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Host–Parasite Interactions and the Evolution of Immune Defense

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1. INTRODUCTION

Parasites are both numerous and ubiquitous, and so all living organisms face a continual struggle to fend off a constant barrage of immunological insults within their environment. The mechanisms for doing so are many and varied; they include physical and chemical defenses, such as thick skin, fur, or cuticle; behavioral defenses, such as grooming, behavioral fever, and self-medication; and immune defenses, including the innate immune system common to all animals and the vertebrate-specific acquired immune system (Schmid-Hempel, 2011; Wilson, 2005). Since the advent of molecular advances such as whole-genome sequencing and next-generation techniques, our mechanistic understanding of immune defenses has grown considerably for both vertebrates and invertebrates. Amongst the many things these have revealed is the similarity between many aspects of innate immune defenses of vertebrates and invertebrates (Vilmos & Kurucz, 1998), and this has highlighted the utility of using insects, especially *Drosophila*, as model hosts for understanding the evolution of, and plasticity in, the innate immune response. The 1990s saw the emergence of the new discipline of “ecological immunology” (Rolf & Siva-Jothy, 2003; Schmid-Hempel, 2005; Sheldon & Verhulst, 1996), which employs the robust experimental designs of evolutionary and behavioral ecology to dissect and understand patterns in disease prevalence and immune function. In particular, well-developed theoretical frameworks borrowed from areas such as epidemiology, life-history theory and sexual selection have been utilized to understand phenomena such as the proximate and ultimate costs of evolving, maintaining and deploying the immune system and other antiparasite defenses. The ecoimmunology approach has also resulted in the formulation of new, testable hypotheses about the plasticity of immune defense, including the notion of density-dependent prophylaxis (DDP), transgenerational immunity, nutritional immunology, and specific memory without conventional antibodies (e.g. Kurtz & Franz, 2003; Lee, Cory, Wilson, Raubenheimer, & Simpson, 2006;

Moret & Schmid-Hempel, 2001; Ponton, Wilson, Cotter, Raubenheimer, & Simpson, 2011; Wilson & Reeson, 1998).

In this chapter, we explore some of these approaches and phenomena, focusing particular attention on our own research, which highlights especially the value of using invertebrates to tackle key questions about the evolution, plasticity, and consequences of antiparasite defenses. In so doing, we aim to show that the host's armory against parasites consists not only of a simple yet sophisticated suite of interconnected immune effectors that can be mobilized to kill the parasite or reduce its impact (typically referred to as "immunity"), but also a range of behavioral and physical mechanisms to limit the likelihood of the animal becoming exposed to the parasite in the first place. In 1999, Owens & Wilson argued that there was a need for a broad definition of *immunocompetence*, to accommodate these additional, often neglected, aspects of an organism's defenses against parasites, and offered the following solution: "*immunocompetence is a measure of the ability of an organism to minimize the fitness costs of an infection via any means, after controlling for previous exposure to appropriate antigens*" (Owens & Wilson, 1999). This definition reflects the fact that parasites impact negatively on host fitness and that the host has a variety of mechanisms, including but not limited to classical immunity, to reduce those costs. It also recognizes that the response of the host is modulated by its previous exposure to "appropriate antigens", which could include those associated with a specific parasite strain or species, but could also include those of others that are not related. This definition was sufficiently broad to accommodate subsequent phenomena to emerge in the following decade of studies, including immune-priming and specific immune responses in invertebrates. Despite this, the use of the term immunocompetence has dropped in popularity in the intervening years, due in part to a greater appreciation of the fact that different components of the host's antiparasite defenses are subject to their own selection pressures and hence do not necessarily change in unison, either evolutionarily or phenotypically, in response to previous experience or aging (Cotter, Kruuk, & Wilson, 2004). The conflicting uses of the term, and definitions by other related disciplines, also led to confusion and misunderstandings (Viney, Riley, & Buchanan, 2005), which has also restricted its use. For these reasons, we avoid the use of the term immunocompetence here, and instead use the terms "immunity" or "immune function" when referring to attributes of the innate or adaptive immune responses, and use "antiparasite defenses" to encompass other nonimmunological parasite resistance mechanisms.

A key concept underpinning the ecoimmunology approach, and evolutionary ecology in general, is the notion of the *tradeoff*, i.e. that the expression of one trait (such as immune function) necessarily results in the reduction of one or more other traits (such as lifespan or reproductive output). Throughout this review, we identify some of the key genetic and phenotypic tradeoffs constraining the expression of antiparasite defenses, including tradeoffs among different immunological effectors; between antiparasite defenses and antipredator defenses; between nutrients required to enhance immunity and those required for reproduction or growth; between personal immunity and immunity for the offspring or social group; and tradeoffs between immunity and the capacity to attract mates. We also highlight, where possible, the epidemiological and population dynamical consequences of variation in the evolution or expression of antiparasite defenses.

We start by outlining the variety of different transmission strategies by which parasites may infect their hosts and the range of behavioral, physical, and immunological antiparasite mechanisms that have evolved to combat different types of parasites and how these are measured (Section 2). Most parasites are assumed to be transmitted in a broadly density-dependent manner (Anderson & May, 1991) and so for animals living in groups, or those for whom population densities fluctuate significantly within or between generations, parasites may pose a particular challenge. These challenges, and the host's response to them, are discussed with particular reference to insects, in which such effects have been most clearly documented (Section 3). Underpinning the notion of phenotypic tradeoffs is the realization that nutritional and other resources are not limitless. This means that animals must forage for essential nutrients in their environments and those that have too few nutrients, or the wrong blend, will be constrained in the options for their use. Immunological effectors (antimicrobial peptides (AMPs), immune cells, enzyme cascades, etc.) require micro- and macronutrients to fuel them, but different effectors may require different amounts or mixtures of these nutrients. And the nutritional resources required to power the immune system may be very different from those needed for other activities such as growth, reproduction, or general maintenance. This generates tradeoffs that may be resolved by preingestive behavioral plasticity and/or by postingestive resource allocation strategies. Some of these are discussed in detail here, as well as a robust conceptual framework for their elucidation—the geometric framework for nutritional ecology (Raubenheimer & Simpson, 1999; Raubenheimer, Simpson, & Mayntz, 2009; Simpson & Raubenheimer,

1995) (Section 4). In the final section, we discuss the implications of antiparasite defenses for the evolution of social behaviors, including parent–offspring interactions, sociality, and sexual behavior. We focus particularly on the risk of disease and its effect on reproductive investment, particularly whether organisms will “terminally invest” in reproduction if they perceive the risk of death due to parasitism to be high. In addition to personal immune responses, we also look at investment in “social” immune responses, i.e. those responses that have been selected to provide benefits to one or more recipients in addition to the challenged individuals, and also the potential for conflict between investment in the personal and social immune responses. We also consider gender-specific differences in immunity; in polygynous mating systems, at least, the different life-history strategies of the two sexes establishes different selection pressures on their antiparasite defenses. This may result in males compromising their antiparasite defenses and so sacrificing their long-term survival chances by instead investing in behaviors, physical structures, and lifestyles that enhance their short-term mating efforts. In contrast, females may invest in immunological, physical, and behavioral mechanisms that protect them from parasites to ensure they live long enough to realize their full breeding potential. These interactions within social groupings are discussed and their impact on immune function are illustrated (Section 5). We end by trying to draw some general conclusions about the evolutionary ecology of host–parasite interactions and identifying fruitful areas for future study (Section 6).



2. IMMUNITY AND OTHER ANTIPARASITE DEFENSES

2.1. Parasites Defined

We start by defining what we mean by the term “parasite”. Here, we use this as a generic term for any organism that lives within or on a host animal to the detriment of that host. This broad classification can be further divided into “macroparasites” and “microparasites” (Watt, Dobson, & Grenfell, 1995). For reasons of brevity, we exclude from our review any discussion of kleptoparasites, brood parasites, or parasites of organisms other than animals.

Macroparasites include parasitic helminths, such as nematodes, tapeworms, and flukes, as well as parasitic arthropods, including parasitoids, and ectoparasites, such as ticks, fleas, and biting flies that might act as vectors of microparasites. Macroparasites are multicellular organisms that typically do not multiply within their final or definitive host, but instead produce transmission stages (eggs and larvae) that pass into the external environment.

They usually exhibit an aggregated distribution on their host, with some hosts having lots of parasites, but most having few or none. Immune responses elicited against macroparasites are generally quite transient and depend on the number of parasites present in a given host, and so the impact of the parasite on its host tends to be dose-dependent—the more parasites, the greater the impact. There is an important group of macroparasites that are specific to invertebrate hosts, especially insects, and that is the *parasitoids*. These are mostly wasps or flies that live in or on a single host individual for most of their preadult lives and ultimately either kill or sterilize the host prior to maturation. Importantly, hosts generally become attacked by parasitoids when the highly mobile adult female parasitoid locates the host and lays its eggs in, on or near their hosts either singly (solitary parasitoids) or in groups (gregarious parasitoids). There is a vast literature on the biology and behavior of parasitoids, and the reader is referred to monographs by Godfray (1994), Hochberg and Ives (2000) and Wajnberg, Bernstein, and van Alphen (2008).

Microparasites (or “pathogens”) include viruses, bacteria, fungi, and protozoa. They are small organisms with short generation times that multiply within their definitive or final hosts. In contrast to what is observed in most macroparasites, the impact of microparasites on the host is primarily determined by whether or not the individual host is infected, rather than the number of parasites present per host (but see e.g. Graham, Grzywacz, Mushobozi, & Wilson, 2012). The duration of microparasitic infections are generally short in relation to the host lifespan and they are often virulent so may kill or sterilize their hosts. Those hosts that survive infection tend to develop immunity to reinfection that may be life-long.

Parasites, as described above, are transmitted by a variety of mechanisms, and the transmission route impacts on the defense mechanisms that are deployed by the host to combat them. Here, we briefly review the transmission process before exploring some of these defense mechanisms in detail. We focus particularly on the defenses that have evolved in insect hosts, since these are generally simpler and less well documented.

2.2. Parasite Transmission

The starting point for understanding the evolution and plasticity of antiparasite defenses is a good appreciation of the natural history of the host–parasite interaction. Epidemiology theory generally assumes that most parasites are transmitted in a positively density-dependent manner, such that as local density increases, so too does the *per capita* risk of an

individual encountering an infectious conspecific or a parasite's infective stage, though tests of this fundamental assumption are relatively rare (McCallum, Barlow, & Hone, 2001). This is based on the “mass action” assumption that infectious and susceptible individuals within a population behave like the molecules in a closed vessel, moving randomly in space such that the encounter rate between two types of molecules is directly proportional to their relative densities. Thus, if the density of susceptible hosts in a population is S , and the density of infectious hosts (or other infectious agents) is I , then the number of new infected hosts per unit area per unit time will be βSI , where β is the *transmission coefficient*—a constant representing the probability of a new infection arising *per contact* between a susceptible and infectious host. This notion identifies an important attribute of the infection process, namely that the probability of a host becoming infected depends on two processes—(1) the probability of a susceptible host encountering an infectious agent, which is some function of the densities of both entities, $S * I$, and (2) the probability of a host–parasite interaction resulting in a successful infection, β . The former attribute determines the host's rate of *exposure* to a parasite threat, whilst the latter, in part at least, is a function of the host's *susceptibility* to infection, which is determined by its immune response and other (mostly nonbehavioral) antiparasite defenses. It is often important to distinguish between these two components of the infection process—exposure and susceptibility—because they may have very different implications for the evolution of host defenses, and the two interact to determine the outcome of an infection process (see Section 3).

Of course, not all parasites are expected to be transmitted in a linear density-dependent manner. For some host–parasite interactions, the contact rate between susceptible and infected individuals is predicted to be independent of host density (i.e. “density-independent” or “frequency-dependent”) and for others there may be an asymptotic relationship between contact rate and host density (Ardia, Parmentier, & Vogel, 2011; Begon et al., 2002; McCallum et al., 2001). In models of sexually transmitted diseases (STDs), for example, it is often assumed that parasite transmission is only weakly related to host density because the number of sexual partners an individual has depends on their attractiveness and/or the species' mating system (Knell & Webberley, 2004; Sheldon, 1993), but see Ryder, Webberley, Boots, & Knell (2005) for an experimental test of this STD assumption using the two-spot ladybird, *Adalia bipunctata*, and its sexually transmitted parasitic mite, *Coccipolipus hippodamiae*. In such circumstances, the number of new

infections per unit time and area will approximate $\beta SI/N$, where $N = S + I$. In practice, there are a range of transmission processes observed in the wild, and intermediate situations between frequency and density-dependent transmission, or a combination of the two, may not be uncommon (Ryder et al., 2005, 2007; Smith et al., 2009). Indeed, nonlinear relationships between host and/or parasite density and the number of new infections can be expected due to a variety of mechanisms, including phenotypic plasticity in the host (Section 3.2).

2.3. Behavioral Defenses

A first line of defense for many organisms is to use behavioral mechanisms to avoid contact with parasites, to kill parasites before infection occurs, or to limit their spread. These can include activities carried out individually or collectively. For example, badgers have extensive setts with many possible sleeping chambers and they move between these chambers frequently to avoid the build-up of ectoparasites (Butler & Roper, 1996). Many mammals twitch or use their tails to deter biting flies that may transmit parasites (Hart, 1997). Similarly, some caterpillars thrash around and attempt to bite parasitoids that are trying to oviposit in them (S.C. Cotter, pers obs *Spodoptera littoralis* and its parasitoid *Microplitis rufiventris*). This could deter a parasitoid, encouraging it to find a less troublesome host.

Grooming behavior to remove parasites is well established in vertebrates, and includes preening and dust bathing in birds, for example, and mutual grooming in primates (Hart, 1997; Nunn & Altizer, 2006). In insects, it can also be particularly effective against parasites that enter the body by penetrating the cuticle, such as fungi and parasitoids. For example, termites groom themselves and their nest-mates to remove fungal spores from the cuticle (Rosengaus, Maxmen, Coates, & Traniello, 1998). A similar study found that when fungus-inoculated *Acromyrmex echinatior* ants were kept with groups of nest-mates they showed reduced mortality compared to those kept in isolation (Hughes, Eilenberg, & Boomsma, 2002). It was proposed that this was due to a combination of hygienic allogrooming and increased antibacterial secretions stimulated by the presence of fungal spores.

Behavioral fever is another mechanism by which individuals or groups can attempt to overcome parasitic infection. Fever is well established in homeotherms as a mechanism to fight infection, but poikilotherms are not able to increase their body temperature using physiological mechanisms. It was demonstrated first in fish that poikilotherms are able to behaviorally modify their temperature to achieve a fever-like state (Reynolds, Casterlin, &

Covert, 1976) and it has since been shown to occur in a number of different insect species (e.g. Adamo, 1998; Blanford, Thomas, & Langewald, 1998; Bronstein & Conner, 1984; Campbell, Kessler, Mayack, & Naug, 2010; McClain, Magnuson, & Warner, 1988). For example, locusts will choose to bask at a temperature that is higher than optimal when infected with the fungus *Metarhizium anisopliae*. The locusts can cope with this raised temperature but it results in arrest of the growth of the fungus (Elliot, Blanford, & Thomas, 2002). Crucially, even though all locusts eventually succumb to infection, those that exhibit the fever are still able to produce viable offspring, thereby salvaging some fitness before they die (Elliot et al., 2002). Some social insects can also induce behavioral fever, by acting collectively; individuals huddle together to raise temperatures beyond those optimal for pathogens (Wilson-Rich, Spivak, Fefferman, & Starks, 2009). Cold temperatures can also be used as a parasite defense. For example, when workers of the bumblebee, *Bombus terrestris*, are parasitized by conopid flies, they typically stay out in the cold overnight rather than returning to their warm nest; a behavior that slows down the development of the parasitoid and reduces the chances of its successful development (Muller & Schmid-Hempel, 1993).

Hygienic behaviors are typical of many social insect species. For example, ants have been found to remove infected cadavers from the colony, and both infected ants and honeybees will remove themselves from the colony in an apparently altruistic act of suicide (Heinze & Walter, 2010; Rueppell, Hayworth, & Ross, 2010). Ants also collect resins with antimicrobial properties to prevent the growth of microbes within the nest (Chapuisat, Oppliger, Magliano, & Christe, 2007; Christe, Oppliger, Bancala, Castella, & Chapuisat, 2003). A similar behavior occurs in some bird species that have been shown to bring aromatic plants to the nest to act as fumigants (Gwinner & Berger, 2005; Lafuma, Lambrechts, & Raymond, 2001). Animals thus use a range of mechanisms to avoid parasitism, by reducing contact with parasites and tackling parasite propagules in the environment before they reach the body. If these behavioral mechanisms fail, many species have external defenses that can be deployed before the parasite enters, or attaches to, the body.

