP–SA, a soluble protein that has a high affinity for G^+ bacteria in *Drosophila*. PGRP–SA mutants are unable to secrete drosomycin, a potent antifungal peptide, and do not respond to G^+ infections, although they can clear fungal and G^- infections easily (Michel *et al.*, 2001).

The second important class of molecules that detect non-self are the Gram-negative binding peptides. As their name suggests, Gram-negative binding peptides detect and bind to G^- bacteria, principally targeting the lipopolysaccharide-rich and β-1,3 glucans component of the cell wall, resulting in the production of the potent antimicrobial peptides drosomycin, attacin and cecropin in *Drosophila* (Kim *et al.*, 2000).

Other potential pattern recognition peptides are some thioester-containing peptides (TEPs) (which bear similarities to the vertebrate complement component C3) and hemolin (an immunoglobulin superfamily protein, Lanz Mendoza and Faye, 1999). An insect thioester-containing peptide (probably secreted in the fat-body, Lagueux *et al.*, 2000) has been shown to act as an opsonin, promoting phagocytosis of G^- bacteria (Levashina *et al.*, 2001) and suggesting it may have pattern recognition abilities. Studies of *Manduca sexta* hemolin have shown it to be an immune surveillance protein (Kanost *et al.*, 2004) expressed in the gut of diapausing moths. It presumably affords them immune protection during this vulnerable life history stage (Lee *et al.*, 2002). Hemolin also plays vital roles in development (e.g. Bettencourt *et al.*, 2000, 2002) where precise mechanisms coordinating cell–cell recognition and interaction are as important as they are in immunity. This observation emphasises the important point that immune effector systems can be influenced by selection on other functions because of the tendency of these effector systems to be multifunctional.

4.3 SIGNAL TRANSDUCTION

Once non-self has been identified and signalled by conformational change in the detection molecules, the signal needs to be translated into an appropriate biological action (transduction).

Soluble, humoral-based, signal transducers are responsible, among other things, for triggering the fast-acting 'constitutive' immune responses, the most important of which is prophenoloxidase (see Gorman and Paskewitz, 2001). The best understood of the humoral transducers are the serine proteases, a group of enzymes that mediate a range of physiological functions (Rawlings and Barrett, 1994). Immunologically functional serine protease proenzymes are activated by conformational changes in pattern recognition molecules (see above): the active serine protease then cleaves proenzymes in other controlling cascades (by targeting peptide bonds with a catalytic serine-containing domain). However, serine proteases (and other transducers such as 'Persephone', Ligoxygakis *et al.*, 2002a,b) also activate the cell-based signal transduction pathways (see below) in response to microbial infection (see Hultmark, 2003) and so act as intermediaries for the activation of the slower responding 'inducible' defences as well.

At the core of the insect immune response (Hultmark, 2003) are two cell-based signal transduction pathways referred to by the name of the transmembrane proteins that mediate them: Toll and Imd (Fig. 3). The biochemical details of these pathways have recently been reviewed (see Hultmark, 2003; Hoffmann, 2003): we will summarise the generic aspects of these pathways.

Toll's only known ligand is the protein Spätzle but, because null *Spätzle* mutants are less impaired at responding to microbial insult than are *Toll* mutants (Lemaître *et al.*, 1996), there are likely to be other Toll ligands. Cleavage

of Spätzle by serine proteases (which were activated by certain G^+ bacteria and/or fungi) in the haemolymph activates the Toll pathway (see Gobert *et al.*, 2003; Weber *et al.*, 2003 for details), resulting in the synthesis and secretion of the potent antifungal peptide drosomycin (Lemaître *et al.*, 1997) and the activation of haemocytes (Qiu *et al.*, 1998).

The Imd pathway is activated by G^- bacteria and/or fungi and is probably the principle regulator of inducible antimicrobial peptides directed at G^- bacteria and fungi (see Hultmark, 2003). Stimulation of the Imd pathway in *Drosophila* results in the synthesis and secretion of drosomycin, cecropin and diptericin. Stimulation of Imd also switches on the downstream JNK pathway (Sluss *et al.*, 1996), a mitogen-activated protein kinase that forms the 'front-end' of vertebrate immune signalling pathways. In *Drosphila* this JNK activation results in the expression of cytoskeletal genes (Boutros *et al.*, 2002) that are probably involved in wound healing (Rämet *et al.*, 2002a).

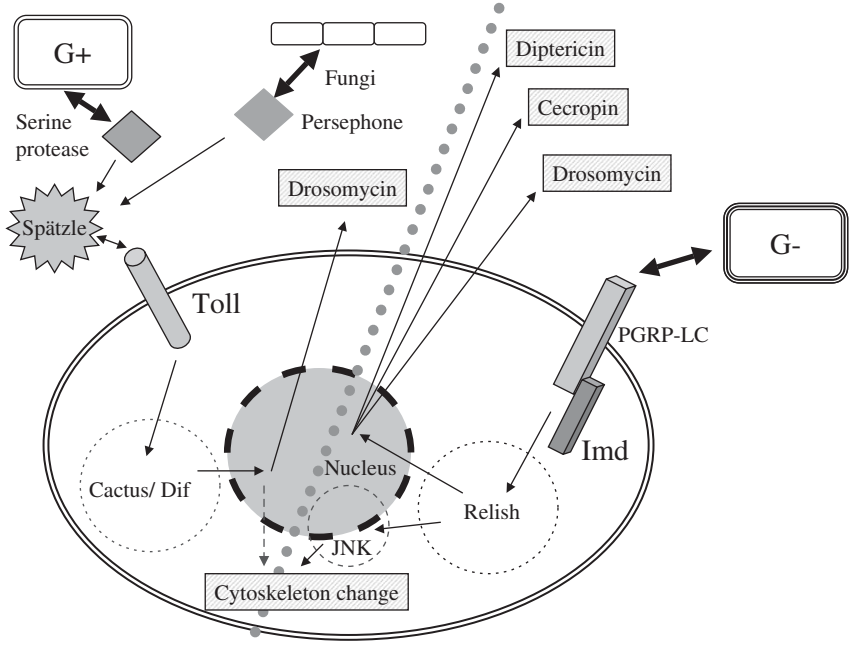

FIG. 3 A simplified schematic of the activation of the Toll and Imd pathways in *Drosophila* immunity.