2.4. External Defenses

External defenses are seen in both vertebrates and invertebrates. For example, birds preen their feathers with a secretion from the uropygial gland, which has been shown to have antimicrobial activity and so protects the feathers from feather-degrading bacteria (Martin-Vivaldi et al., 2010;

Shawkey, Pillai, & Hill, 2003). Several animal species also use self-produced antimicrobials in the fabric of a nesting structure. For example, tungara frogs cover their eggs in a foam that contains a cocktail of chemicals that protect the eggs from microbes (Fleming, Mackenzie, Cooper, & Kennedy, 2009), and several nest-building fish produce antibacterial mucus or glue which protects eggs from microbial contamination (Giacomello, Marchini, & Rasotto, 2006; Knouft, Page, & Plewa, 2003; Little, Perutz, Palmer, Crossan, & Braithwaite, 2008). Many invertebrates have similar nest-protection mechanisms, for example, termites coat the inside of their chambers with antifungal fecal pellets (Rosengaus, Guldin, & Traniello, 1998). Bark beetles use oral secretions that contain a fungus-inhibiting bacterium to coat the inside of the chambers in which they lay their eggs, thus protecting them from invasive fungi (Cardoza, Klepzig, & Raffa, 2006), whilst burying beetles prepare a vertebrate carcass for their offspring by covering it with antibacterial exudates (Cotter & Kilner, 2010b); see Sections 5.3 and 5.4 for more details.

The next line of defense is the physical barrier of fur, feathers, scales, and skin that make it difficult for parasites to penetrate the body, and these can be further enhanced by chemical protection mechanisms. Sweat and organic acids on the skin, melanin in the skin, antimicrobials in the respiratory tract, and lysozymes in tears and saliva all help to kill microbes before they enter the body (Janeway, Travers, Walport, & Shlomchik, 2001; Mackintosh, 2001). Insects also benefit from the protection provided by either a cuticle strengthened by melanin, or from a hardened exoskeleton. In insects, the first, and probably best, lines of defense against most parasites are the cuticle and the midgut. Thus, any modifications that enhance their ability to act as physical or chemical barriers to penetration by entomopathogens are likely to be favored. The fatty acids and hydrocarbons present on the cuticle form the insect's first defensive mechanism. If a fungal pathogen is unable to utilize these compounds, it will be unable to grow through the cuticle. The entomopathogenic fungi *Beauveria bassiana*, *M. anisopliae*, and *Nomuraea rileyi* are able to utilize some of the hydrocarbons and fatty acids, allowing them to penetrate cuticles refractory to most other microorganisms (Bidochka & Khachatourians, 1992; Briese, 1981; St Leger, 1993).

The pigment responsible for darkening the cuticle of many insects is melanin. Melanin is a polymer and so may act to strengthen the cuticle, making it more resistant to pathogens such as fungi and parasitoids that attack via that route (St Leger, Cooper, & Charnley, 1988; Hajek & St Leger, 1994). Melanin is also toxic to microorganisms (e.g. Montefiori & Zhou,

1991; Ourth & Renis, 1993; Sidibe et al., 1996), as it can bind a range of proteins (e.g. Doering, Nosanchuk, Roberts, & Casadevall, 1999) and it inhibits many of the proteases and chitinases produced by microorganisms to enable penetration of the cuticle (Bull, 1970; Kuo & Alexander, 1967). In addition to melanin, also present in the cuticle is the endpoint of the prophenoloxidase (proPO) cascade, phenoloxidase (PO). This is activated when the cuticle is punctured or stimulated by microbial cell wall components (including peptidoglycan, β -1,3 glucan and possibly lipopolysaccharide), resulting in the local production of toxic quinones and melanin in the cuticle, which can reduce fungal growth and immobilize bacteria (St Leger, 1991; Marmaras, Bournazos, Katsoris, & Lambropoulou, 1993). There is also evidence that the level of PO activity in the cuticle is correlated with resistance against *B. bassiana* fungus in *S. littoralis* (Cotter, Hails, Cory, & Wilson, 2004) (Section 3.3).

The midgut is the only part of the insect not covered by cuticle and so is an important route of entry for many parasites including protozoa, viruses, and pathogenic bacteria. A thin envelope called the peritrophic membrane separates food and gut flora from the midgut epithelia. Although the membrane must be permeable in order to digest food, the pores are too small for most bacteria, so providing some protection against infection (Chapman, 1998). There are a number of mechanisms by which the host can resist viral infection in the midgut. Infected midgut cells can be sloughed, and sometimes replaced with immune cells (Briese, 1981; Keddie, Aponte, & Volkman, 1989). PO activity (see Section 2.5.1.3) has been reported from the midgut, and in *Helicoverpa zea* refractory to *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) infection, virally infected midgut cells were found to be melanized, presumably via the action of midgut PO (Washburn, Kirkpatrick, & Volkman, 1996). Similarly, in mosquitoes, ookinetes of the malarial parasite, *Plasmodium*, may become encapsulated in the midgut by a humoral sheath of melanin that ultimately kills them (Gorman, Cornel, Collins, & Paskewitz, 1996). If these external defenses fail, and parasites manage to gain entry to the body, then the classical internal immune system is brought into play to isolate and eliminate parasites, or at least hold them at bay, allowing the animal time to reproduce.

2.5. Internal Defenses

The typical immune response consists of a range of effectors, targeted at organisms that have invaded the body. These can be either *constitutively expressed*, analogous to a standing army ready to do battle, or *induced* upon infection, analogous to calling up the reserves. The immune response can also be separated into *innate* responses, which are present in all animals, and

acquired responses, which were thought to be restricted to vertebrates, but evidence suggests that invertebrates may also have some form of specific immune memory (Section 2.5.2).

2.5.1. Innate Immunity

2.5.1.1. Recognition Mechanisms

Many of the recognition mechanisms are conserved across vertebrates and invertebrates. With microparasites, such as bacteria or fungi, recognition of the invaders as non-self occurs via *pathogen-associated molecular patterns* (PAMPs). PAMPs include bacterial or fungal cell wall components such as lipopolysaccharides (LPS), peptidoglycans (PEP), or β 1-3 glucans. In insects, these PAMPs activate either the *Toll* (fungi and gram positive bacteria) or *Imd* pathways (gram negative bacteria), via host *pattern recognition receptors* (PRRs) that result in a systemic antimicrobial response. In vertebrates, these PRRs are found on the surface of immune cells found in tissues, such as macrophages (Janeway et al., 2001). The recognition of non-self via PRRs initiates inflammation, which starts a cascade of events in which inflammatory chemicals (e.g. histamine) attract other immune cells, further triggering other parts of the immune system (see Section 2.5.1.2) (Janeway et al., 2001). Interestingly, Toll-like receptors are involved in pathogen recognition in both invertebrates and vertebrates, as part of the innate immune response, though whether this suggests an ancient evolutionary origin to the immune system (Vilmos & Kurucz, 1998) or convergent evolution is not yet clear (Leulier & Lemaitre, 2008).

In insects, *Imd* additionally stimulates the production of *peptidoglycan recognition proteins*, which break down PEP into nonimmunogenic fragments to limit the response. PAMPs also trigger a serine protease cascade that results in the cleavage of inactive proPO to the active form of PO, which plays many roles in cuticle strengthening, wound repair, and the control of both micro- and macroparasites (see the Sections 2.5.1.2 and 2.5.1.3). The proPO cascade also produces intermediates that can stimulate the *Toll* pathway, allowing cross talk between effectors (Ragan, An, Jiang, & Kanost, 2009). With a few exceptions, viruses do not appear to upregulate either the *Toll* or *Imd* pathways in insects and may instead interact with the *JAK/STAT* pathway, which is stress-induced and responds to wounding (Imler & Eleftherianos, 2009).

2.5.1.2. Cellular Responses

In vertebrates, the leucocytes, or white blood cells make up the cellular response to parasites. Leucocytes are not strictly associated with a particular

tissue but can migrate around the body, moving to sites of infection. Leucocytes include phagocytic cells such as monocytes, which are present in the blood, macrophages, which can move beyond the vascular system seeking out parasites to engulf, and dendritic cells that patrol the skin and mucus membranes (Janeway et al., 2001). Neutrophils are also phagocytic but additionally employ a respiratory burst to attack parasites, producing strong oxidizing agents and damaging free radicals. Mast cells, eosinophils, and basophils release histamines, initiating inflammation. Eosinophils and basophils, along with neutrophils, are known as granulocytes due to their grainy appearance. Upon activation, they also release toxins and free radicals to help kill parasites, and so must be tightly regulated to avoid autoimmune damage (Janeway et al., 2001).

Like vertebrates, insects possess blood cells (hemocytes) but they play no role in oxygen transport and instead are used for nutrient storage and immune responses (Chapman, 1998). Hemocytes are differentiated into different cell types, which differ between insects and may or may not have the same names. For simplicity, hemocytes can be described by their functions, i.e. those that act as: (1) phagocytes, (2) spreading cells that are involved in wound healing and encapsulation, and (3) PO-containing cells (Strand, 2008), though some cells may perform more than one of these functions. Most insects have cells described as plasmatocytes (lamellocytes in *Drosophila*), which are spreading cells with phagocytic capabilities, and granulocytes (plasmatocytes in *Drosophila*) which, like the vertebrate cells, are named for their grainy appearance and are adhesive cells (Strand, 2008). Other cell types are oenocytoids (crystal cells in *Drosophila*) and spherulocytes, and are thought to produce humoral immune effectors (Ashida & Brey, 1997; Ashida, Ochiai, & Niki, 1988; Lavine & Strand, 2002; Soderhall & Smith, 1986; Strand, 2008).

Once an invader has been recognized by the humoral system, hemocytes are recruited to the site of infection. In insects, components of the *Toll* and *Imd* recognition pathways have been shown to be expressed in hemocytes (Irving et al., 2005), and there is also evidence for chemotactic attraction of hemocytes to wound sites (Stramer et al., 2005). When the hemocytes come into contact with microparasites, such as bacteria, they phagocytose, or ingest them (Tanada & Kaya, 1993). Invading organisms that are too large to be phagocytosed are encapsulated by the formation of an envelope of hemocytes (cellular encapsulation) and/or melanin (humoral encapsulation). Cellular encapsulation involves the formation of a multilayered envelope typically by plasmatocytes and granulocytes

(Gotz, 1986). The first stages of non-self recognition and attachment are the same as for phagocytosis. Melanization commonly occurs soon after the onset of encapsulation. It does not always occur, however, and the level of melanization is variable and dependent on the foreign body; biotic factors provoke a greater level of melanization than abiotic factors. In humoral or melanotic encapsulation, typical of most dipteran insects that have low densities of hemocytes, such as anopheline mosquitoes and some species of *Drosophila*, the invading macroparasite is engulfed by an envelope of melanin without the adhesion of hemocytes (Gorman, Schwartz, & Paskewitz, 1998).

2.5.1.3. Humoral Responses

Humoral responses comprise a cascade of peptides and proteins that complement the cell-based immune response. In vertebrates, these are typically produced in the liver and they help to tag parasites for destruction by cells, or damage parasite cell membranes, killing them directly (Janeway et al., 2001). These proteins include specific antimicrobial responses such as the production of AMPs and lysozymes, both of which are primarily manufactured in the fat body in insects—the insect equivalent of the vertebrate liver. However, in Lepidoptera, AMPs and lysozymes have also been found in hemocytes, with granulocytes and plasmatocytes expressing multiple AMPs, and spherulocytes and oenocytoids expressing lysozyme (Lavine, Chen, & Strand, 2005). Lysozyme is constitutively expressed in many insects but is also upregulated upon recognition of PAMPs (e.g. *S. littoralis*, (Cotter, Simpson, Raubenheimer, & Wilson, 2011)), whereas AMPs are typically produced only in response to parasite recognition, though they may be generated prophylactically in response to wounding to protect against bacteria that may opportunistically invade via the open wound (e.g. *Drosophila*, (Lemaitre, Reichhart, & Hoffmann, 1997), *Nicrophorus vespilloides*, (Cotter, Ward, & Kilner, 2011)).

Because of the delay in AMP production, lysozyme probably plays a major role in the first line of defense against bacterial parasites in insects (Haine, Moret, Siva-Jothy, & Rolff, 2008). Haine, Moret, et al. (2008), working with *Tenebrio molitor* beetles showed that following infection, 99.5% of the bacteria are cleared by constitutive lysozymes and cell-mediated responses (Fig. 3.1). Thus, the function of the AMPs may well be to mop up any resistant bacteria that have evaded the first line of defense and so reduce the likelihood of resistant strains persisting.

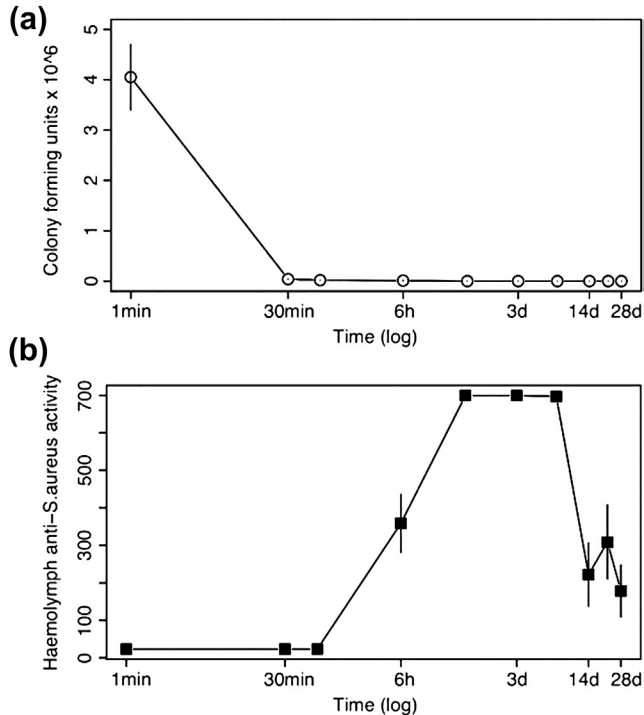


Figure 3.1 The number of *Staphylococcus aureus* bacteria (as measured by the number of colony-forming units, CFU) recovered from *Tenebrio molitor* hemolymph over 28 days (a), and the hemolymph anti-*S. aureus* activity from the same individuals (b). Induced hemolymph anti-*S. aureus* activity was measured as the number of *S. aureus* colony-forming units (CFUs) killed during 2 h of exposure to *T. molitor* hemolymph and is shown as CFU \times 10³. Each point represents the mean number of CFUs from 7 to 10 beetles (T1 SEM). (Reproduced from Haine, Moret, et al. (2008). Reprinted with permission from AAAS.).

POs also form a key part of the insect humoral response. POs are highly reactive and are stored as an inactive precursor, proPO. They are activated via a serine protease cascade and this is kept in check by serpins (serine protease inhibitors). POs oxidize tyrosine derivatives to their corresponding quinones and their polymerization product, melanin (Hiruma & Riddiford, 1988; Mason, 1955; Nappi & Vass, 1993). Many of the intermediates produced within the PO cascade are cytotoxic and can aid in killing parasites but can also be toxic to the host if not tightly regulated (Nappi & Vass, 1993). This is analogous to the tight regulation required of eosinophils and basophils in the vertebrate innate response, and indeed both types of response produce dangerous reactive oxygen species (Gonzalez-Santoyo & Cordoba-Aguilar, 2012). Whilst the direct action of PO on microparasites such as bacteria is

not clear (Cerenius, Lee, & Soderhall, 2008), there is evidence that it plays a role in the insect's antimicrobial response; for example, one pathogenic bacterium, *Photorhabdus luminescens*, is known to produce a compound that inhibits PO production (Eleftherianos & Revenis, 2011; Liu et al., 2007). PO has also been shown to play a role in potentiating cell-based responses such as phagocytosis (Liu et al., 2007; Nappi, Carton, Li, & Vass, 1992) and it is involved in hemolymph coagulation (Bidla, Lindgren, Theopold, & Dushay, 2005; Nagai & Kawabata, 2000), which can help to compartmentalize the hemocoel, restricting immune cascades to the site of infection/wounding (Haine, Rolff, & Siva-Jothy, 2007).

2.5.2. Acquired Immunity and Specific Immune Memory

2.5.2.1. Vertebrates

While an innate system of immunity is common across all animals, it was thought that the acquired immune response, known as the adaptive or specific immune system, was restricted to vertebrates. With the exception of red blood cells, all cells in the vertebrate body express the major histocompatibility complex (MHC), which can bind fragments of molecules, known as antigens, and present them on their surface. These antigens can be self or non-self, and it is the non-self antigens that are recognized by the adaptive system. The adaptive immune system comprises types of leucocytes called lymphocytes, which come in two major forms, B- and T-cells.

Macrophages and dendritic cells that have engulfed parasites, chop them up and display the antigens on their surface via the MHC, presenting them to the T-cells (Janeway et al., 2001). Antigen recognition activates specific T-cells that then either form cytotoxic T-cells or helper T-cells. Upon activation, cytotoxic T-cells mature and replicate, producing an army of lymphocytes that actively seek out body cells infected with parasites and kill them. In contrast, helper T-cells have no phagocytic or cytotoxic function and instead act as mediators of the immune response by secreting cytokines to mature other T-cells and B-cells or recruit cells of the innate system to the site of infection (Janeway et al., 2001).

B-cells differ from T-cells in that they will recognize antigens in their native form, not only when they are bound to the MHC. Upon recognition of an antigen, B-cells will engulf the antigen and display it on its own MHC. This will then be recognized by matching helper T-cells, which will produce cytokines to help the B-cells to replicate and mature into antibody-producing plasma cells. These plasma cells live for 2–3 days, secreting antibodies that bind to antigens, making them easier targets for the killer

cells of the innate system and the humoral response (Janeway et al., 2001). Some of the activated B- and T-cells form memory cells that remain in the body and can be quickly activated upon subsequent infection by the same parasite. This produces a faster, stronger response to secondary infection. But how can the vertebrate-adaptive immune system recognize every possible pathogen that could be encountered? The system is incredibly powerful; a combination of somatic mutations and genetic recombination of antigen receptor gene segments allows a small number of genes to produce over a trillion different antibody molecules. These are randomly generated before birth and enable the immune system to react to an almost unlimited array of antigens (Rajewsky, 1996).

2.5.2.2. Invertebrates

It is now recognized that insects may also have some form of adaptive immune response. There have been a number of examples of an upregulated immune response providing long-term resistance against future infection (Moret & Siva-Jothy, 2003; Rosengaus, Traniello, Chen, Brown, & Karp, 1999), but this was thought to be persistence rather than specific immune memory. Indeed, insects do not appear to have anything that resembles the vertebrate antibody response. However, evidence for some form of specific immune memory is growing. The first clear example of invertebrate immune memory was found when the copepod *Macrocylops albidus* was challenged with its tapeworm, *Schistocephalus solidus* (Kurtz & Franz, 2003). Three days later, hosts were exposed to either siblings of the first exposure (i.e. antigenically similar) or unrelated tapeworms (i.e. antigenically dissimilar). In line with a specific immune memory, the infection success of antigenically similar tapeworms was reduced compared to that induced by antigenically dissimilar parasites (Kurtz & Franz, 2003).

A similar result was found in the bumblebee, *B. terrestris*. Sadd and Schmid-Hempel (2006) challenged bees with a low, nonfatal dose of one of three pathogenic bacteria, 8 or 22 days later the bees were then challenged a second time with a pathogenic dose of the original or a different bacterium and bee survival was measured. At eight days, all previously challenged bees showed greater survival than controls, irrespective of the nature of the bacterial combination, but by 22 days, it was only the bees that received the same bacteria in the second challenge that showed increased survival. Bees that received a congeneric or unrelated bacterium survived no better than the control bees (Sadd & Schmid-Hempel, 2006). The most parsimonious explanation for this finding is that there are two mechanisms in place: immune

upregulation and memory: challenging an insect with any bacterium upregulates an immune response, and this may remain active and protect the individual for some time after the original infection (Haine et al., 2008a). In the bee example, individuals were protected for up to eight days after the original challenge against all bacteria, but by 22 days, bees showed immunity only against the same strain as the original infection, suggesting immune memory (Sadd & Schmid-Hempel, 2006).

So what form might this specific immune memory take? Studies have suggested that the *Down syndrome cell adhesion molecule* (Dscam) might play a key role (Dong, Taylor, & Dimopoulos, 2006; Watson et al., 2005). Through alternative splicing, Dscam is able to produce tens of thousands of different proteins (Crayton, Powell, Vision, & Giddings, 2006; Dong et al., 2006), each with different adhesive domains and interaction specificities (Dong et al., 2006). Dong et al. (2006) showed that the mosquito, *Anopheles gambiae*, produces different splice variants in response to different pathogens, and that specific splice forms show greater protection against the eliciting pathogen. This suggests that insects are indeed able to produce a diversity of specific pattern recognition proteins in response to infection, analogous to the vertebrate, T-cell mediated antibody response (see Kurtz & Armitage, 2006 for an in depth review).

2.6. Assaying Immune Defense

The rapid growth of ecological immunology studies amongst behavioral ecologists and evolutionary biologists has been strongly underpinned by the use and development of relatively simple physiological assays to quantify immune function and antiparasite defenses. These assays can be divided into three main types: (1) those that measure *constitutive* levels of immune effectors (i.e. those that are expressed even in the absence of an immune challenge); (2) those that measure *induced* immune responses following challenge by a pathogenic or nonpathogenic immune elicitor; and (3) those that measure the behavioral or life-history responses to lethal or sublethal *infections* with known or putative pathogens. Here, we briefly outline some of the more commonly employed assays that are discussed in later sections. It is worth noting, however, that in addition to these more traditional physiological assays of immune function, there is a growing trend for ecoimmunologists to measure both constitutive and induced immune responses via a variety of protein and gene expression assays (e.g. Freitag, Heckel, & Vogel, 2009; Freitag, Wheat, Heckel, & Vogel, 2007; Jackson et al., 2011). Readers are referred to the following reviews for a discussion of the pros and cons

of some of these assays and their interpretations: (Adamo, 2004b; Boughton, Joop, & Armitage, 2011; Graham et al., 2011; Norris & Evans, 2000).

2.6.1. Constitutively Expressed Defenses

Assays of immune defenses that are constitutively expressed (or “baseline” immune measures) may include behavioral, external, and internal defenses. For insects and other invertebrates, these typically include assays of cuticular melanization (see Wilson & Cotter, 2009 and references therein), hemolymph lysozyme activity (e.g. Adamo, 2004a; Anderson & Cook, 1979; Arce, Johnston, Smiseth, & Rozen, 2012; Cotter, Hails, et al., 2004; Freitag, Wheat, et al., 2007; Gershman, 2008; Lee, Simpson, & Wilson, 2008; Powning & Davidson, 1973), hemolymph PO activity (see Gonzalez-Santoyo & Cordoba-Aguilar, 2012 and references therein), nitric oxide (NO) activity (e.g. Cordoba-Aguilar, Jimenez-Cortes, & Lanz-Mendoza, 2009; Rivero, 2006), and hemocyte density (e.g. Miller & Simpson, 2010; Ryder & Siva-Jothy, 2000; Wilson, Knell, Boots, & Koch-Osborne, 2003).

Cuticular melanization has been measured both qualitatively by eye (Barnes & Siva-Jothy, 2000; Robb, Forbes, & Jamieson, 2003; Wilson, Cotter, Reeson, & Pell, 2001) and quantitatively using either a fiber-optic spectrometer for living insects (Cotter, Myatt, Benskin, & Wilson, 2008; Lee & Wilson, 2006) or digital image analysis of photographs for dead insects (Barnes & Siva-Jothy, 2000; Wilson et al., 2001). Lysozyme-like antibacterial activity is measured as the ability of lysozyme in the sample to break down bacterial cells. This can be measured using a lytic zone assay in a Petri dish, whereby samples are placed in holes cut into agar seeded with freeze-dried bacteria; the strength of the antibacterial response is then measured by the size of the clear zone of dead bacteria around each hole compared against a lysozyme standard (Kurtz & Sauer, 2001; Cotter, Hails, et al., 2004). Alternatively, lysozyme can be assayed with a turbidity assay in a plate reader, where samples are added to a liquid suspension of bacteria and the increase in light absorbance over time indicates the activity of lysozymes breaking down the bacterial cells (Adamo & Parsons, 2006; Steiger, Gershman, Pettinger, Eggert, & Sakaluk, 2011). Hemolymph PO activity is assayed spectrophotometrically, using a colorimetric method in which the hemolymph sample is mixed with one of the enzyme's substrates, such as L-Dopa or dopamine, and the amount of enzyme present determined by the level of melanization of the sample (Barnes & Siva-Jothy, 2000; Cotter, Beveridge, & Simmons, 2008; Reeson, Wilson, Gunn, Hails, & Goulson, 1998; Wilson et al., 2001). The amount of the enzyme's inactive precursor, proPO, can also be measured by

converting the proPO to PO beforehand using a detergent or some other activator, such as trypsin or chymotrypsin (e.g. Adamo, Jensen, & Younger, 2001; Freitag et al., 2009; Mydlarz & Palmer, 2011). Similarly, NO activity can be measured spectrophotometrically by mixing the hemolymph sample with sulfanilamide and naphthylethylenediamine and measuring the color change (Cordoba-Aguilar et al., 2009). Hemocyte density is measured by taking a known volume of hemolymph and counting the number of hemocytes either under a microscope and hemocytometer (Kurtz, 2002; Ryder & Siva-Jothy, 2000; Wilson, Knell, et al., 2003) or electronically using an automated cell counter (Randall, Tummala, & K, 2013).

In vertebrates, there are a number of assays for constitutively expressed immune defenses that have been modified for use in wild or captive animals from assays originally developed for humans, livestock or nonhuman model organisms. Those used by ecoimmunologists include various hematological measurements (Beldomenico et al., 2008; Nunn, Lindenfors, Pursall, & Rolff, 2009), and the hemolysis-hemagglutination assay (Matson, Ricklefs, & Klasing, 2005). Total and differential blood cell counts of vertebrates are usually done via microscopy with gridded slides or using an automated cell counter or flow cytometry (Beldomenico et al., 2008; De Boever et al., 2010; Ruiz, Rosenmann, Novoa, & Sabat, 2002). Low concentrations of red blood cells (erythrocytes) may be due to infection by some parasites; lymphocytes are the effectors of acquired immunity and low counts may indicate immunosuppression or low immune investment, whilst high counts may indicate parasitic infection; elevated neutrophil counts may be an indication of acute inflammatory response to cytokines released during tissue injury and bacterial infection; and high monocyte counts may reflect chronic inflammatory response caused by bacterial or protozoan infections (Beldomenico et al., 2008). The hemolysis-hemagglutination assay measures constitutive innate humoral immunity in vertebrates by quantifying natural antibody-mediated complement activation and red blood cell agglutination (Matson et al., 2005; Mauck, Matson, Philipsborn, & Ricklefs, 2005). Natural antibodies are unique among immunoglobulin molecules because their presence does not require previous exposure to a particular antigen, and a range of functions have been assigned to them, including directly controlling novel bacterial and viral disease challenges and regulating self-reactive B- and T-cells (Matson et al., 2005). The assay involves collecting a heparinized blood sample from the animal, which is mixed in a dilution series to a microtiter plate with a dilute allo-specific blood cell suspension (e.g. rabbit), then digitally scanned twice at intervals to quantify agglutination and lysis, respectively.

2.6.2. Induced Defenses

Induced defenses are any that are upregulated by the host in response to an immune insult. In invertebrates, this is generally quantified using assays measuring the production of AMPs (Haine, Moret, et al., 2008; Povey, Cotter, Simpson, Lee, & Wilson, 2009), hemocyte phagocytic activity (Kurtz, Nahif, & Sauer, 2000) and the encapsulation response (Gorman et al., 1996; Gotz, 1986). In addition, the upregulation of PO and lysozyme activity can also be measured after infection. Most AMPs are not constitutively expressed and so can only be measured after first challenging the invertebrate with a microbial (bacterial or fungal) infection either orally or via injection. After 6–96 h, when the AMPs have become upregulated (Fig. 3.1), a blood sample is taken from the animal and inserted into a small hole in a Petri dish containing a live test bacterium in semisolid agar; AMP activity is then measured as the zone of bacterial growth inhibition around the hemolymph sample (e.g. Povey et al., 2009). The phagocytic activity of hemocytes, i.e. their capacity to phagocytose small alien bodies such as dead bacteria, yeast cells, or even silica beads, can be measured both *in vivo* and *in vitro* (Ehlers, Zosel, Mohrig, Kauschke, & Ehlers, 1992; Kurtz et al., 2000). In the latter case, a monolayer of hemocytes is produced on a microscope slide and the cells incubated for several hours in Grace's medium containing small (5 μm diameter) silica beads or other microparticles to allow phagocytosis to proceed; by fluorescently labeling both hemocytes and silica beads, it is possible to determine the proportion of hemocytes that have successfully ingested the particles as a measure of phagocytic activity (Kurtz et al., 2000). Finally, the encapsulation response can be measured by inserting into the hemocoel of the live insect either multiple Sephadex beads (40–120 μm diameter) or a small piece (100–200 μm diameter; 1–2 mm length) of nylon filament. In both cases, the inserted object(s) provide a sufficiently large novel antigen to trigger an encapsulation response around them that comprises hemocytes and/or melanin; the area and darkness of the retrieved capsule can then be measured either visually or via image analysis software (Paskewitz & Riehle, 1994; Cotter & Wilson, 2002; König & Schmid-Hempel, 1995; Ryder, 2007).

The two most commonly employed assays used by ecoimmunologists to test for induced responses in vertebrates are arguably the general inflammation response to a mitogen stimulant (PHA test) and the antibody production response to an antigen challenge. In the former assay, a mitogen stimulant, such as phytohemagglutinin (PHA), is injected into the skin of the animal (such as the wing web of birds) and immune inflammation activity

is measured indirectly as the magnitude of the swelling response several days later using a spessimeter compared to saline-injected controls (Martin et al., 2006; Verhulst, Riedstra, & Wiersma, 2005). In the latter assay, a novel antigen, such as keyhole limpet hemocyanin or sheep red blood cells, is injected into the animal and antibody production is measured several days later via hemagglutination to test for the development of specific, inducible humoral immunity compared to saline controls (Adriaansen-Tennekes, Reilingh, Nieuwland, Parmentier, & Savelkoul, 2009; Verhulst et al., 2005).

2.6.3. Disease Resistance Measures

A third key type of assay used by ecoimmunologists is to challenge a host animal with a lethal or sublethal dose of a parasite and to record its response in terms of specific behavioral or life-history responses, including growth, fecundity, and survival. Indeed, some authors have questioned the relevance of immune function assays in isolation of these disease resistance assays when hosts are challenged with live pathogenic parasites (Adamo, 2004b; Nieman & Pedersen, 1999). These assays typically include an LD₅₀ test in which the animals are given an oral or injected dose of the lethal parasite that would be expected to kill about 50% of those inoculated, and then subsequent mortality across a range of environments tested. Alternatively, a lower parasite dose (<LD₁₀) may be used to minimize parasite-induced mortality so as to better quantify the effects of infection on subsequent behaviors (e.g. anorexia, self-grooming, mating behavior, or self-medication), or effects on growth and fecundity (e.g. Boots & Begon, 1993; Little & Killick, 2007; Povey et al., 2009). In some studies, the pathogen load or the rate at which the infection is cleared is also measured (Ayres & Schneider, 2009; Graham et al., 2012; Hill & Farmer, 2005; McKean & Nunney, 2001).

2.7. Counting the Costs of Defense

A fundamental assumption of life-history theory as applied to the ecological immunology approach is that antiparasite defenses are costly, such that they may reduce the expression of another component of the host's fitness. This will result in tradeoffs between different fitness- and immune-related components, leading to variation between individuals and genotypes in how much they invest in immunity and other traits. The costs of immune defense can be classified in a number of ways. Schmid-Hempel (2011) argues for a classification according to the cause (evolutionary costs, maintenance costs, and deployment costs) and their implementation (genetic vs physical mechanisms) (Figure 3.2).