4.4 EFFECTOR SYSTEMS – ENZYME CASCADES AND CYTOTOXINS

4.4.1 *Phenoloxidase*

Perhaps the most important constitutive immune effector system in insects is the tyrosinase (Chase *et al.*, 2000) PO. This enzyme catalyses the initial steps in the production of the biopolymers melanin and sclerotin (see Sugumaran, 2002). As well as its core role in immunity, it also plays an ontogenetic role by the iterative production of melanin and sclerotin during exocuticle manufacture after ecdysis (Neville, 1975) as well as reproductive roles during the production of species-specific visual signals (e.g. True *et al.*, 1999; Siva-Jothy, 2000). The production of PO from its inactive precursor PO is triggered via a serine protease cascade initiated by the detection of PAMPs (see Söderhäll and Cerenius, 1998; Cerenius and Söderhäll, 2004 for reviews). Immunological PO activity produces melanin, which is used to form one of two types of capsule around a pathogen. Cell-free, inert, melanotic capsules are found in a range of insects, including bumblebees (e.g. Allander and Schmid-Hempel, 2000) and mosquitoes (e.g. Gorman and Paskewitz, 2001). The second type of melanic capsule occurs in immune responses where haemocytes smother the invader and phenoloxidase activity melanises the resultant cell mass, forming a melanised cell-mass (e.g. Lackie *et al.*, 1985). In both cases, the insect 'externalises' the intruder behind an inert and impermeable barrier. Interestingly, despite (a) the observation that PO activity is correlated with pathogen death and isolation and (b) the incredibly detailed dissection of the molecular mechanisms releasing and regulating the prophenoloxidase cascade (Söderhäll and Cerenius, 1998; Cerenius and Söderhäll, 2004), it remains to be empirically demonstrated how PO activity deals the fatal blow to the pathogen. It seems most likely that certain products of PO activity (quinones, phenols and reactive oxygen species) are utilised for their cytotoxic effects and that the consequently moribund (or dead) pathogen is finally smothered and externalised in the melanised capsule: if it is still alive the barrier will deprive it of oxygen and nutrients.

An exciting recent development in the study of PO is a growing body of evidence that the cascade is not just a fast-acting blunt instrument of defence. It appears to be subtly integrated into other mechanisms, with suggestions that the 'cross talk' aids clotting (Li *et al.*, 2002) and microbial peptide synthesis (Braun *et al.*, 1998).

4.4.2 *Nitric oxide*

Nitric oxide is a soluble, highly reactive gas synthesised within cells by the enzyme nitric oxide synthase (NOS). Nitric oxide's cytotoxic activity arises from its ability to combine with superoxide radicals and produce highly reactive peroxynitrite groups that are particularly effective at oxidising lipids.

Insect haemocytes are capable of generating nitric oxide in response to immune insult (Weiske and Wiesner, 1999) and NOS activity has been identified in the midgut epithelium of mosquitoes (Luckhart *et al.*, 1998) and the cardiac valve (the junction between the foregut and midgut) of Tsetse flies (Hao *et al.*, 2003). In both cases, NOS plays a defensive role, reducing the ability of the parasite to move though the NOS-active tissue and so gain access to the host.

4.4.3 *Reactive oxygen species*

The 'respiratory burst' is an NADPH oxidase-driven conversion of oxygen into the so-called 'reactive oxygen species', a group of highly reactive oxygen radicals. Reactive oxygen species are highly cytotoxic and have recently been identified in the haemolymph of immune-challenged insects (e.g. Whitten and Ratcliffe, 1999; Dettloff *et al.*, 2001; Glupov *et al.*, 2001). Because these radicals are unstable, and therefore very transitory, mechanistic detail about their production and control is lacking. It seems likely, however, that they are produced by haemocytes in the vicinity of microbial insult, and their synthesis and release are very tightly controlled in order to limit auto-reactive damage.

4.5 EFFECTOR SYSTEMS – ANTIMICROBIAL PEPTIDES

In comparison with other humoral immune effector systems, antimicrobial peptides are highly specific in their effects. However, that specificity comes with the cost of slow responsiveness (see Section 5.3). *Drosophila* shows three main structural groups of these peptides in seven distinct families (reviewed in Bulet *et al.*, 1999). They are mostly relatively small, often membrane-bound, and are extremely effective at neutralising G^+ bacteria, G^- bacteria and fungi in the haemolymph. They are synthesised mainly via signals transmitted through the Imd pathway (but the Toll pathway is also important) and are manufactured in the fat body (Hoffman and Reichhart, 2002), in haemocytes (Lowenberger, 2001) and in the epithelium (e.g. Tzou *et al.*, 2000).

Although these peptides are produced in quantity after microbial insult, and details of the pathways leading to the synthesis of these peptides are being rapidly uncovered (see Hoffmann, 2003 for review), we have little understanding of their coordinated role and mode of action.

In a similar vein, almost nothing is known about insect immunological defences against viruses. There is some evidence that hosts produce proteins that interfere with viral replication (Wyers *et al.*, 1995) , and one would additionally assume that boundary defences (Section 3) are critical.

4.6 EFFECTOR SYSTEMS – HAEMOCYTES

Our understanding of the insect haemopoietic system (see Lavine and Strand, 2002 for review) is derived largely from classifications based on haemocyte

morphology and/or behaviour. Almost nothing is known about the cell surface or genetic markers that control (and so define) haemocyte function (but see Chain *et al.*, 1992; Mullet *et al.*, 1993; Willot *et al.*, 1994; Strand and Johnson, 1996; Lebestky *et al.*, 2000). Recent studies by Hou *et al.* (2002) and Sorrentino *et al.* (2002, 2004) are, however, providing real insight into functional polymorphisms in haemocytes in relation to coordinated cellular responses to insult. Despite this, haemocyte lineage relationships and haemopoiesis remain, for the moment at least, poorly understood phenomena. One major drawback with the historical use of haemocyte morphology to define function types has been the abundance of morphology based names derived from studies of different species describing what are probably only a few distinguishable morphotypes. This problem is exacerbated by the fact that these studies are often conducted on monolayer preparations and the distinction between morphotypes made on qualitative criteria. For example, one commonly used division is the separation of immunologically active haemocytes into 'granulocytes' and 'plasmatocytes'. The former, as their name suggests, contain granules or vesicles, the latter, by definition, do not, but additionally show spreading behaviour when in contact with a foreign surface. Most insect immunologists would accept this distinction. However, studies using quantitative techniques such as flow cytometry, where the size and granularity of thousands of haemocytes are measured, reveal a single population of haemocytes showing degrees of granularity (e.g. Chain *et al.*, 1992). This is not to say that haemocytes do not have discrete functions in a coordinated immune response, rather that the morphological approach has some severe limitations in its ability to resolve those functions. This caveat being acknowledged, however, we can make certain generalisations about haemocytes on the basis of their morphology and behaviour.