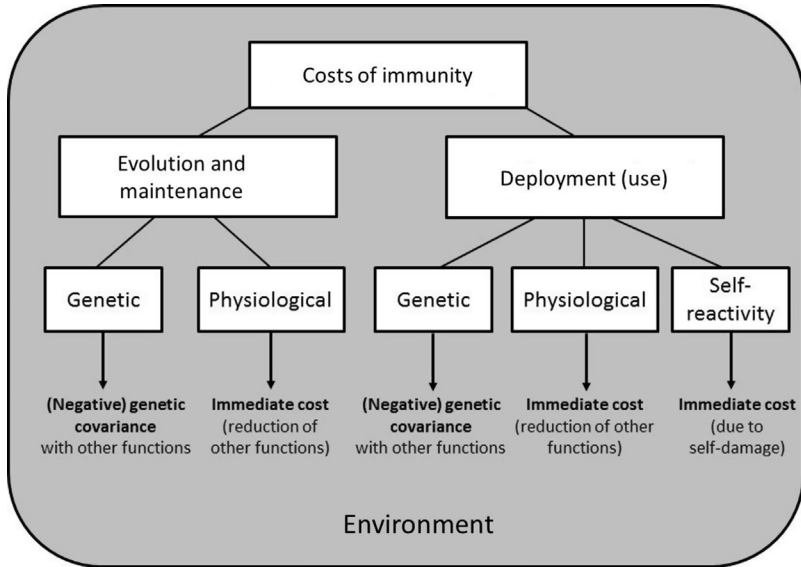


Figure 3.2 Costs of immune defences. Costs can be classified according to cause (evolution, maintenance, deployment) or according to implementation (genetic or physiological). (Adapted after Schmid-Hempel (2011)).

2.7.1. Evolutionary and Maintenance Costs

Parasite load often has a significant heritable component (Bishop, Jackson, Coop, & Stear, 2004; Smith, Wilson, Pilkington, & Pemberton, 1999; Uller, Olsson, & Madsen, 2003), which suggests that at least some of the parasite resistance mechanisms, including immunity, also exhibit heritable variation (Coltman, Wilson, Pilkington, Stear, & Pemberton, 2001; Little & Ebert, 2000; Raberg, Stjernman, & Hasselquist, 2003). The mechanisms maintaining this genetic variation are often unclear, but one pervasive element is due to genetic correlations between traits and evolutionary tradeoffs (Bishop et al., 2004; Coltman, Pilkington, Kruuk, Wilson, & Pemberton, 2001; Cotter, Hails, et al., 2004).

Evolutionary costs of immunity can be uncovered in two main ways. The first is by examining the underlying genetic architecture of a suite of traits using quantitative genetics methods. For example, Simmons and Roberts (2005) used a standard half-sib breeding design to explore the tradeoffs between immunity and reproduction in the field cricket, *Teleogryllus oceanicus*. They found that sperm viability and hemolymph lysozyme activity were negatively genetically correlated, consistent with evolutionary reproductive costs of immunity. Thus, genotypes that invested in sperm viability necessarily had reduced antibacterial capacity.

A second approach to uncovering evolutionary costs of antiparasite defenses is by selecting for or against relevant immune traits and looking for corresponding changes in other traits. Conversely, costs of immunity can be established by selecting for or against other fitness-related traits and looking for corresponding changes in immune traits. Hosken (2001) took the latter approach when investigating the costs of enforced polyandry in dung flies, *Scathophaga stercoraria*. After 12 generations of selection, polyandrous lines had invested more heavily in sexual characters: males had increased testes size and females had larger accessory glands (Hosken, Garner, & Ward, 2001; Hosken & Ward, 2001), but they had lower levels of PO activity in their hemolymph, suggesting a cost to immune investment (Hosken, 2001). A complementary approach that selected for PO levels in dung flies before measuring reproductive investment found different results (Schwarzenbach & Ward, 2006). Here, it was shown that individuals from high PO lines were more fecund, at least in their first clutch, but that longevity under starvation was compromised (Schwarzenbach & Ward, 2006). Thus, both studies uncovered costs to immune investment, but the first was driven by investment in reproduction and the second by investment in longevity. The difference in the results from the two experiments may reflect the fact that multiple mating, which occurred only in the first experiment, places different selection pressures on individuals, thus altering their resource allocation strategies (Schwarzenbach & Ward, 2006).

That there are costs associated with having the immune system in a state of readiness can be inferred from observations that many organisms prophylactically increase their investment in this “standing army” when the risks of parasitism are high, and reduce it again when those risks decrease. For example, a number of studies have shown that constitutive immune function is upregulated in many species when population density increases (e.g. Barnes & Siva-Jothy, 2000; Reeson et al., 1998; see Section 3.2 for details). Similarly, at the start of winter, it has been shown that immune function is upregulated in rodents (Nelson & Demas, 1996). *Maintenance costs* can also be inferred from studies that have examined immune capability in organisms engaged in other costly activities. For example, in the wolf spider, *Hygolycosa rubrofasciata*, males “drum” to attract females, an energetically costly activity, and these drumming males show reduced lytic activity when presented with females to court (Ahtiainen, Alatalo, Kortet, & Rantala, 2005). Similarly, in humans, it appears that immune function is downregulated in high-performance athletes relative to nonathletes (Kumae, Kurakake, Machida, & Sugawar, 1994).

2.7.2. Deployment Costs

In addition to the evolutionary and maintenance costs of immunity, there can also be costs associated with using the immune system. Deployment costs of immunity can be uncovered by looking for negative correlations, genetic or phenotypic, between the expression of induced immune responses to artificial or live parasites and other life-history traits, such as reproductive effort. However, phenotypic correlations can vary from positive to negative depending on their relationship with other traits, their condition-dependence and the age or state of the individual at the time of testing (Cotter, Ward, et al., 2011; Westneat & Birkhead, 1998). For example, in the lizard, *Zootoca vivipara*, polyandrous females have a *stronger* inflammatory response to PHA challenge than monandrous females despite having higher reproductive costs (Richard, Massot, Clobert, & Meylan, 2012). Thus, to uncover deployment (usage) costs of immunity, it is often necessary to force individuals to over-invest in one trait (e.g. immunity) and measure changes in another (e.g. reproduction).

For example, Jacot, Scheuber, and Brinkhof (2004) activated the immune system of male field crickets, *Gryllus campestris*, by injecting them with LPS and found that this decreased their daily calling-rate relative to control males. Immune activation also reduced lifespan, suggesting that the reduction in calling-rate was not sufficient to offset the costs of the immune response. Similar results have been reported in vertebrates. For example, in the great tit, *Parus major*, immune activation by LPS results in a reduction in sperm swimming velocity (Losdat, Richner, Blount, & Helfenstein, 2011).

Costs of immunity may also be inferred from studies that have forced over-investment in a costly trait, such as reproduction, and then measured the deployed immune response. For example, in collared flycatchers (*Ficedula albicollis*), an experimental increase in the number of chicks in the nest resulted in a decline in several serological parameters indicative of immune function (Gustafsson, Nordling, Andersson, Sheldon, & Qvarnstrom, 1994). Similarly, McKean and Nunnery (2001) provided male fruit flies, *Drosophila melanogaster*, with different numbers of mates and found that as the number of females increased, so the ability of males to clear bacteria from their hemolymph decreased, suggesting that mating is costly in terms of the deployed immune response.

The preceding sections outline the wide range of defense mechanisms potentially at the disposal of animals living in a parasitic world, as well as some of the epidemiological factors that contribute to variation in the risk of becoming parasitized. It also provides an introduction to some of the

tools available to ecoimmunologists to tease apart variation in the evolution, maintenance, and deployment of those defenses, and experimental approaches for determining the constraints underpinning this variation. In the following sections, we use this framework to understand three areas of ecology that have benefitted from this ecological immunology methodology. Specifically, we focus on the consequences of living in crowds (Section 3); the interaction between nutrition and disease ecology (Section 4); and social variation in antiparasite defenses (Section 5).



3. POPULATION DENSITY, GROUP-LIVING AND PARASITE DEFENSE

3.1. Fluctuating Populations

Most pest species, especially insect pests, tend to have high reproductive rates and short generation times, and under favorable environmental conditions this leads to a high intrinsic rate of population growth, r (Barbosa, Letourneau, & Agrawal, 2012). As a consequence, many pest species exhibit sporadic high-density population outbreaks or eruptions that may cause serious economic damage if they occur on food crops, feed stores, or in other managed environments. During periods of high population density, the biotic and abiotic environment may be very different from that experienced when population densities are low. As a result, many species of insects have evolved an adaptive response to crowding known as *density-dependent phase polyphenism*, a form of phenotypic plasticity in which a single genotype may exhibit different phenotypes depending on the degree of crowding experienced (Pener & Simpson, 2009). Specifically, tactile, visual, and/or olfactory cues associated with local population density may trigger in the insect a suite of developmental changes that alter its metabolism, behavior, color, and morphology, some of which may occur within a matter of hours of switching (Simpson, Despland, Hagele, & Dodgson, 2001). Population density not only affects the quality and quantity of food available to individuals, with knock-on effects for fueling the immune system and resisting disease (see Section 4), but may also influence a range of other factors, including the individual risk of predation and parasitism.

3.2. Density-Dependent Prophylaxis

As discussed earlier (Section 2.2), disease transmission is often assumed to be positively density-dependent, such that as population density (and the

degree of intraspecific crowding) increases, so contact between infectious and susceptible individuals increases, leading to a predicted increase in the *per capita* risk of infection (Anderson & May, 1981; McCallum et al., 2001). The DDP hypothesis argues that since disease resistance mechanisms are costly to maintain (e.g. Wilson, 2005), in order to counter this density-dependent increase in infection risk, individuals should tailor their investment in prophylactic disease resistance mechanisms to meet this predictable threat (Wilson & Reeson, 1998). In other words, it predicts that as population density increases, so organisms will increase their investment in immunological, behavioral, chemical, and/or physical disease resistance mechanisms, and that this will result in a positive relationship between population density and *per capita* (dose-dependent) parasite resistance (reviewed by Wilson & Cotter, 2009).

The first direct test of the DDP hypothesis was conducted using larvae of the African armyworm, *Spodoptera exempta*, an important lepidopteran pest of staple crops such as maize, wheat, sorghum, rice, and pasture grasses in sub-Saharan Africa (Reeson et al., 1998, 2000), but see also (Kunimi & Yamada, 1990). Larval densities of this moth may exceed 1000 caterpillars per m² (Fig. 3.3a), though this varies considerably both spatially and temporally, and densities within outbreaks of around 100 per m² are more common (Graham et al., 2012; Rose, Dewhurst, & Page, 2000). In response to this unpredictable variation in population density, the African armyworm, just like the infamous desert locust, has evolved a form of density-dependent phase polyphenism, with extreme phenotypes adapted to both high- and low-density conditions (Section 3.3). Reeson et al. (1998) experimentally tested the DDP hypothesis in the laboratory by rearing *S. exempta* larvae under either high- or low-density conditions for three larval instars and then orally challenged early fourth-instar larvae with one of a range of doses of *S. exempta* nucleopolyhedrovirus (SpexNPV), a host-specific baculovirus of armyworms that is assumed to be transmitted between larvae in a density-dependent manner (Grzywacz, Mushobozi, Parnell, Jolliffe, & Wilson, 2008). As predicted, they found that the LD₅₀ (i.e. the viral dose required to kill 50% of larvae orally inoculated) was much higher for larvae that had been crowded prior to viral inoculation than for those that had been reared solitarily: c. 14,000 viral occlusion bodies per larva for crowd-reared larvae vs c. 1600 for solitary-reared larvae; (see Section 3.3). Lethally-infected larvae that had been raised in crowds also took significantly longer to die of infection, suggesting a more resistant phenotype (Reeson et al., 1998).

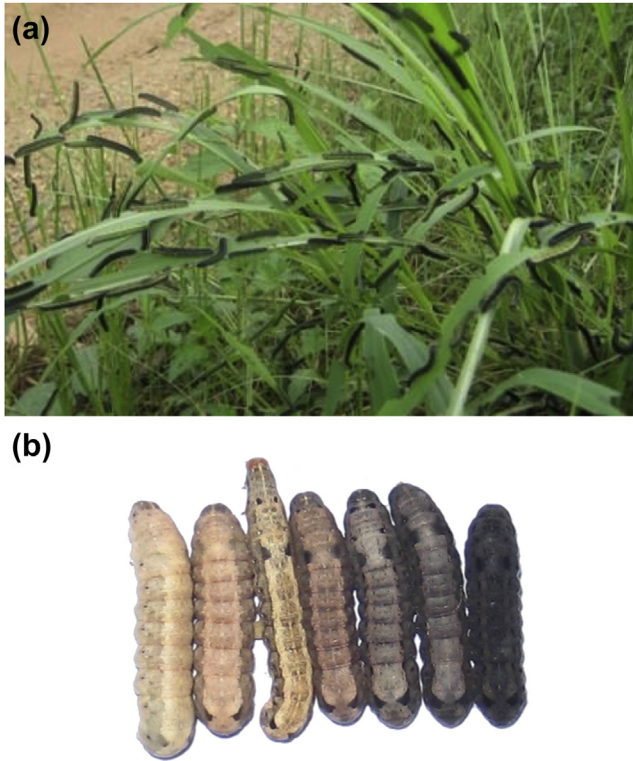


Figure 3.3 Melanization of *Spodoptera* caterpillars in response to crowding. (a) A high-density aggregation of *S. exempta* larvae on *Cynodon* grasses (photo courtesy of W.L. Mushobozi). (b) The gradation in melanization occurring in *S. littoralis* larvae. In both species, pale larvae are more likely to occur at low population densities and dark larvae at high population densities. (For color version of this figure, the reader is referred to the online version of this book.)

In a follow-up study, Reeson et al. (2000) showed that this phenomenon may also be important in the field. Larvae were again reared under solitary or crowded conditions in the laboratory until the start of the fourth instar, at which point they were moved into 1 m³ mesh cages in the field, into which had been placed nine small maize plants, each hosting two *S. exempta* larvae that had recently died of SpexNPV. Thus, the live larvae were free to move about the cages and become orally infected naturally by encountering virus-killed cadavers. Live larvae were placed in the cages at one of three densities (one, three or nine larvae per plant) and the retrieved three or five days later to determine their infection status back in the lab. Using this approach, it was possible to determine the relative importance of both the

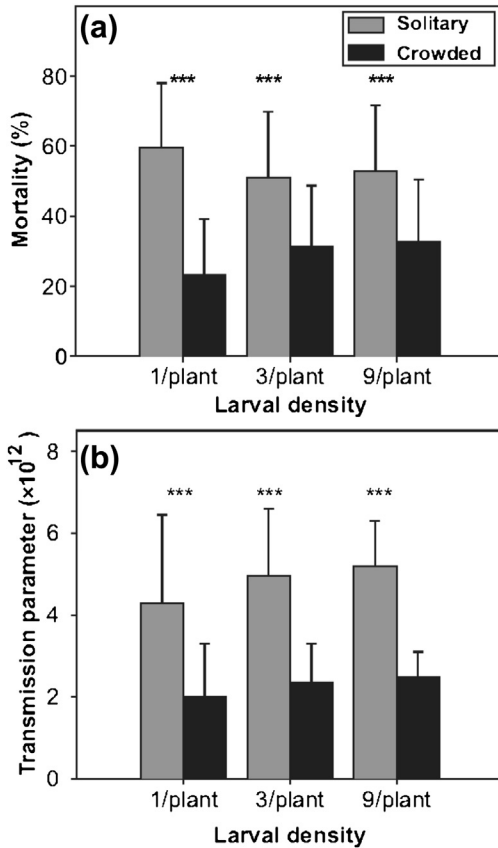


Figure 3.4 Relationship between larval density and (a) average mortality and (b) the transmission parameter, for larvae of the African armyworm, *Spodoptera exempta*, exposed to its nucleopolydovirus (NPV) under field conditions. Mortality and viral transmission were greatest for larvae reared under solitary, rather than crowded conditions until the start of the fourth instar, but did not vary significantly with the density of larvae during the few days that the larvae were exposed to the virus. (Reproduced from Reeson et al. (2000).). Means \pm s.e. are shown.

larval-rearing density prior to the expression of phase polyphenism (up to fourth instar) and the local density of larvae per cage during the short infection process (the first 3–5 days after the start of the fourth instar). The results showed quite clearly that although the density of live larvae per plant had a negligible effect on virus-induced mortality and virus transmission, both metrics were significantly higher among larvae reared in isolation prior to being moved outside than among those raised in crowds, so providing further support for the DDP hypothesis (Fig. 3.4). This is despite that fact that

gregaria phase larvae are much more mobile than their *solitaria* counterparts and so presumably are more likely to encounter viral cadavers (Reeson et al., 2000). Of course, in the field, whether or not we see a positive, negative, or neutral relationship between population density and disease prevalence will depend on the relative magnitudes of density-dependent risk of exposure to disease (due to density-dependent transmission processes) and DDP (limiting susceptibility to a given level of exposure) (Wilson & Cotter, 2009). An analysis of SpexNPV disease in natural field populations of *S. exempta* indicated a negative relationship between larval density and viral load, consistent with the DDP response being sufficiently strong to overcome the (putative) density-dependent transmission process (Graham et al., 2012).

Studies on *S. exempta*, and the closely related Egyptian cotton leafworm, *S. littoralis*, have explored the effects of rearing density on a suite of immune traits in order to establish whether all immunological effectors are upregulated to the same degree in response to larval crowding. In *S. exempta*, an increase in larval-rearing density was associated with an increase in the activity levels of PO in the hemolymph, midgut, and cuticle (Reeson et al., 1998; Wilson et al., 2001). Since all three of these tissues are sites for parasite invasion (Section 2.4), and PO is a key component of the melanization and encapsulation cascade, it seems likely that this upregulation of PO is a coordinated response to resist parasites that invade their hosts via the cuticle and orally, including viruses, bacteria, fungi, and parasitoids. In *S. littoralis*, it was established that despite food being provided to insects ad libitum, variation in larval density was reflected in significant variation in body condition, as measured by larval weight and levels of protein in the hemolymph. Thus, solitary larvae tended to be heavier than their gregarious counterparts and also had higher levels of hemolymph protein, though it is unclear whether these differences are an adaptive response to crowding, possibly related to DDP, or simply a (nonadaptive or neutral) bi-product of intraspecific competition. Regardless, for most immune traits, the effects of rearing density outweighed these condition-dependent effects. Crowded *S. littoralis* larvae had significantly higher levels of PO activity in their cuticles, but levels of PO in the hemolymph were not significantly higher and neither was hemocyte density (Cotter, Hails, et al., 2004; Wilson & Cotter, 2009). However, contrary to the DDP hypothesis, crowded larvae also had lower levels of melanotic encapsulation of an artificial parasite (nylon monofilament) and lower levels of lysozyme-like antibacterial activity (Cotter, Hails, et al., 2004; Fig. 3.5). This suggests that there is not a uniform upregulation of all constitutive immune traits in response to crowding and that there are potential tradeoffs between immune traits.

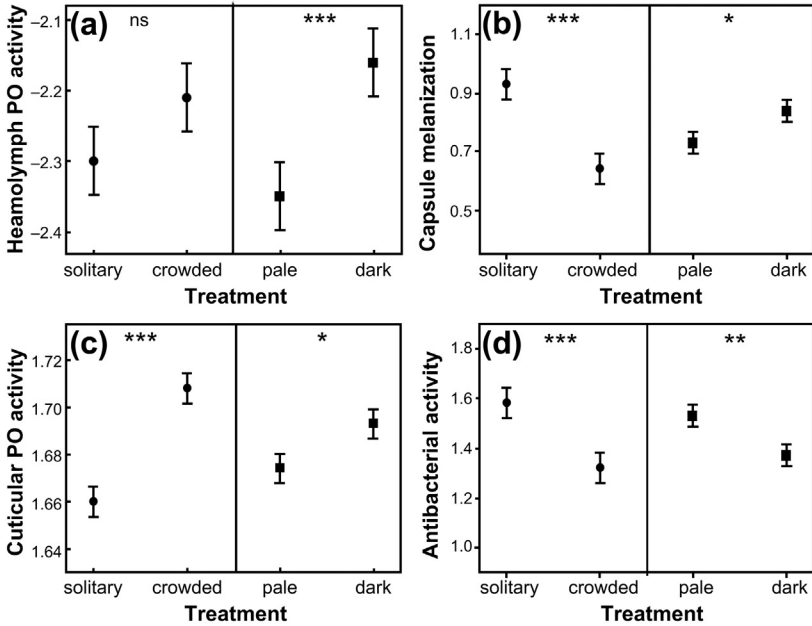


Figure 3.5 Effect of color and larval density on immune function parameters in larvae of the Egyptian cotton leafworm, *Spodoptera littoralis*. Larvae were reared in solitary or crowded conditions and scored for color: pale or dark. The figures show mean (\pm s.e.) levels of investment in four different immune parameters: (a) hemolymph phenoloxidase activity, (b) cuticular phenoloxidase activity, (c) capsule melanization of a foreign implant, and (d) lysozyme-like antibacterial activity. (Reproduced from Cotter, Hails, et al. (2004)).

The notion of tradeoffs was explored further in a quantitative genetic analysis of immune and life-history traits in *S. littoralis* (Cotter, Kruuk, et al., 2004). This revealed that all of the traits measured were heritable, with narrow-sense heritability estimates (h^2) for immune traits in the range $h^2 = 0.36$ – 0.65 , and for life-history traits in the range $h^2 = 0.20$ – 0.85 . As expected, there were significant positive and negative genetic correlations (r_A) between life-history traits and immune traits. For example, lysozyme-like antibacterial activity was positively genetically correlated with pupal development rate ($r_A = 0.35$), but negatively genetically correlated with larval development rate ($r_A = -0.29$). Importantly, there were also significant genetic correlations among the four immune traits examined. For example, there were significant positive genetic correlations between hemocyte density and both hemolymph PO activity ($r_A = 0.21$) and the degree of cuticular melanization ($r_A = 0.55$). In contrast, the genetic

correlation between hemocyte density and antibacterial activity was significantly *negative* ($r_A = -0.23$), indicative of a possible genetic tradeoff between these two traits. Subsequent studies in a range of other organisms have revealed similar negative correlations among immune traits, suggesting physiological and/or genetic tradeoffs within the immune system (Fedorka, Zuk, & Mousseau, 2004; Freitak, Heckel, & Vogel, 2007; Povey et al., 2009; Rantala & Roff, 2005; Simmons & Roberts, 2005); but see (Lambrechts, Vulule, & Koella, 2004).

It is now more than a decade since the DDP hypothesis was first proposed and the evidence to date is generally supportive from across a range of insect taxa, including Lepidoptera, Orthoptera, Coleoptera, Hymenoptera, and Isoptera (see Table 2 in Wilson & Cotter, 2009), as well as from a number of other non-arthropod taxa, such as the coral-eating crown-of-thorns sea star, *Acanthaster planci* (Mills, 2012) and a range of breeding bird species in Europe (Moller, Martin-Vivaldi, Merino, & Soler, 2006). However, there are a number of notable exceptions to these general trends. For example, Miller and Simpson (2010) conducted a field test of the DDP hypothesis using the Australian plague locust, *Chortoicetes terminifera*, and found that total hemocyte counts (THCs) were *negatively* correlated with field population densities. Moreover, when locusts were isolated from marching bands of conspecifics, their THC *increased* relative to the group-housed controls, contrary to predictions of the DDP hypothesis. Miller and Simpson suggest that their counter-intuitive results may be explained as a response by isolated locusts to increase their hemocyte densities to counter potentially greater exposure to parasitoids and nematodes. In another study, Triggs and Knell (2012) showed that whether positive or negative DDP is observed may depend critically on other environmental conditions, such as food availability and temperature. Using the Indian meal moth, *Plodia interpunctella*, they showed that when the food provided to the larvae was of good quality (high levels of yeast and glycerol), the larvae showed the predicted positive density-dependent prophylactic response, increasing their investment in immunity when population density was high. However, when food quality was poor and temperature low, larvae that had been reared at high densities invested relatively less in immunity, reversing the previous effect of population density. The role of nutrition in immune function and disease resistance is discussed in Section 4.

It should be noted that DDP will not apply to all diseases; indeed it is predicted only for those that are generally transmitted in a positively density-dependent manner. Also, it is not expressed in all immune traits;

again, this is to be expected, not least because of intrinsic tradeoffs between different immune traits, as discussed above (Cotter, Kruuk, et al., 2004; Cotter, Simpson, et al., 2011). It should also be emphasized that the DDP hypothesis applies not only to immune-related traits, but also to physical, chemical, structural, behavioral, and other traits that may limit the impact of infectious disease (Owens & Wilson, 1999; Wilson & Reeson, 1998). For example, Elliot and Hart (2010) have argued that a greater emphasis should be placed on prophylactic *behavioral* responses that limit the potential for infections to spread, especially for social species (see below).

Since, by definition, DDP responses vary in a density-dependent manner, they have the potential to impact on the dynamics of the host–pathogen interaction at a population scale (Wilson & Cotter, 2009). There have been several modeling studies, using a range of approaches, that have examined the consequences of DDP for host–parasite dynamics. White and Wilson (1999) used a discrete-time host–pathogen model, representing nonoverlapping insect generations, and found that if the effect of DDP was sufficiently small, it *stabilized* the host–pathogen dynamics. In contrast, Reilly and Hajek (2008) developed a continuous-time model for the host and pathogen within a season and a discrete-time map between seasons, and found that DDP had a *destabilizing* effect on the population dynamics. Reynolds, White, Sherratt, and Boots (2011) used a more general continuous-time model framework and found that the ability of DDP to drive population cycles was critically dependent on the time-delay between the change in population density and the subsequent phenotypic change in the level of resistance: when the time-delay was short or absent, DDP *destabilized* the system, but as the delay increased its destabilizing effect reduced and then became increasingly *stabilizing*. Thus, it appears that the dynamical consequences of DDP is not easily predicted and probably depends on specific details of the host–pathogen system.

3.3. Melanism

One of the clearest manifestations of phase transformation in many polyphenic insects, such as armyworms and locusts, is a change in color. For example, in the desert locust *S. gregaria*, nymphs of the low-density *solitaria* morph are green and cryptically colored, whereas those of the high-density *gregaria* morph are conspicuous yellow-and-black. In African armyworm, *S. exempta*, larvae reared at low population densities are usually green or pale brown, whereas those reared at high densities are invariably jet black (Wilson et al., 2001). In *S. littoralis* larvae, the color variation between

low- and high-density phenotypes is more gradual (Fig. 3.3b), but in each of these cases, and for other lepidopteran insects in particular, the darkening of the cuticle is mainly due to the deposition of the pigment melanin (Lee & Wilson, 2006; Tawfik et al., 1999; Wilson et al., 2001).

The degree of cuticular melanization can be viewed as a highly plastic trait. In *Spodoptera* caterpillars, the degree of cuticular melanism has a significant genetic component (Cotter, Kruuk, et al., 2004; Lee & Wilson, 2006; Cotter, Myatt, et al., 2008), with a heritability of around $h^2 = 0.18\text{--}0.36$, but the predisposition to respond to crowding by melanizing the cuticle (i.e. the phase polyphenism trait) is also genetically regulated and responds to selection (Cotter, Myatt, et al., 2008). However, cuticular melanism also varies in response to diet, with cuticles being more melanized on diets containing high levels of protein (Cotter, Simpson, et al., 2011), and proteins of high quality (Lee et al., 2008). Thus, cuticular melanization may also be viewed as a condition-dependent trait. Moreover, although the amount of melanin in the cuticle is fixed at each molt, in Lepidoptera and other taxa in which the insect's exoskeleton expands within each larval stage as it grows, the perceived darkness of the cuticle varies depending on time since molting, with it appearing darker immediately postmolt and gradually becoming paler as the insect grows and the cuticle stretches (Lee & Wilson, 2006), analogous to the change in the color of a balloon as it is inflated. This may have implications for predators or parasitoids that hunt for insects based on their colors or darkness (see below).

It has been proposed that melanization of the cuticle could be an adaptive response to enhance resistance to parasites and pathogens (Wilson et al., 2001; see Section 2.4). The predicted functional association between melanism, PO activity, and parasite resistance has now been tested in a range of insect species. As indicated above, in general, PO activity and the degree of cuticular melanism tend to increase with increasing population density. Therefore, a robust test of the association between melanism and disease resistance must control for the confounding influence of density. In the African armyworm, *S. exempta*, around 15% of larvae reared under low-density conditions express a high-density, melanic phenotype (Reeson et al., 1998), reflecting in part the underlying genetic variation in expression of phase polyphenism (Cotter, Kruuk, et al., 2004; Cotter, Myatt, et al., 2008). When solitary-reared larvae were challenged with SpexNPV, resistance was higher in melanic, solitary-reared larvae than in nonmelanic larvae (average mortality over a range of viral doses = 59% in melanic larvae, 70% in nonmelanic; LD₅₀ melanic = 3082 OB/larva,

LD₅₀ nonmelanic = 1325 OB/larva). Moreover, as predicted by the DDP hypothesis, resistance was highest in (melanic) crowd-reared larvae (average mortality = 42%; LD₅₀ = 14,188 OB/larva; Fig. 3.6a; Reeson et al., 1998). Thus, the typical nonmelanic solitary form of *S. exempta* was approximately twice as susceptible to SpexNPV virus as the atypical melanic solitary form, and 10 times more susceptible than the typical melanic gregarious form. The fact that this trend was mirrored by a gradual increase in hemolymph PO activity across these three phenotypes suggests a possible causal relationship (Figure 3.6b).

Similar correlations between melanism and parasite resistance were observed when this species was challenged with the ectoparasitoid

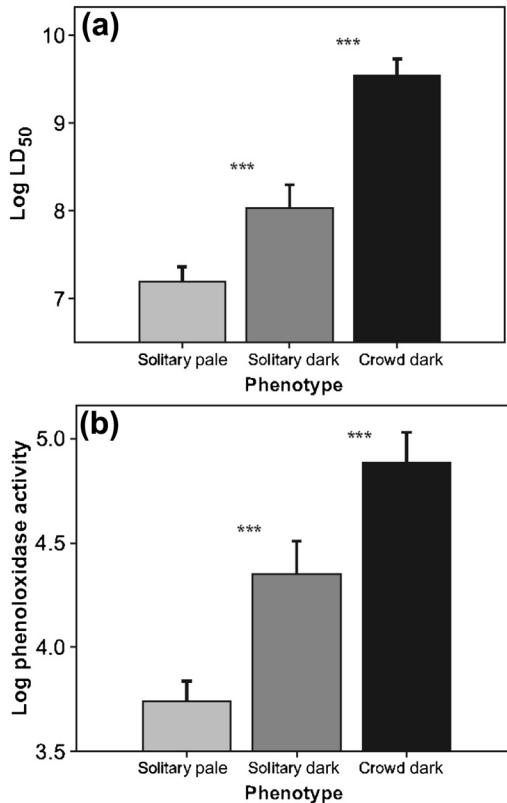


Figure 3.6 The relationship between larval density/phase and (a) resistance to a *Spodoptera exempta* nucleopolyhedrovirus (SpexNPV), measured as the dose of virus required to kill 50% of the larvae, LD₅₀ (Reeson et al., 1998), (b) immune function measured as hemolymph phenoloxidase activity (Wilson et al., 2001). Bars show means \pm s.e. (Reproduced from Wilson and Cotter (2009)).

Euplectrus laphygmae, with the proportion of parasitoid eggs that were melanized by the host caterpillar (mediated via the proPO cascade) increasing as the degree of host cuticular melanism increased (Wilson et al., 2001; Fig. 3.7). When the larvae were challenged with an entomopathogenic fungus, *B. bassiana*, there was no effect of cuticle color on fungus-induced mortality and an inconsistent effect of larval-rearing density. However, in the closely related species, *S. littoralis*, larvae with melanic cuticles, and those reared at high-density, were consistently more resistant to the fungus than those that were reared at low density and/or were nonmelanic, and the effects of rearing density and cuticle color on resistance were additive (Wilson et al., 2001).