Small, cytoplasm-deficient cells are usually termed 'prohaemocytes' and are widely believed to differentiate into other haemocyte types. The best evidence for this function comes from studies showing that they (and other morphotypes) undergo mitosis (e.g. Gardiner and Strand, 2000) and from *in vitro* studies, which suggest that they differentiate into other haemocyte types (e.g. Yamashita and Iwabuchi, 2001).

Haemocytes that contain granules and vesicles are usually referred to as 'granulocytes' but their behaviour varies across insect taxa (reinforcing the notion that morphology is not a good basis for inferring function). They are capable of phagocytosis in some insect species (Lavine and Strand, 2002) and are the first haemocytes to form an encapsulation response to a large invader (Pech and Strand, 1996) – quickly followed by other cell types. Pech and Strand (1996, 2000) show that the granulocytes attach and then apoptose (undergo programmed cell death). In this context, it is interesting that Chain *et al.* (1992) identified an epitope stuck to the contact surface that appeared to be released from blebbed granulocytes (Fig. 2d in Chain *et al.*, 1992). The fact that they contain granules suggests granulocytes are involved in the synthesis and

storage of bioactive compounds involved in immunity, but little information exists about the details of this role and their coordination in an immune response.

The other easily identifiable haemocyte morphotype is the plasmatocyte, a cell characterised by its propensity to spread on contact with a foreign surface. Plasmatocytes are capable of phagocytosis (e.g. Elrod-Erickson *et al.*, 2000) and are the mainstay of the coordinated cellular attacks directed at haemocoelic intruders discussed below (see also Lackie *et al.*, 1985).

These cell types, and others (see Lavine and Strand, 2002), are not only intimately involved in the manufacture and secretion of many of the compounds already discussed, but also coordinate a number of distinct responses to septic insult. As with other components of the immune system, it is important to bear in mind that haemocytes have vital tasks other than defence: they are intimately involved with rebuilding during metamorphosis (e.g. Wigglesworth, 1965), cuticle manufacture (Sass *et al.*, 1994) and basement membrane formation (Ball *et al.*, 1987), among other things.

Phagocytosis occurs when a haemocyte encounters and recognises a small (i.e. smaller than itself) pathogen. The pathogen is engulfed by the cell and is killed. Mammalian phagocytes kill the pathogens they engulf with nitric oxide (e.g. Nathan and Hibbs, 1991) and reactive oxygen species (e.g. Robinson and Badwey, 1994). Both of these effector systems have been associated with immune-challenged insect haemocytes (see Section 4.4) and probably perform a similar function. Phagocytosis can be promoted by certain cytokines (a thioester-containing peptide identified by Levashina *et al.*, 2001 promotes phagocytosis of G^- bacteria), suggesting that pathogen-naïve haemocytes can be 'switched on' during an insult, presumably making haemocoel clearance more rapid.

Targets that are too large for a single haemocyte to phagocytose (e.g. clusters of localised microbes) are smothered by layers of haemocytes which become melanised, a process known as nodule formation (because of the small, dark 'nodules' that appear within the haemocoel). Nodule formation requires that haemocytes must not only 'recognise' that phagocytosis is not an option, but must instead become adhesive and spread over the target as well as one another. The processes that control and regulate nodule formation are probably mediated by cytokines and cell adhesion molecules, and although nodule formation has not been mechanistically dissected, a number of candidate cytokines are known. The most potent insect cytokine known is plasmatocyte spreading peptide (Clark *et al.*, 1997), a peptide which causes plasmatocytes to spread and externalise adhesion molecules (Strand and Clark, 1999). Plasmatocyte spreading peptide homologues have been identified from a number of Lepidoptera and some have similar activity (Wang *et al.*, 1999; Strand *et al.*, 2000). A *Drosophila* protein, peroxidasin, stimulates haemocyte adhesion and spreading (Nelson *et al.*, 1994) and is similar to the well-characterised crustacean cell adhesion molecule peroxinectin (Johansson, 1999). Another

important aspect of nodule formation is the aggregation of haemocytes, a phenomenon stimulated by the soluble lectin-like protein hemocytin (Kotani *et al.*, 1995). Once the haemocytes have been stimulated to stick and spread, and have formed a covering over the agglutinated microbes, the nodular cell mass is usually melanised by the activity of PO. The control of this process in the context of melanising self is a mystery, but the consequences are that the pathogens are neutralised and externalised. Once formed the nodules remain in the insect until it dies.

Insects that are subjected to parasitoid attack face relatively large intruders in their haemolymph which need to be neutralised. In the case of parasitoid attack the consequences of failure to neutralise the parasite is host death, so the selection pressure for an effective response is strong. The response to large intruders is termed 'encapsulation' since the phenotypic consequence is usually spectacular (Fig. 4) and often visible without dissecting the insect. However, there is probably very little, if any, difference between cellular encapsulation and nodule formation apart from the scale of the process. Not surprisingly, most studies of insect cellular immunity focus on encapsulation because of its amenability to study. Haemocytes still need to identify the target, stick and spread and recruit other haemocytes to the task (see Lackie *et al.*, 1985). Integrins, a class of vertebrate cell adhesion molecules, are probably involved in encapsulation (Pech *et al.*, 1995; Lavine and Strand, 2001) and are expressed on the surface of haemocytes attached to a foreign surface (Nardi *et al.*, 2003). Lavine and Strand's (2003) recent work shows that at least one integrin plays an important role in regulating haemocyte adhesion during encapsulation.

Encapsulation is a mainstay of ecological immunity assays because it is easy to measure the single phenotypic outcome of the coordination of several

FIG. 4 The cellular encapsulation response directed at a 1 mm length of nylon monofilament implanted in the haemocoel of an adult *Tenebrio molitor* beetle for 20 h. Slight melanisation is apparent over the surface of the encapsulating haemocyte mass covering the nylon. There are easily distinguishable areas where the melanisation is more intense.

branches of an individual's immune system. Studies have revealed that the magnitude/speed of encapsulation is correlated with an individual's haemocyte load (the total number of haemocytes in the haemocoel) and that haemocyte load is a variable that responds to selection from parasitoids (e.g. Kraaijeveld and Godfray, 1997; Kraaijeveld *et al*., 2001). As mentioned above (see Section 4.4.1), encapsulation does not always involve a cellular reaction to non-self: some insects produce a cell-free, homogeneous melanic capsule around the intruder. However, as with nodule formation, when there is a cellular response it is finally melanised, forming a dark, impermeable barrier around the insult, which remains in the host until death.