The experiment by Wilson et al. (2001), demonstrating that melanic larvae may be more resistant to parasitoids (Fig. 3.7), probably mediated by the functional link between cuticular melanism and PO activity in the cuticle and hemolymph, leads to the intriguing possibility that cuticular melanism could act as an honest signal to foraging parasitoids of a host that has high PO activity and a tough cuticle, and hence one that should

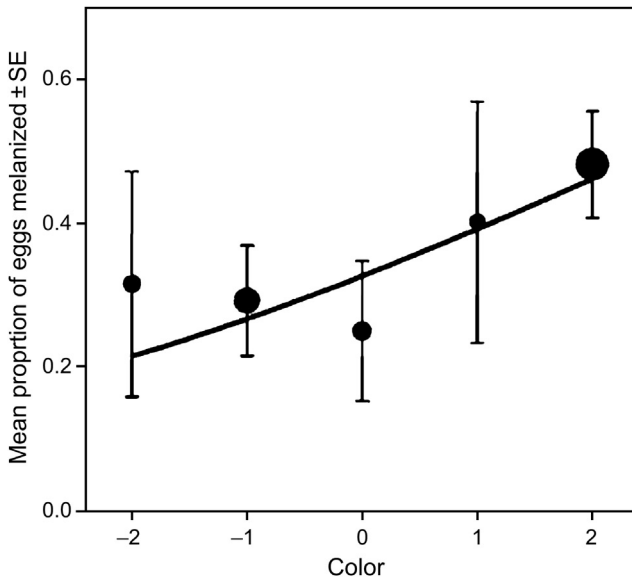


Figure 3.7 Relationship between cuticular melanization and resistance to the ectoparasitoid *Euplectrus laphygmae*. The vertical axis shows the proportion of melanized eggs (\pm s.e.) as a function of degree of cuticular melanization, scored on a scale from ± 2 (very pale) to $+2$ (very dark). Symbol size reflects sample size. The line is the fitted logistic regression to the raw data. (Reproduced from Wilson et al. (2001)).

be avoided. In support of this notion, there is evidence that parasitoids can distinguish between different color morphs of aphids (e.g. Ankersmit, Acreman, & Dijkman, 1981; Ankersmit et al., 1986). Moreover, Verhoog, Boven, and Brakefield (1996) noted that the parasitoid wasp *Venturia canescens* was “not eager” to parasitize a melanic strain of the Mediterranean meal moth, *Ephestia kuehniella*, and its ovipositor “sometimes appeared to become stuck in the cuticle,” suggesting that the melanized cuticle might present a tougher physical barrier. As far as we are aware, this prediction has not yet been experimentally tested, though Hagen, Sorlibraten, Ims, and Yoccoz (2006) found that in larvae of the winter moth, *Operophtera brumata*, there was a *positive* relationship between cuticular melanism and the incidence of parasitoid attack, contrary to expectation.

In some circumstances, melanism may aid crypsis, a classic example being the rise of the melanic form of the peppered moth, *Biston betularia*, during the industrial revolution (Cook, Grant, Saccheri, & Mallet, 2012; Kettlewell, 1973). It has also been shown that melanic forms of the pygmy grasshopper, *Tetrix subulata*, suffer reduced predation risk in postfire environments (Karpestam, Wennersten, & Forsman, 2012). Under these circumstances, selection by predators and parasites will reinforce each other and favor melanism. Alternatively, melanism may reduce crypsis and increase conspicuousness to visually hunting predators and parasitoids, leading to a potential tradeoff. This may help to explain the findings of Hagen et al. (2006), above, with the increased conspicuousness imposed by melanism offsetting the increased parasite resistance. Although melanism may often be costly in terms of increased conspicuousness to natural enemies, when it is manifested in response to increased population density, these costs may be greatly reduced by the dilution effects associated with being in a crowd (Wilson, 2000). Thus, whether or not melanism is beneficial will depend on the relative balance between these two selection pressures. However, the balance may be shifted in favor of melanism (and increased conspicuousness) if this is also associated with an increase in unpalatability to predators, such that melanism becomes an aposematic signal (Wilson, 2000). An example of this is seen in nymphs of the locusts, *Schistocerca emarginata* and *S. gregaria*, which preferentially feed on plant species that confer on them unpalatability to lizard predators when they switch into the yellow-and-black morph typical of high-density populations (Sword, 1999, 2000). A similar phenomenon may also occur in the African armyworm, *S. exempta*, which prefers to feed on cyanogenic grasses belonging to the genus *Cynodon* when in high-density aggregations and expressing the melanic phenotype (Wilson, 2000).

Overall, the evidence across a broad range of different insect species, including Lepidoptera, Coleoptera, Odonata, and Orthoptera overwhelmingly support a positive relationship between cuticular melanization and both immune function and disease resistance (see Shlichta & Smilanich, 2012 for a recent review). However, as the previous examples illustrate, the benefits of melanism for increased immune function may be traded-off against other functions and selection pressures. Amongst those studies that have controlled for the potentially confounding effects of rearing density, most also reveal a positive association between these traits (e.g. Barnes & Siva-Jothy, 2000; Kunimi & Yamada, 1990; Mitsui & Kunimi, 1988). However, there are notable exceptions to these general patterns. For example, Goulson and Cory (1995) found that when tested at fifth instar, melanic *Mamestra brassicae* larvae were significantly more susceptible to nucleopolydnavirus (NPV) than nonmelanics (though not when tested at fourth instar), and Robb et al. (2003) observed that darker individuals of the polymorphic mountain stone weta, *Hemideina maori*, showed lower encapsulation rates and melanization than paler individuals. In the latter case, this may be because *H. maori* is a *genetically polymorphic* species (as opposed to a *polyphenic* species), and so does not alter its phenotype in response to population density, suggesting that the benefits of cuticular melanism may be restricted to phase-polyphenic species.

3.4. Group-Living and Immune Defense

Since most infectious diseases are expected to be transmitted in a density-dependent manner, a logical extrapolation of the DDP hypothesis outlined above (Section 3.2) is that we might expect group-living species to invest more in prophylactic disease resistance than solitary-living species. This is because group-living species will typically experience higher local densities than solitary-living ones and so experience a higher *per capita* risk of infection from conspecifics. It has long been assumed that increased parasitism is a cost of group-living, and hence that group-living species should invest more in antiparasite defenses, but there is a shortage of good evidence to support this assumption from either vertebrate or invertebrate systems (Freeland, 1979; Coté and Poulin, 1995; Hochberg 1991; but see Spottiswoode, 2008; for a comparative study of PHA responsiveness in pair-breeding vs cooperatively breeding African bird species).

To test this hypothesis experimentally, Wilson, Knell, et al. (2003) measured several aspects of immune function in the larvae of 12 species

of Lepidoptera grouped into six phylogenetically matched pairs. The larvae were reared on the same diet and in the same environmental conditions assayed at similar times in order to reduce as far as possible any other confounding variables. They found that, although there was considerable variation across species in the density of hemocytes in the hemolymph (i.e. THC), across the six species-pairs, the THC of the solitary species was 40% higher, on average, than that of the gregarious species. Moreover, all six solitary species had THC estimates that were higher than their paired gregarious species (Fig. 3.8a), reflecting a stronger encapsulation response (Fig. 3.8b). Similar results were apparent in the levels of PO

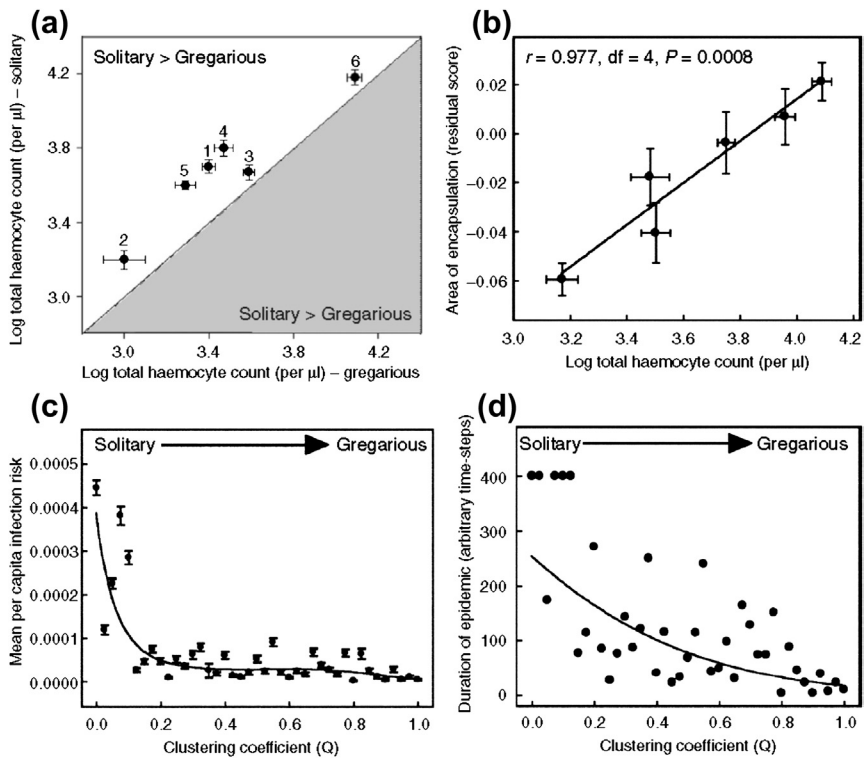


Figure 3.8 Group-living and risk of disease in lepidopteran larvae. (A) Relationship between feeding style and total hemocyte count (THC); gregariously feeding species have significantly lower THC than solitary-feeding species (means \pm SE shown). (B) Relationship between THC and magnitude of encapsulation response directed against a nylon implant by six species of lepidopteran larvae (means \pm s.e. shown). Relationship between the degree of host-clustering and (C) mean *per capita* infection risk (\pm s.e.) and (D) duration of epidemic. Data are output from a dynamic, susceptible/infected spatially explicit model. (Reproduced from Wilson, Knell, et al. (2003)).

activity, where PO levels were higher in the solitary species in five of the six species-pairs. Thus, counter to the expectation, solitary species appear to be investing relatively more in immune function than gregarious species.

In order to try to explain this counter-intuitive finding, Wilson, Knell, et al. (2003) developed a dynamic, susceptible/infected spatially explicit model in which different degrees of host “clustering” were created by allowing different proportions of distant (random) and local (nearest-neighbor) reproduction. In the model, there is a uniform network of sites, each taking one of three possible states: empty, occupied by a susceptible host, or occupied by an infected host. A susceptible host becomes infected when it “contacts” a neighboring infected individual, and a site becomes empty when the occupant dies, and the site is then available to be reoccupied by the offspring of other individuals. The key variable in this model for testing the effects of group-living on the infection risk is the clustering coefficient, Q , which determines the proportion of offspring born into neighboring sites, as opposed to randomly across the whole lattice. By changing this clustering coefficient, Wilson et al. were therefore able to produce populations with different average local clustering. This showed that, for a significant region of model parameter space host-clustering could, in fact, *reduce* an individual’s risk of becoming infected, both in epidemic and endemic host–pathogen interactions. For example, in the epidemic situation, in which the disease spreads rapidly through a susceptible population, the model revealed that as hosts cluster into groups (i.e. as Q increases), so the mean *per capita* risk of becoming infected declines (Fig. 3.8c), and the duration of the epidemic decreases (Fig. 3.8d).

The mechanism explaining this decline in an infection risk with host aggregation can be explained by the theoretical framework known as *percolation theory*. If the disease transmission requires close proximity between potential hosts, then any process that increases the distance between infected and susceptible hosts will lead to reduced transmission. Thus, by increasing the *variance* in nearest-neighbor distance, host-clustering increases the probability that the pathogen will fail to breach the gap between the host it is infecting and the nearest (group of) susceptible hosts, just as a forest fire will eventually extinguish if the distance between neighboring trees is too great for the flames to spread (Wilson, 2009). This threshold degree of host-clustering is known as the *percolation threshold* (Wilson, 1983, 2009). Thus, part of the advantage of group-living is attributable to the fact that any disease epidemics will tend to fade-out faster within populations

of group-living organisms than within populations of solitary hosts due to a failure to transmit between groups (Wilson, Knell, et al., 2003). Watve and Jog (1997) came to similar conclusions using a much simpler epidemiological model, and Davis, Trapman, Leirs, Begon, and Heesterbeek (2008) applied formal percolation theory to explain the epidemiological pattern of plague (*Yersinia pestis*) in the burrow-dwelling great gerbil (*Rhombomys opimus*) and its fleas (mainly *Xenopsylla* spp.) in Kazakhstan (see also Reynolds, Sword, Simpson, & Reynolds, 2009; Wilson, 2009 to see how the same theory might explain the evolution of phase-polyphenism in locusts, with predators replacing parasites). It is worth noting, however, that theory suggests that group-living will fail to be beneficial for disease evasion when the parasite is highly mobile (e.g. parasitoids) or is transmitted by a mobile vector (e.g. mosquitoes). In these circumstances, of course, the parasite is no longer constrained by the spatial distribution of its host. Group-living will also fail to be advantageous in this context when hosts are highly mobile or at such low densities that the infection risk is low for all hosts.

Within group-living species, we might expect the risk of disease to increase with group size if this increases the frequency of interactions between susceptible hosts and infectious conspecifics or propagules. Rifkin, Nunn, and Garamszegi (2012) conducted a meta-analysis of 69 studies examining the relationship between group size and both parasitism and immune defenses across a range of (mostly vertebrate) animal taxa. Overall, there was a consistent positive effect of group size on parasitism risk, i.e. parasitism rates tended to increase with group size. However, contrary to expectation, they also found that the relationship was positive for *all* types of transmission process, including vector-borne (average effect size = +0.396), parasitoid (+0.222), contagious (+0.213), environmental (+0.179), and searching parasites (+0.046), though the trend was nonsignificant for the latter. Thus, in this meta-analysis, there was no evidence for the negative relationship between group size and parasitism risk predicted by percolation theory and the models of Watve and Jog (1997) or Wilson, Knell, et al. (2003). Rifkin et al. found that the effect size also varied with host taxon, with a stronger relationship between group size and parasite risk in birds than in mammals, which they suggested may be driven by ecological and social factors. Social factors are likely to be particularly important for the evolution of immune function and antiparasite strategies in insects, which have evolved a broad range of social systems including eusociality (see Section 5.3).



4. FORAGING, NUTRITION, AND IMMUNITY

4.1. Resource Limitation

In humans, it is widely recognized that diet is paramount to health and that malnutrition and obesity are major contributors to disease (Gross & Newberne, 1980). This is no less true of other animals, including insects. The resources gained from the diet is key to maintaining the immune system and to activating an effective immune response when challenged (Lochmiller & Deerenberg, 2000; Schmid-Hempel, 2003). If resources are limiting or inadequate, especially those resources required to fight off infections, then the immune response could be compromised. A number of studies in both vertebrates and invertebrates have shown that nutrient limitation or starvation can constrain the immune response. For example, starved mice showed a reduced ability to develop a T-cell mediated immune response after infection with *Listeria monocytogenes* (Wing, Magee, & Barczynski, 1988), whilst starvation in cats reduced monocyte phagocytic activity and MHC expression (Simon, Saker, & Thomas, 2000). Starved bumblebees, *B. terrestris*, showed reduced survival compared to controls when their immune systems were activated by an artificial immune challenge (Moret & Schmid-Hempel, 2000). Moreover, their parasites were more virulent when growing in starved hosts (Brown, Loosli, & Schmid-Hempel, 2000). Similarly, when deprived of nutrients, mealworm beetles, *T. molitor*, exhibited a downregulation of immune system function (Siva-Jothy & Thompson, 2002), suggesting that immunity is costly.

However, there is an added complication with understanding how resources affect immunity, in that some animals seem to reduce their intake of food in response to infection. This “illness-induced anorexia” is common amongst vertebrates (Kyriazakis, Tolkamp, & Hutchings, 1998), but has also been documented in a number of insects including *Manduca sexta* caterpillars (Adamo, 2005; Bedoyan, Patil, Kyriakides, & Spence, 1992), the cricket *Gryllus texensis* (Adamo, Bartlett, Le, Spencer, & Sullivan, 2010), and *Drosophila* (Ayres & Schneider, 2009). Evidence from vertebrates and invertebrates supports the idea that this is not a manipulation of host behavior by the parasite, but a response mediated by the host immune system (Adamo, Fidler, & Forestell, 2007; Exton, 1997). Caloric restriction, rather than simply starvation, has also been shown to have dramatic effects on immunity and the host’s ability to limit its parasite burden (i.e. *resistance*) and the ability to limit the harm caused by a given parasite burden (i.e. *tolerance*). *Drosophila* reared on a calorie-reduced diet showed an

increase in *tolerance* to *Salmonella typhimurium* but a decrease in *resistance* to *L. monocytogenes* (Ayres & Schneider, 2009). Moreover, a number of studies have shown that there is a metabolic cost of immune defense (see Table 5.7 in Schmid-Hempel, 2011). For example, insertion of a nylon implant into larvae of the cabbage butterfly, *Pieris brassicae*, resulted in an 8% increase in the standard metabolic rate on a day-3 postchallenge (Freitak, Ots, Vanatoa, & Horak, 2003), and blue tits, *Cyanistes caeruleus*, injected with diphtheria-tetanus vaccine showed an 8–13% increase in basal metabolic rate by a day-7 postchallenge (Svensson, Raberg, Koch, & Hasselquist, 1998).