Wound repair bears a lot of physical similarities to encapsulation: haemocytes are recruited to the critical site, become adherent and form a mass, which is eventually melanised. The process probably shares pathways and processes with encapsulation and nodule formation. However, wound healing is usually much more rapid than encapsulation (pers. obs.) and will have a more intimate association with clotting. Moreover, there appear to be distinct peptides associated with wound healing. Paralytic peptide 1, isolated from *M. sexta*, has been shown to speed up the cellular component of wound healing (Wang *et al.*, 1999) while hemokinin is released by damaged cuticular epithelial cells and induces haemocytes to aggregate at the wound site (Cherbas, 1973).

5 Ecological immunology and variation in immune defence

Insect immunity was the exclusive domain of immunologists seeking to understand the mechanistic basis of immune effector systems. However, the last decade has seen the concepts of population biology, ecology and evolutionary biology combine with immunity to produce 'ecological immunology', one of the fastest growing fields of evolutionary ecology (Sheldon and Verhulst, 1996; Rolff and Siva-Jothy, 2003). This field of research examines how and why micro-evolutionary processes generate, and maintain, variation in immune effector systems and the coordinated host response to pathogens (Schmid-Hempel, 2003). Evolutionary ecologists based their reasoning on two main theoretical approaches. The first approach relies on the theory of the evolution of life history traits (Roff, 1992; Stearns, 1992) and assumes that the evolution and the use of immune defences are costly (Sheldon and Verhulst, 1996) (see Section 5.1). The second approach is based on arms-race models of coevolution (Van Valen, 1973), which propose that coevolution between hosts and parasites can lead to sustained oscillations in host genotype frequencies through negative frequency-dependent selection, favouring rare host genotypes (Haldane, 1949; Hamilton, 1980; Frank, 1991, 1993; Thompson and Lymbery, 1996: Peters and Lively, 1999, see Section 5.2). Since one of the biggest problems in combining two, or more, established areas of research is the loss of information through

language differences, we include a table of definitions of frequently used evolutionary terms (see Table 1).

5.1 LIFE HISTORY THEORY AND THE COSTS OF IMMUNE DEFENCE

How does variation in life history traits (the major features of an organism's life cycle that determine fitness, e.g. size at birth, age at maturity, age-specific fecundity, survival rate) translate into variation in fitness among individuals? To examine this question, life history theory assumes the existence of trade-offs between traits that constrain the simultaneous evolution of two or more traits (Roff, 1992; Stearns, 1992). Immune defence should be viewed in this context since if immune defence only provided resistance to pathogens and parasites with no cost, then natural selection would have favoured universally perfect immunity. Since this is not the case (i.e. susceptibility persists), immune defence is probably costly and so is traded off against the need for investment in other important fitness traits: selection will favour individuals with an optimal balance between immune defence and other fitness traits. Several kinds of immune defence costs can be distinguished (Schmid-Hempel, 2003).

TABLE 1 Definitions of evolutionary terminology

Terms used in the text	Definition	Reference
Antagonistic pleiotropy	The genetic correlation between traits is such that selection on one trait is opposed by the consequent selection on the second trait	Roff (1997)
Coevolution	The joint evolution of two or more interacting species, each of which evolves in response to selection imposed by the other	Futuyma (1998)
Frequency-dependent selection	The fitness of a phenotype or a genotype varies with the phenotypic or genotypic constitution of the population	Roff (1997)
Evolutionary trade-off	Occurs when selection on one trait decreases the value of a second trait, i.e. a negative genetic correlation	Stearns (1992)
Fitness	The average contribution of an allele or genotype to the next generation. Usually, only correlates can be measured	Futuyma (1998)
Life history trait	A trait that is directly connected with fitness, such as development time, fecundity and viability	Roff (1997)
Physiological trade-off	Two or more traits compete for resources within a single organism	Stearns (1992)
Adaptation (adaptive)	A process of genetic change, owing to selection, whereby the average state of a character becomes improved with reference to a specific function	Futuyma (1998)

5.1.1 *Evolutionary cost of immune defence*

The evolutionary cost of immune defence relies on negative genetic covariance between a component of the immune system and another fitness-relevant trait of the organism or even another component of the immune system (Stearns, 1992). This phenomenon is assumed to result from antagonistic pleiotropy, where a gene that has a positive effect on one component of fitness (i.e. immune defence) has a negative effect on another. These genetic relationships between traits cannot be changed during the lifetime of the organism. Therefore, high expression of immune defence may negatively affect fitness by constraining other correlated fitness traits, especially in the absence of parasites or pathogens. These genetic trade-offs between immune defence and other fitness parameters are usually investigated through quantitative genetic estimation of trait covariance and selection experiments (see Table 2). Studies manipulate variation in host immune defence and then observe the correlated response in other important traits. For instance, Kraaijeveld and Godfray (1997) selected replicate lines of *Drosophila melanogaster* for increased resistance to the parasitoid wasp *Asobara tabida* and measured the correlated response on other important fitness parameters ranging from egg viability to female fecundity. Encapsulation ability was increased by 55% in five generations in their selection experiment. Compared to control lines, the resistant-selected lines were characterised by a twofold increase in circulating haemocytes and a reduced competitive feeding ability of the larvae under crowding. In contrast to this direct approach, other studies have selected for change in host traits and measured the corresponding change in immune defence. This 'indirect' approach was used by Koella and Boëte (2002) who selected lines of the mosquito *Aedes aegypti* for earlier or later age at pupation. They measured the extent to which selection changed the mosquito's ability to encapsulate and melanise Sephadex beads. The authors obtained mosquito lines with early and late age at pupation and found that encapsulation ability, as well as adult body size, were positively correlated with age at pupation.

The evolutionary cost of immune defence is assumed to affect the dynamic of resistant and susceptible genotypes in a host population according to parasite prevalence. Resistant host genotypes should only be maintained when parasites are abundant. Yan and Severson (2003) tested this assumption using the mosquito *A. aegypti* and the malaria parasite *Plasmodium gallinaceum*. The authors created experimental mosquito populations by mixing susceptible and resistant strains in equal proportions and then determined the dynamics of markers linked to loci for *Plasmodium* resistance and other unlinked neutral markers over 12 generations. They found that when a mixed population was maintained under parasite-free conditions, the frequencies of alleles specific to the susceptible strain at markers closely linked to the loci for resistance (QTL markers), as well as other unlinked markers, increased in the first generation and then fluctuated around equilibrium frequencies for all of those markers.