However, using starvation, or indeed calorie restriction, to understand the contribution of diet to immunity assumes that “resources” equate to “energy” and that the diet is essentially a univariate currency, with animals foraging to maximize their energy intake alone. But food comprises far more than its caloric content, it is a complex mix of macro- and micro-nutrients, all of which may independently, or in combination, affect the animal’s health. This has been recognized more recently by manipulating specific components of the diet rather than simply its energy content or overall amount. Due to the costs involved in maintaining and activating the immune system, it is likely that the requirement for specific nutrients would change in the face of an immune challenge, opening up the opportunity for animals to modulate their feeding behavior to gain those nutrients when infected.

4.2. Nutrient Manipulation

A number of studies have manipulated the nutrient content of the diet and looked for effects on the immune system. For example, Lee et al. (2006) who manipulated the ratio of protein (P) to carbohydrate (C) in a range of diets provided to virally challenged *S. littoralis* caterpillars. Because protein and carbohydrate provide roughly the same number of calories per unit mass, the diets were *isocaloric*, and any effects on immune function of varying their ratio could not be due to variation in the availability of energy, but rather to the availability of the specific macronutrient, P or C. Uninfected larvae performed best on diets that had an even P:C ratio, but virally infected larvae were much more likely to survive if they were restricted to a diet that was protein-biased (i.e. P:C = 5:1) (Lee et al., 2006). Indeed, survival declined steadily as the protein content of the diet decreased (and the carbohydrate content increased, Lee et al., 2006). This result has since been repeated in the congeneric species, *S. exempta*, with both the bacterium, *Bacillus subtilis*, (Povey et al., 2009) and the host-specific virus, SpexNPV

(Povey et al., submitted for publication). In both cases, infected *S. exempta* larvae performed best on the extremely protein-rich diet, even though uninfected caterpillars prefer a carbohydrate-biased diet (Lee, Simpson, & Raubenheimer, 2004).

So what are the mechanisms underpinning these findings? Lee et al. (2006) found that cuticular melanism and lysozyme activity levels in *S. littoralis* larvae were affected by the protein *quality* of the diet (based on amino acid composition), though PO activity levels were unaffected. They also showed that high-protein diets allowed caterpillars to maintain higher levels of lysozyme and PO than low-protein diets, and to mount a stronger encapsulation response. Povey et al. (submitted for publication) also found that as the amount of protein in the diet of *S. exempta* increased, so too did PO activity, hemolymph protein levels, AMP activity, and hemocyte density. AMPs and lysozymes work directly against bacteria and so should contribute to the increased resistance seen against the *B. subtilis* infection (Povey et al., 2009). PO activity and encapsulation ability are likely to contribute to the ability to resist viral infection, but other mechanisms may also come into play, such as the ability to slough infected midgut cells and replace them with resistant ones (Briese, 1981; Keddie et al., 1989) (see Section 2.4), a response that is likely to be demanding of protein.

Nutrient manipulation has also been used to try to understand illness-induced anorexia. *Manduca* caterpillars reduce their intake of food in response to bacterial infection (Adamo, 2005; Bedoyan et al., 1992) and caterpillars infected with the parasitoid wasp, *Cotesia congregata*, reduce feeding during the wasp's emergence from the body. But what is the function of this response? Adamo et al. (2007) tested 4 key hypotheses, that illness-induced anorexia: (1) starves pathogens of nutrients such as protein or iron; (2) reduces the likelihood that the individual will continue consuming contaminated food; (3) reduces the risk of pathogens moving from the gut into the blood during a systemic infection; and (4) enhances immune function. They also tested a further hypothesis that anorexia reduces conflicts between digestive processes and immunity. They found no evidence for the first four hypotheses, but did show that force-feeding infected larvae with lipids reduced their resistance to bacterial infection, suggesting that there may be a conflict between lipid metabolism and immunity (Adamo et al., 2007). Lipid metabolism relies on the transport molecule *high-density lipophorin* (HDL), but after a high-fat meal, if the capacity of HDL is exceeded, *apolipophorin III* can bind with HDL to form *low-density lipophorin*, which can carry more lipid. However, apolipophorin III also functions as an immune molecule:

it can bind with bacteria and fungi in the hemolymph and trigger an immune response (Weers & Ryan, 2006). Adamo et al. (2010) tested the hypothesis that this dual function of apolipoprotein III leads to a mechanistic tradeoff between lipid digestion and immunity. They found that bacterially infected crickets were less likely to survive on a high-lipid diet and, when given a choice, actively preferred a low-fat diet after infection (Adamo et al., 2010).

4.3. Self-Medication

So it seems that it is the specific nutrients ingested, rather than simply their energy content, that most influences the ability to mount an immune response and fight off the disease. We might expect therefore that if specific nutrients, or nutrient-ratios, are beneficial to the host in mounting an immune response against a parasite, infected hosts would actively select these beneficial diets, thereby engaging in a form of “self-medication”, defined as behavior that increases the defense against parasites by one species by using substances that have been produced by another (Clayton & Wolfe, 1993). Circumstantial evidence for self-medication through ingestion comes from several studies of vertebrates, most notably from chimpanzees using plant-derived medicinal substances to combat protozoan or helminth infections (Fowler, Koutsioni, & Sommer, 2007; Huffman & Seifu, 1989). There are also examples where medicinal chemicals or specific micronutrients have been shown to be utilized by insects infected with bacteria or parasitoids (e.g. Bernays & Singer, 2005; Krishchik, Barbosa, & Reichelderfer, 1988). However, until recently, there was little evidence that animals used macronutrients to self-medicate.

To test this idea, Lee et al. (2006) provided infected and uninfected caterpillars with a choice between a protein-rich diet and one that had equal amounts of protein and carbohydrate. These choices equated to the diet that either infected (protein-rich) and uninfected (balanced) caterpillars performed best on. Infected caterpillars chose a more protein-rich diet than controls and, within the infected group, those that survived the infection chose a more protein-rich diet than those that succumbed (Lee et al., 2006). Similarly, Povey et al. (2009) found that when given a choice between two diets varying in their P:C ratios, *S. exempta* larvae injected with a sublethal dose of bacteria increased their protein intake relative to control larvae. Larvae infected with NPV showed a large increase in relative protein consumption immediately postinfection, with protein intake subsequently decreasing to match the intake of control caterpillars by the time they pupated (Fig. 3.9a; Povey et al., submitted for publication). These results suggest that both *S. littoralis* and *S. exempta* larvae alter their feeding

behavior in response to infection to improve their survival prospects, possibly by enhancing the levels of protein available for producing the immune system components and other effectors required to resist infections (i.e. a form of “self-medication”, *sensu* Clayton & Wolfe, 1993).

Macronutrient self-medication in response to immune challenge has been established in a number of other systems. For example, mealworms challenged with LPS, when given a choice between high- and low-protein diets, greatly increased their intake of the protein-rich food, resulting in improved antibacterial activity and hemocyte density (Catalan, Barcelo, Niemeyer, Kalergis, & Bozinovic, 2011). Of course, an alternative explanation for the behavioral changes observed following infection is that they are not adaptive responses by the host to infection, or even nonadaptive artifacts of parasitism, but are instead due to the manipulation of host behavior by the parasite. There is a vast literature on putative examples of manipulation of host behavior by parasites, and the challenges faced in trying to distinguish this from other explanations for changes in behavior (e.g. Eberhard, 2000;

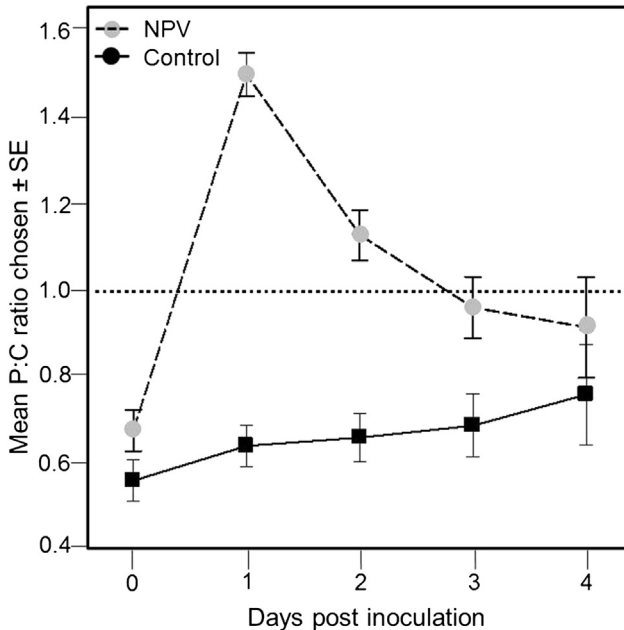


Figure 3.9 The effects of virus (SpexNPV) treatment on the mean protein to carbohydrate ratio (P:C) of the diet selected by caterpillars pre- (day 0) and post (days 1–4) treatment. Caterpillars were inoculated with an LD10 dose of virus. (Reproduced from Povey *et al.* (In Revision, *Journal of Animal Ecology*)).

Holmes & Bethel, 1972; Moore, 2002; Poulin, 2010; Wilson & Edwards, 1986). However, in instances like those described above, where the host clearly benefits from changing its behavior, the parasite suffers reduced fitness following the behavioral change, and a potential mechanism for the altered fitness-states is established, the most parsimonious explanation for the behavior change is that it is regulated by the host.

4.4. The Geometric Framework

Whilst informative, the approaches to understanding the impact of resources on immunity described thus far have the drawback of being unable to address the potentially interactive effects of varying the energy *and* nutrient content of the diet. By considering isocaloric diets, one essentially takes a single slice across nutrient space rather than looking at the whole picture. Depending on the relationship between the diet components and the trait of interest, this can generate very different results (consider the effect of taking different slices diagonally across Fig. 3.10c). The geometric framework for nutritional analyses provides a framework for modeling nutrition as a multidimensional

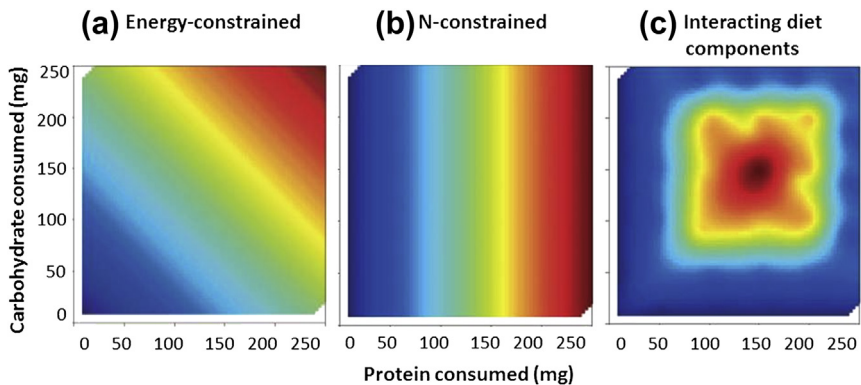


Figure 3.10 Hypothetical surfaces showing how a trait might be expected to vary with carbohydrate and protein intake. Blue colors represent low values of the trait and red colors high values. Because P and C are isocaloric, an insect that consumes 200 mg of P and 50 of C gets the same amount of energy as one that consumes 50 of P and 200 of C. (a) Energy constrained—as the energy content of the diet increases the trait increases, irrespective of whether that energy comes from P or C. (b) Nitrogen-constrained N is found only in P, therefore, as P increases, the trait would increase, irrespective of how much C is consumed. (c) Interacting diet components—this results in an optimal combination of the two diet components at which the trait shows peak performance. (*Reproduced from Cotter, Simpson, et al. 2011*). (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.)

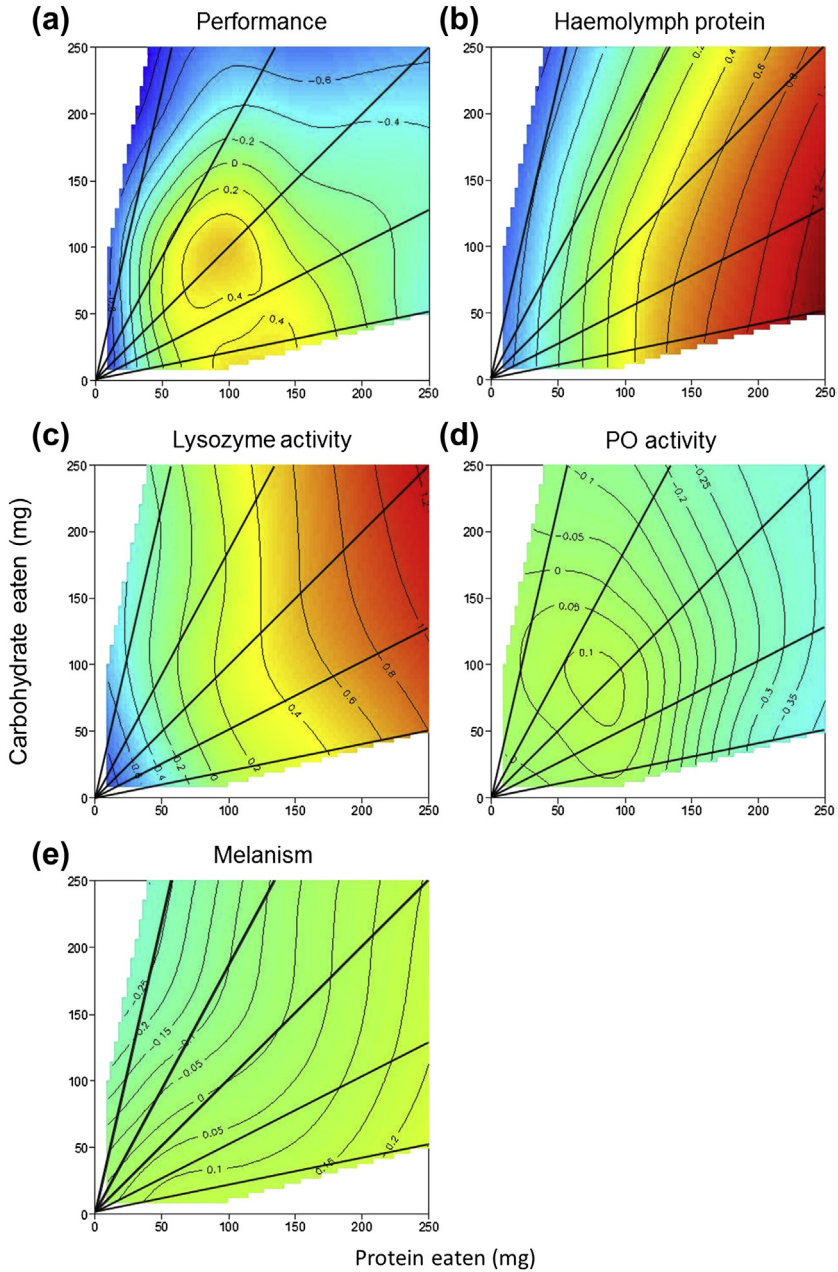


Figure 3.11 Response surfaces showing the effects of protein (P) and carbohydrate (C) intake on (a) larval performance, (b) hemolymph protein levels, (c) lysozyme activity, (d) PO activity, and (e) cuticular melanism. Consumption was recorded for individual caterpillars confined to 1 of 20 diets varying in both the %P and the total amount of P and C.

phenomenon, allowing the consideration of the effects of energy and nutrient composition simultaneously (Simpson & Raubenheimer, 1995). Briefly, animals are manipulated to consume over a large area of nutrient space by restricting them to a range of diets that vary in both total nutrient content and composition. Dietary intake is measured and the trait of interest recorded. The trait of interest is then mapped onto the intake coordinates producing a form of contour plot (Figure 3.10).