TABLE 2 Examples of studies of the cost associated with the evolution of immune defence

Insect species	Selective regimes	Effects	References
(a) Select for increased resistance and measure corresponding changes in other traits			
Honeybee (*A. mellifera*)	Increased resistance to bacterial disease	Higher larval mortality	Rothenbuhler and Thompson (1956)
Honeybee (*A. mellifera*)	Increased resistance to bacterial disease	Slower larval growth	Sutter *et al.* (1968)
Indian meal moth (*Plodia interpunctella*)	Selection for increased resistance to granulosis virus	Slower development, lower egg viability, but increased pupal mass	Boots and Begon (1993)
Mosquito (*A. aegypti*)	Selection for increased resistance to malaria parasite	Decreased adult body size, fecundity and longevity	Yan *et al.* (1997)
Mosquito (*A. aegypti*)	Increased resistance to nematode infections	Reduced reproductive success	Ferdig et al. (1993)
Fruitfly (D. melanogaster)	Increased encapsulation to larval parasitoids (Asobara tabida)	Reduced competitive ability	Kraajeveld and Godfray (1997)
Fruit fly (*D. melanogaster*)	Increased encapsulation to virulent larval parasitoids (*Leptopilina boulardi*)	Lower survival rate of larvae	Fellowes *et al.* (1998)

(b) Select for change in host trait(s) and measure corresponding change in immune defence			
Dung fly (*Scatophaga stercoraria*)	Selection for polyandry leading to larger reproductive organs	Correlated reduction of PO activity	Hosken (2001)
Mosquitoes (*A. aegypti*)	Selection for earlier or later age at pupation (i.e. age at reproduction)	Earlier reproduction correlates with lower encapsulation response, the opposite for later reproduction reduced reproductive success	Koella and Boëte (2002)
(c) Experimental competition between resistant and susceptible genotypes			
Mosquito (*A. aegypti*)	Mixing plasmodium-susceptible and resistant mosquito populations in equal proportion and comparing frequencies of resistance and susceptible alleles after 12 generations under parasite-free or parasite-exposure conditions	In parasite-free conditions frequencies of susceptible alleles increased and under parasite exposure allele frequencies did not change	Yan and Severson (2003)

Conversely, when the mixed population of mosquitoes was exposed to an infected blood meal every generation, allele frequencies at the QTL markers for resistance were not significantly changed. In other words, resistant genotypes are competitive only under parasite pressure. When parasite pressure was removed, resistant genotypes suffered from a lower competitive ability.

5.1.2 *Physiological cost of immune defence*

The physiological costs of immune defence results from resource-based trade-offs between the immune system and other important functions. Assuming that the different functions of an organism compete for the same pool of resource, the allocation of resource to the immune system is expected to constrain other functions that are sustained simultaneously and *vice versa*. These resource costs have two components (Schmid-Hempel, 2003). First, the cost of maintenance of the immune system corresponds to the cost of keeping the machinery at a given level of readiness; second, the cost of using the immune system when responding to a challenge.

The magnitude of resource trade-offs in the maintenance of the immune system is determined by constraints that result from the evolved physiology. However, maintaining immune defence is still a plastic trait (see Section 6.4) that shows variation influenced by individual decision. For instance, in the armyworm *Spodoptera exempta*, the basic level of PO activity in the cuticle, haemolymph and midgut is upregulated at high population density (Wilson *et al.*, 2001). Similarly, mating activity is known to lead to non-resource-dependent downregulation of the immune function (Siva-Jothy *et al.*, 1998; Rolff and Siva-Jothy, 2002). Measuring the resource cost of maintenance of the immune function is difficult, as many regulatory processes may interfere with it. For example, immune-depression under food stress (or an increase in other demanding activities) may reflect the occurrence of physiological regulation avoiding self-damage rather than a resource-based trade-off. As long as the regulatory mechanisms between functions are unknown, measures of the cost associated with the maintenance of the immune system will remain difficult to quantify.

Unlike the cost of maintenance, the cost associated with producing an immune response is relatively easy to measure and has been the target of several studies (Table 3). An immune response is assumed to use up part of an organism's energy budget. Demonstration of this cost consists of challenging a host immunologically and measuring the corresponding changes in other traits (including immune defence) compared to controls. For example, mosquitoes (*Armigeres subalbatus*), which have encapsulated micro-filarial parasites show reduced and delayed egg-laying (Ferdig *et al.*, 1993). Similarly, fruit flies (*D. melanogaster*), which succeeded in encapsulating the eggs of the parasitoid wasp *A. tabida* during the larval stage, show reduced adult survival (Hoang, 2001).

TABLE 3 Examples of studies of the physiological cost of immune defence

Insect species	Protocol	Effects of treatment	References
(a) Nutrition and general stress (cost of the maintenance of the immune system)			
Bumblebee (*B. terrestris*)	Restricted access to food in captivity	Reduction of the reproductive success but no effect on encapsulation response	Schmid-Hempel and Schmid-Hempel (1998)
Mealworm beetle (*T. molitor*)	Short-term nutritional deprivation	Downregulation of the PO activity, but rapid upregulation when beetles reaccess to food	Siva-Jothy and Thompson (2002)
(b) Manipulation of the workload (cost of the maintenance of the immune system)			
Bumblebee (*B. terrestris*)	Clipping wings to prevent foraging and flying	Foraging bees show reduced encapsulation response	König and Schmid-Hempel (1995); Doums and Schmid-Hempel (2000)
Damselfly (*Matrona basilaris*)	Observation of activity in the wild	Reduction of the encapsulation response after copulation or oviposition	Siva-Jothy *et al.* (1998)
Fruit fly (*D. melanogaster*)	Increased reproductive activity	Reduction of resistance against bacteria	McKean and Nunney (2001)
Mealworm beetle (*T. molitor*)	Comparing experimentally mated and unmated beetles	Mating reduces PO activity through juvenile hormone	Rolff and Siva-Jothy (2002)
(c) Activation of the immune system (cost of the immune response)			
Mosquito (*A. suballatus*)	Experimental infection with micro-filariae taken from mammalian host	Reduced egg development owing to common biochemical pathway	Ferdig *et al.* (1993)
Bumblebee (*B. terrestris*)	Antigenic challenge by injection of (LPS the surface molecules of Gram-negative bacteria) and latex beads	Reduced survival to starvation	Moret and Schmid-Hempel (2000)

(*continued*)

TABLE 3 Examples of studies of the physiological cost of immune defence (*continued*)

Insect species	Protocol	Effects of treatment	References
Damselfly (*Mnais costalis*)	Activation of the immune system by insertion of small nylon monofilaments	Negative correlation between PO activity and chronic burden of gut parasites (eugregarine trophozooites)	Siva-Jothy *et al.* (2001)
Fruit fly (*D. melanogaster*)	Infection by the parasitoïd *A. tabida*	Survivors of the parasitism had a reduced survivorship under both unstressed and stressed conditions	Hoang (2001)
Bumblebee (*B. terrestris*)	Antigenic challenge by injection of LPS (the surface molecules of Gram-negative bacteria)	Reduction of the reproductive success	Moret and Schmid-Hempel (2001, 2004)
Mosquito (*A. gambiae*)	Antigenic challenge by injection of LPS (the surface molecules of Gram-negative bacteria)	Females show reduced number of eggs produced and ovarian total protein content	Ahmed *et al.* (2002)
Leaf-cutting ant (*Acromyrmex octospinosus*)	Secretion of antibiotic compounds by the exocrine metapleural glands was prevented using nail polish to close them	Reduction of the respiration rate	Poulsen *et al.* (2002)
Mealworm beetle (*T. molitor*)	Activation of the immune system by insertion of small nylon monofilaments	Reduced longevity under *ad libitum* feeding conditions	Armitage *et al.* (2003)
Honey bee (*A. mellifera*)	Antigenic challenge by injection of LPS (the surface molecules of Gram-negative bacteria)	Reduced capacity of associative learning	Mallon *et al.* (2003a)

However, it is difficult to distinguish the cost of the immune response from the negative effect of the parasite in these experiments. The use of non-living and non-pathogenic immunogens (like nylon filaments, latex micro-beads or lipopolysaccharides (bacterial cell-surface molecules)) helps to avoid the potential confounding effect of parasitism. For instance, the immune response to an implanted nylon monofilament was shown to reduce longevity in the mealworm beetle *T. molitor* (Armitage *et al.*, 2003). Bumblebee (*Bombus terrestris*) workers challenged with either lipopolysaccharides extracted from *Escherichia coli,* or bacteria-sized latex micro-beads show a reduced survival under starvation (Moret and Schmid-Hempel, 2000). The use of lipopolysaccharides as an immunogen recently helped to demonstrate a broad range of costs associated with the immune response (Table 3). In insects, the immune response to lipopolysaccharides is relatively specific and involves both the PO cascade and antimicrobial immune pathways. Bumblebee workers, which have been challenged with lipopolysaccharides, show an increased antibacterial activity but a reduction in PO activity (Moret and Schmid-Hempel, 2001), suggesting a trade-off between the two immune pathways (however, a better understanding of the physiological links between these immune pathways is required for a more robust conclusion). Lipopolysaccharide-challenged female mosquitoes (*Anopheles gambiae*) had a lower ovarian total protein concentration and produced fewer eggs (Ahmed *et al.*, 2002). In the honeybee (*Apis mellifera*) producing an immune response to lipopolysaccharides has been claimed to negatively affect associative learning (Mallon *et al.*, 2003a).

In addition to resource-based trade-offs, physiological costs of immune defence also involve the self-damage caused to host tissues by the activated immune system. For instance, upon challenge, the activation of the PO cascade generates a variety of cytotoxic substances (Nappi and Ottaviani, 2000; Carton and Nappi, 2001) inside the open haemocoel of the insect. These molecules are toxic to pathogens, but may also cause cell damage and cell death in the host (Sugumaran *et al.*, 2000). Fortunately for the insect host, mechanisms exist to limit or prevent self-reactivity in the open haemocoel. Some of these mechanisms are passive (e.g. melanin deposited during the encapsulation response serves as a trap for reactive oxygen species and helps to localise the immune response to the pathogen surface in *Drosophila*, Nappi *et al.*, 1995). Other mechanisms are active such as the production of the serine protease inhibitor proteins that restricts PO activity to the site of infection in *Drosophila* (De Gregorio *et al.*, 2002) and *M. sexta* (Zhu *et al.*, 2003). These active mechanisms are also likely to be costly and therefore individual insects will have to balance the benefit of successful defence with the cost of self-reactivity. The life history consequences of self-reactivity are not yet known. However, assuming a cost to self-reactivity and/or its prevention for a particular component of the insect immune system, one would predict a switch to other, less costly, immune components when the prevalence of challenges is increased (Moret, 2003). This

maybe why locusts (*Schistocerca gregaria*) exposed to a high risk of infection exhibited greater antibacterial activity, while PO activity remains constant (Wilson *et al.*, 2002).

5.2 SPECIFIC RELATIONSHIPS BETWEEN HOSTS AND PARASITES

Another approach to explain variable levels of immune defence in populations is that specific interactions between hosts and parasites themselves generate variable immune responses. Independent of any cost of immune defence, arms-race models of host–parasite coevolution (often referred to as the 'Red Queen Hypothesis' (Van Valen, 1973; Peters and Lively, 1999), suggest that parasites and pathogens become rapidly adapted to those host genotypes that are the most frequent in the population. This would favour rare host genotypes through negative frequency-dependent selection and would consequently maintain genetic variation among a host population. Such a coevolutionary dynamic (over the timescale of a few generations), where parasites and pathogens continuously track host defences in order to bypass them, should result in variable degrees, and success, of host defence. This hypothesis predicts parasites should become adapted to their local hosts (Hamilton *et al.*, 1990; Ebert, 1994; Ebert and Hamilton, 1996; Imhoof and Schmid-Hempel, 1998; Lively and Dybdahl, 2000) and that parasites cannot infect different host types with the same efficiency (Jaenike, 1993; Ebert, 1998). However, the physiological mechanisms by which adapted parasites managed to overcome local host resistance remain unknown. Its existence suggests specificity in the innate system (see Sections 6.1 and 6.2).

Studies from the host's perspective have demonstrated that hosts also vary in the response of their specific immune responses when differentially susceptible to different parasite species, or different strains of the same parasite (Schmid-Hempel *et al.*, 1999; Brown *et al.*, 2001; Carius *et al.*, 2001). Hosts can show both specific and non-specific responses to parasite infections. Investigations about the relationship between these two components of the immune system are rare since addressing this question requires the combination of the defence cost approach (see Section 5.1) with an understanding of the nature and degree of specificity in insect immunity (Fellowes *et al.*, 1998; Webster and Woolhouse, 1998; Frank, 2000; Jokela *et al.*, 2000).

6 Outlook

In the last section of this review, we examine topics that emerge from the synthesis between the mechanistic approach and the evolutionary ecological approach. These issues are mainly derived from research and theory in evolutionary ecology, but require an understanding of the underlying physiology.

6.1 MEMORY IN INSECT IMMUNITY?

Adaptive (acquired) immunity is restricted to vertebrates and comprises 'antigenic specificity, diversity, immunologic memory, and self/non-self recognition' (Goldsby *et al.*, 2000). This is 'unlike innate immune responses' (Goldsby *et al.*, 2000, p. 10). Goldsby *et al.* correctly assume innate responses are less mechanistically sophisticated than acquired responses, but equate this with a lack of functional sophistication. A recent study on copepods (Kurtz and Franz, 2003) demonstrated a remarkable degree of memory in invertebrate immunity. Copepods were infected with tapeworms and subsequently reinfected either with tapeworms that were genetically similar to the first infection, or genetically dissimilar to the first infection. Copepods reinfected with a genetically similar parasite were much more successful in clearing the infection. The immune response of the copepod was specific and was based on the primary infection. The mechanism remains unclear (it is unlikely that parasite-borne substances caused the differential infection success, because of the design of the study), but there are candidate compounds on which a mechanism for this ability might rest. For example, lectins occur in almost all animals; they are proteins that lack catalytic activity but bind to specific carbohydrates on cell surfaces (Marques and Barraco, 2000). Quantitative variation in different sugar motives (PAMPs – see Section 4.2) on the surface of the parasite might stimulate a specific quantitative response to a particular combination of sugars. This would constitute a type of dose-dependent recognition whereby bacterial strains which differed in their cell wall composition elicited a different, specific, combination of responses. Identifying the causal basis of this specificity is an important goal for ecological immunologists.

6.2 HIGH SPECIFICITY, FEW RECEPTORS

Two main pathogen receptor pathways are known from insects: Toll and Imd (Hoffmann, 2003) (see Section 4.3 and Fig. 3). They are assumed to be specific to either G^+ or G^- bacteria (see however Gobert *et al.*, 2003) and produce a rather coarse level of discrimination (in sharp contrast to the sophisticated specificity of vertebrate immunity). However, a study on bumblebees and their trypanosome parasites (*Crithidia bombi*) casts a different light on specificity in insect immunity. Mallon *et al.* (2003b) infected nine different colonies of the bumblebee *B. terrestris* with four different strains of *C. bombi*. All combinations were examined and the results show all host colonies differed in their susceptibility to the parasite. However, the response depended strongly on the pathogen isolate. There were no resistant or susceptible colonies, and the response depended on individual combinations. A similar study has been conducted on the water flea, *Daphnia*, and the picture that emerged from that study was the same. The combination of host clone and parasite strain (in this case a bacterium) was of central importance for the infection success of the

parasite (Carius *et al.*, 2001). There is clearly a huge gap between our knowledge of the molecular mechanisms that enable differentiation between G^+ and G^- bacteria and the results of these infection studies, which suggest the existence of a much higher degree of specificity (see Watson *et al.,* 2005).

Taken together with the findings on specific memory by Kurtz and Franz (2003) and the phenomenon of trans-generational transfer of immunity (Moret and Schmid-Hempel, 2001; Little *et al.*, 2003), it seems likely that there are undiscovered mechanism(s) that allow insects to resolve different pathogens with relatively high resolution. Classical immunology has built up a large body of evidence that such specificity is unlikely to exist, but Hultmark (2003) recently highlighted the fact that most studies of immune function in *Drosophila* use non-pathogenic bacteria. Consequently, the immune phenomena identified might represent host responses to saprophytic microorganisms rather than responses to virulent infections. Furthermore, Oliver *et al.* (2003) recently demonstrated that facultative bacterial symbionts may additionally confer resistance in their hosts, making this issue even more complicated to resolve.

6.3 MULTIPLE INFECTIONS

Another problem with the way mechanistic studies are conducted is that the consequence of single infections is usually examined (but see Hurst *et al.*, 2003; Hughes and Boomsma, 2004). Given the omnipresence of pathogens and parasites in the natural world, the most likely scenario is that concomitant infections are prevalent. A recent study by Hughes and Boomsma (2004) shows that avirulent microorganisms out-compete virulent parasites in simultaneous infections once the virulent parasite breaks down the host's immune defence. Therefore, the 'mix' of the pathogen cocktail will be crucial to the infection (and host response) outcome. This observation poses considerable challenges for studies of insect immunity. How are concomitant infections dealt with by the host? Can resources for defence (e.g. essential amino acids), be depleted during these complex insults? How is the immune system upregulated after the first infection? An intriguing finding in the context of this last question is the enhanced resistance of mosquitoes against *Plasmodium* after prior systemic infection with bacteria (Lowenberger *et al.*, 1999). If *A. gambiae* or *A. aegypti* were immune activated with bacteria before they obtained an infectious blood meal (either *P. berghei* or *P. gallinaceum*), they showed a significant reduction in parasite oocysts on the midgut. This finding is supported by the observation that insect immune responses can outlast the insult that stimulated them (Moret and Siva-Jothy, 2003).

Signalling pathways and antimicrobial peptides are usually regarded as being highly conserved (Zasloff, 2002; Hoffmann, 2003). The fact that these pathways are conserved is surprising given the strong selection exerted by pathogens and the subsequent fast evolution of resistance genes (Hurst and Smith, 1999). However, as pointed out by Zasloff (2002) the use of

antibacterial peptides by hosts probably exploits a constraint in the design of bacterial cell walls. In contrast to multicellular organisms, bacterial cells are usually positively charged. Antimicrobial peptides bind to the charged component and destroy the cell wall. It is likely that evolutionary constraints are in place that prevent most bacteria from evolving resistance to this host response, but some bacteria, such as resistant forms of *Serratia,* have managed to reduce the concentration of negatively charged binding sites.

6.4 PLASTICITY OF IMMUNE FUNCTION

Insect immunology is traditionally a laboratory-based biological discipline. This constraint was probably imposed by the sophisticated and sensitive methodologies used to study it. Immunological studies also try to control conditions in order to reduce the variation in the studied immune trait. One effect of this tight control (and one reason for doing it) is that laboratory practitioners rarely observe phenotypic plasticity. Phenotypic plasticity is defined as 'the property of a genotype to produce different phenotypes in response to different environmental conditions' (Pigliucci, 2001). Among the best-known examples is Woltereck's Daphnia, a species that produces a defensive 'helmet', in response to the odour of predatory fish. Phenotypic plasticity is likely to be a very important feature of immune defence. Table 4 lists studies that have measured components, or correlates, of immune defences in different environments and which suggest a role for phenotypic plasticity in immunity (although most of the cited studies were not examining phenotypic plasticity directly).

The examples in Table 4 mainly look at haemocyte densities and PO: hardly any information is available on the plasticity of other components of the immune system. Overall, the picture that emerges suggests that immune defence in insects is highly plastic, although the adaptive value of this plasticity still needs to be demonstrated. Key questions are 'Do the measured differences in defence traits translate into higher or lower survival and reproduction in the presence of parasites?' and 'Are the costs associated with maintaining and employing immune defence different in different environments?'

7 Conclusions

We started off by describing the insect host's defence (see Fig. 1) by behavioural means, via body surfaces, to the interior. Although the physiological, molecular and genetic understanding of the mechanisms of insect immunity has vastly increased, it has come at the price of stripping study organisms of their 'natural' environments. One aim of this review has been to integrate immunity with environment, and to achieve this end we conclude by extending the defence component model of Schmid-Hempel and Ebert (2003), to include and integrate these two different approaches (Fig. 5).

TABLE 4 Plasticity of immune function and resistance

Species	Environment	Immune trait	Reference
Immune function			
Rhodnius prolixus	Diet	Haemocyte density, lysozyme, antimicrobial activity	Feder *et al.* (1997)
T. molitor	Diet	PO	Siva-Jothy and Thompson (2002)
Chorthippus biguttulus	Habitat	Phagocytosis	Kurtz *et al.* (2002b)
S. gregaria	Population density	Antimicrobial activity	Wilson *et al.* (2002)
Spodoptera	Population density	Cuticular colour, PO, Encapsulation	Wilson *et al.* (2001)
T. molitor	Population density	Cuticular colour	Barnes and Siva-Jothy (2000)
Lestes viridis	Time stress	PO, Haemocyte density	Rolff *et al.* (2004)
Coenagrion puella	Risk of predation and parasitism	PO, Haemocyte density	Joop and Rolff (2004)
Termites	Social environment		Traniello *et al.* (2002)
D. melanogaster	Temperature	Encapsulation lower	Fellowes *et al.* (1999)
B. terrestris	Temperature	Encapsulation	Benelli (1998)

As reviewed here and elsewhere (e.g. Hoffmann, 2003; Hultmark, 2003; see Nicolas Vodovar *et al.,* 2005), we now have considerable knowledge of the mechanisms of insect immune defence but we still do not know what causes variation in immune defence (Schmid-Hempel, 2003). One relatively intensively studied source of variation is the examination of evolutionary and/or physiological costs of immune defence (see Section 5), but most of the other areas we highlight are relatively poorly studied.

To illuminate the importance of combining immunological and ecological/evolutionary perspectives, we will consider some scenarios from the extended defence component model (Fig. 5). The three major sources of variation considered here are host type, parasite type, and environment. We refer to host- and parasite type, respectively (rather than purely genotypes) as this also applies to species with plastic polyphenisms such as darker cuticles under higher densities (see Reeson *et al.*, 1998; Barnes and Siva-Jothy, 2000). Our scenario is the most parsimonious as it only requires two host types, two parasite types and two environments, respectively. Despite this simplicity, the model produces eight different combinations at the three distinguished levels of host defence: behavioural avoidance, avoiding penetration by the parasite/pathogen

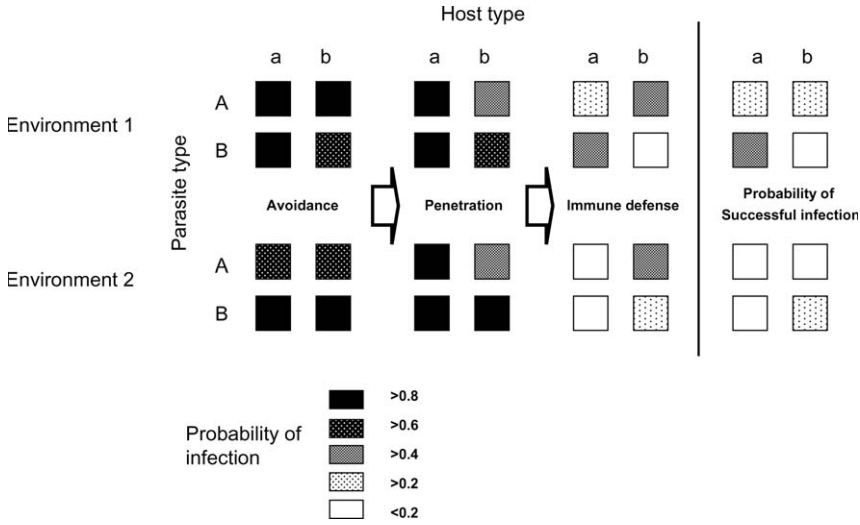

FIG. 5 The extended defence component model with the host (geno-) type and two environments. Shown are three steps in a hypothetical host–parasite/pathogen interaction. First, the parasite has to overcome avoidance behaviour by the host, then it has to enter the host by overcoming the external body walls and finally it has to overcome the immune defence. Shown are two hypothetical host (geno-) types (a and b) and two hypothetical parasite (geno-) types (A and B). The shading shows the probability of the parasite overcoming the different levels of host defence, for example, for the aA combination in environment 1 the probabilities are >0.8, >0.8, >0.2 and we calculated the probability of successful infections using intermediate levels, so here $0.9 \times 0.9 \times 0.3 = 0.24$. For simplicity, we assume a multiplicative model to calculate the probabilities of successful infections (see Schmid-Hempel and Ebert, 2003). More explanation may be found in the text.

and using the immune system. There are four important conclusions to be immediately drawn from this. First, the probability of infection for the same genotype depends on the environment, even within the same host-parasite-type combination (bB) (see Stacey *et al.*, 2003 for a real example). Second, it is possible to invest differently in different levels of the defence system yet yield the same outcome (see, for example, aA and bA in Environment 2). Third, host-type b is more resistant in environment 1, but host-type a is on average more resistant in environment 2. Fourth, knowing the mechanisms is very important (level 3 'immune defence' and to a lesser extent level 2 'penetration') but variation also needs to be understood (see Schmid-Hempel, 2003). This latter view has recently been supported by a genetic study on variation in antibacterial immunity in *D. melanogaster* (Lazarro *et al.*, 2004). They reported naturally occurring polymorphisms of genes involved in antibacterial immunity, primarily those genes that are related to recognition of pathogens and intracellular signalling.

In general, the combination of environment, host- and parasite-type determines the outcome of the interactions. From an immunological point of view, entering the host and establishing the infection are the important components.

In conclusion, insect immune defence is an exciting field which provides applied benefits and gives valuable insights into developmental, genetic and evolutionary processes. Combining mechanistic understanding with an evolutionary and ecological overview will, we predict, be a fruitful union. Rephrasing Stephen Stearns (1998), we hope that ecological and evolutionary thinking will be regarded, and incorporated, as a useful tool in study of the physiology of insect immune defence and parasite resistance.

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