This approach has been employed to consider the effects of protein and carbohydrate composition and content on larval growth and immune traits in *S. littoralis* caterpillars (Cotter, Simpson, et al., 2011). Individuals were restricted to one of 20 different diets that varied in both the amount and ratios of protein and carbohydrate, thus manipulating nutritional composition and energy content simultaneously. Food consumption, larval growth rate, cuticular melanism, hemolymph protein levels, PO activity, and lysozyme levels were then measured, and the following question asked: Is there a single optimal diet for all of these traits? Cotter et al. found that, with the exception of cuticular melanism, none of the measured traits were limited by either the total quantity of nutrients consumed, or by just the amount of protein consumed. This means that the larvae were not simply energy- or nitrogen-limited (Cotter, Simpson, et al., 2011; see Fig. 3.10); rather, different traits responded differently to variation in the ratios of macronutrients, and peaked in different regions of macronutrient space (Fig. 3.11). Whilst cuticular melanism, hemolymph protein, and lysozyme levels peaked in regions of high protein intake, larval performance peaked at intermediate protein levels and was also strongly affected by carbohydrate intake. In contrast, PO activity was relatively unaffected by dietary composition, though the highest activity did occur at a more carbohydrate-biased intake than the other traits (Fig. 3.11). A similar pattern was found when the caterpillars had their immune systems challenged by injection with dead bacterial cells. However, this treatment changed the region of peak activity for the two hemolymph immune traits, PO activity and lysozyme activity (Cotter, Simpson, et al., 2011).

The above results suggest that no diet can simultaneously optimize all components of the immune system. So which diet do immune-challenged



The solid lines indicate the %P rails that the larvae were restricted to (17, 34, 50, and 67 or 83%). The color scale represents standard deviations from the mean with dark blue below the mean and dark red above the mean. (Reproduced from Cotter, Simpson, et al. (2011)). (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.)

and nonchallenged caterpillars choose? Larvae were allowed to choose between pairs of diets that differed in their protein to carbohydrate ratios, and consumption was measured. Surprisingly, both challenged and nonchallenged caterpillars chose the same diet, one that gave a good growth rate and represented a compromise between the nutritional demands of competing immune traits (see Fig. 3.3 in Cotter, Simpson, et al., 2011). Interestingly, there was no evidence that the nonpathogenic immune challenge resulted in self-medication, in contrast to what was seen when the same species of caterpillar was inoculated with live parasites (Section 4.3) (Lee et al., 2006). So why might this be? One possible explanation for the lack of self-medication in the immune-challenged caterpillars is that whilst the antibacterial response requires protein, it is unlikely to consume as much protein as a real infection; actively replicating bacteria will require protein for growth, in addition to the extra protein required by the host to produce or replace antibacterial peptides and hemocytes to phagocytose and nodulate parasites (Tanada & Kaya, 1993). In contrast, a nonpathogenic immune-challenge stimulates the production of AMPs and upregulates lysozyme activity, but caterpillars may be able to free up the required protein for these responses by simply reallocating existing resources (Braude, Tang-Martinez, & Taylor, 1999). A second possibility is that rather than maximizing the immune response, caterpillars may change their diet to starve the parasite of key nutrients, but this would require insights into the nutritional requirements of the parasite—something that has not yet been established.

The geometric approach has also been used to look at the interaction between nutrition and infection with a live macroparasite. Ponton, Lalubin, et al. (2011) assessed the effect of nutrition on *T. molitor* beetles infected with the tapeworm, *Hymenolepis diminuta*. Infected beetles greatly increased their intake of macronutrients, particularly carbohydrate. This had no effect on PO activity or parasite growth, but allowed females to maintain reproductive output during the first two weeks postinfection, a time during which reproduction in infected individuals is usually severely reduced (Keymer, 1980). In this case, the self-medication did not allow the beetle to fight off the infection, but instead ameliorated its negative consequences.

The geometric framework is a promising approach to understand the role of specific micro- and macronutrients in fueling the immune response. The next step is to analyze in detail how the resource requirements of hosts and their pathogens differ. This will allow us to address whether or not a change in diet after infection reflects the requirements of the host's immune system, or whether hosts are "starving" their parasites of key nutrients.



5. REPRODUCTION, PARENTAL CARE, AND IMMUNITY

Breeding is invariably a costly activity, but the costs of reproduction may vary considerably between species, sexes, and individuals. In polygynous animal species, investment in costly ornaments to attract mates or weaponry to fight for access to mates may, directly or indirectly, result in elevated parasitism risk through either an enhanced exposure to parasites or increased susceptibility. Across species, the level of investment that parents provide to their offspring ranges from the minimal production of eggs and sperm, right through to the elaborate levels of care provided in social species such as primates or honeybees. Whilst within species, the energy and resources that parents devote to their offspring could impact upon the resources available for their own personal defenses against parasites leading to variation in immune responses and parasite load (see Section 2.7). However, reproduction is also associated with “social” immune responses, whereby some parents provide their offspring with antiparasite components. The effect of reproductive investment decisions on immunity and other disease resistance mechanisms is therefore complex and may depend on the level of care, parents provide.

5.1. Sexual Selection and Sex-Biased Parasitism

Over 30 years ago, Bill Hamilton & Marlene Zuk proposed a role for parasitism in the evolution of sexually selected traits, such as the colorful plumage, complex songs, and elaborate courtship displays seen in many birds (Hamilton & Zuk, 1982), arguing that these male traits may have evolved to signal to potential mates, the bearer’s good health, and ability to resist the negative effects of parasitism. As a result, a female choosing a male with bright plumage or an elaborate courtship display would tend to acquire “good genes” for parasite resistance for her future offspring. Since its publication, there have been numerous studies testing this hypothesis (nearly 2000 citations in *Web of Science* by the end of 2012) and developing the theory (e.g. Folstad & Karter, 1992; Wedekind & Folstad, 1994), with variable success, and with some authors even arguing that the hypothesis may not be testable (see reviews by Clayton, 1991; John, 1997; Read, 1990; Hamilton & Poulin, 1997; Zuk, 1992). A vast majority of tests of the Hamilton–Zuk hypothesis have focused on vertebrates, especially birds and fish (Hamilton & Poulin, 1997), but there are some examples using invertebrates. For example, Ryder and Siva-Jothy (2000) found that in the

house cricket *Acheta domesticus*, there was a positive correlation between one aspect of the male courtship song (number of syllables per chirp) and hemocyte density, suggesting that females who choose males that produce more syllables per chirp may also be selecting mates with a greater capacity to resist pathogens. In another study, [Rolff and Kraaijeveld \(2003\)](#) found that in a replicated selection experiment in which *D. melanogaster* was selected for resistance to the parasitoid *Asobara tabida*, males from the lines selected for increased parasitoid resistance achieved a higher mating success compared with nonresistant conspecifics, suggesting a genetic correlation between male “attractiveness” and a male capacity to resist parasites.

It has long been recognized by parasitologists that males and females differ in their parasite prevalences and intensities (reviewed [Zuk & McKean, 1996](#)), and a series of meta-analyses have clearly demonstrated a small but consistent male-bias in parasitism in mammals, including humans, but with variable results for other vertebrate taxa ([McCurdy, Shutler, Mullie, & Forbes, 1998](#); [Moore & Wilson, 2002](#); [Poulin, 1996a,b](#); [Schalk & Forbes, 1997](#); [Wilson, Moore, et al., 2003](#)). A number of proximate and ultimate explanations have been proposed for the observed male-biased parasitism in some vertebrate taxa. One of the most widely discussed proximate mechanisms is the *immunocompetence handicap hypothesis* (ICHH), which was originally proposed as a mechanistic explanation for Hamilton & Zuk’s parasite-mediated sexual selection ([Folstad & Karter, 1992](#)). The original ICHH argued that testosterone (or other related androgenic hormones) provides a proximate mechanism to ensure that sexually selected ornaments, such as elaborate plumage or complex song, act as honest signals of male quality, due to its dual properties of stimulating or maintaining sexual ornaments whilst also being immunodepressive (but see also [Wedekind & Folstad, 1994](#) for a variation of the original hypothesis). Since only males produce significant amounts of testosterone, it logically follows that if testosterone depresses the immune system then male vertebrates will be more susceptible to parasitism than females. However, a meta-analysis failed to find consistent support for the assumption that testosterone suppresses immunity ([Roberts, Buchanan, & Evans, 2004](#)), and attention has shifted away from the requirement to invoke immunodepressive hormones as a general explanation for either sex-biased parasitism (SBP) or parasite-mediated sexual selection.

A particularly fruitful variant of the ICHH argued that the proximate traitlinking immune function and sexual ornamentation was an individual’s condition ([Gustafsson et al., 1994](#); [Moller, 1995](#); [Sheldon & Verhulst,](#)

1996). Thus, the possession or maintenance of the ornament is considered to be costly in terms of some limiting nutritional resource, such that investment in the ornament necessarily means there is less of that resource available to invest in immunity or other important functions. Subsequent studies have provided strong support for the notion that both immune function and sexual ornament development are condition-dependent traits and may be traded-off against each other (Section 4 and 2.7). For example, using male zebra finches (*Taeniopygia guttata*) as a model organism, Blount, Metcalfe, Birkhead, and Surai (2003) found that experimental manipulation of the amount of carotenoids (lutein and zeaxanthin) in the diet generated simultaneous changes in both cell-mediated immune function (as measured by the in vivo response to injected PHA) and sexual attractiveness (as measured by bill color).

Building on Zuk's (1990) argument that there should be a stronger focus on ultimate explanations for the observed sex differences in parasitism, Moore and Wilson (2002) argued that the male-biased parasitism seen in mammals was a consequence of sexual selection driving males to invest in traits that would favor their reproductive success, such as an aggressive behavior and large body size (leading to male-biased sexual size dimorphism, SSD), at the expense of elevated risk of parasitism. A prediction of this hypothesis is that as the strength of sexual selection increases, and the degree of male-biased SSD increases, so the extent of SBP should become increasingly shifted toward males. Here, the extent of SBP was calculated as the "rate difference" in parasite prevalence between male and female hosts (i.e. male prevalence–female prevalence), such that positive values for SBP indicate higher rates of parasitism in males than females and a rate difference of zero indicates no sex-bias. In a large meta-analysis of sex differences in parasite prevalence across 350 studies of mammals, they found a strong positive relationship between SBP and SSD, as predicted, even after controlling for host phylogeny (Fig. 3.12) (Moore & Wilson, 2002). Thus, the evolution of male-biased sexual size dimorphism was accompanied by the evolution of male-biased parasitism. Moreover, when SBP was compared in closely related monogamous and polygynous mammal species, they found that the polygynous species exhibited a much stronger male-bias in parasitism than the monogamous species, consistent with a role for sexual selection in generating these trends.

If sexual selection is the ultimate driving force underpinning sex differences in immunity and parasitism, then it follows that patterns similar to

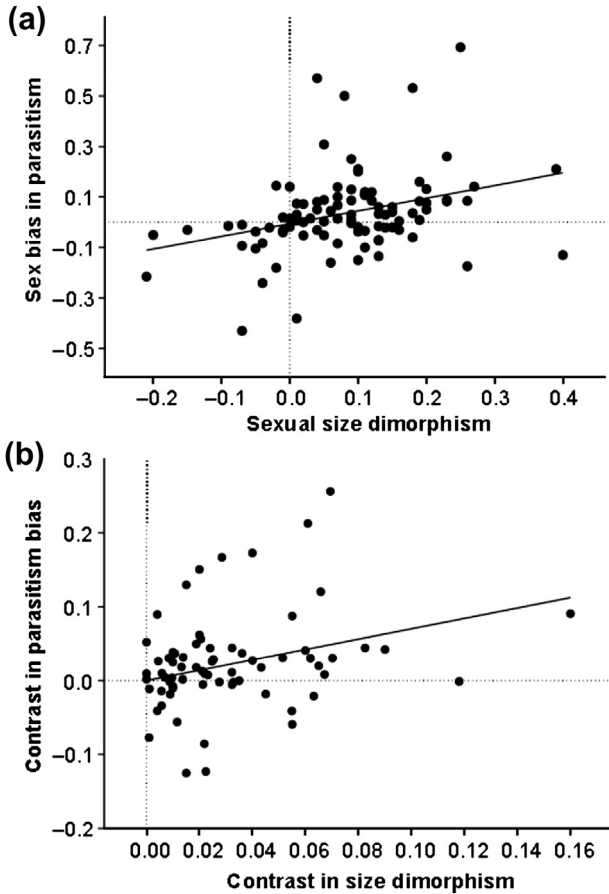


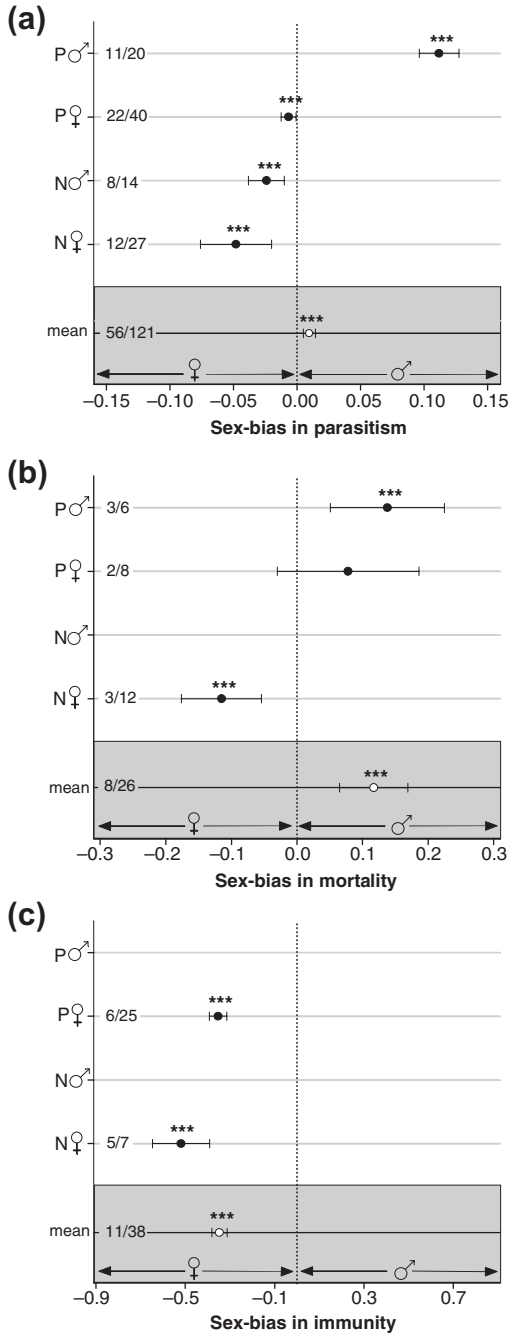
Figure 3.12 Relationship between sexual size dimorphism and sex-biased parasitism in mammals. (a) Raw data, (b) independent contrast scores. Sex-bias parasitism (SBP) rate is calculated as male prevalence–female prevalence; sexual size dimorphism, SSD, is calculated as the logarithm of male mass/female mass. The lines show the least-squares regression lines; in (b), the intercept is forced through the origin. (Reproduced from Moore and Wilson (2002) Reprinted with permission from AAAS).

those found by Moore and Wilson (2002) should also be observed in the invertebrate taxa displaying variation in the strength and direction of sexual selection. To test this, Moore (2003) conducted a meta-analysis of parasitism rates in insects. The study used data extracted from the literature on sex-related incidence of parasitism in 56 species of insects caught in the wild. For each of the 121 host–parasite interactions, the extent of SBP was calculated. She found that, as in mammals and birds (Poulin, 1996a,b; Schalk & Forbes,

1997), but contrary to an earlier meta-analysis across a range of arthropod species including insects (Sheridan, Poulin, Ward, & Zuk, 2000), there was a small but statistically significant male-bias in parasitism overall (Fig. 3.13a). In addition, whereas polygynous species suffered a significant male-bias in parasitism (mean effect size = +0.0094), parasitism was significantly female-biased in nonpolygynous (monogamous and polyandrous) insects (-0.0285). Moreover, as seen in the meta-analysis of SBP in mammals, the extent of SBP increased significantly with a degree of sexual size dimorphism: as the discrepancy in body size increased so too did the discrepancy in parasite prevalence.

In mammals, SSD and mating system strongly covary: most mammals are polygynous and exhibit male-biased size dimorphism and the minority are monogamous and exhibit no consistent sex difference in body mass. Thus, in mammals, it is not possible to dissociate the relative contributions of these two factors in explaining the evolution of SBP. However, in insects this is not the case because the benefits of large body size are often in terms of a fecundity advantage to females (as opposed to the male-fighting advantage seen in mammals). This allowed Moore (2003) to determine the relative contributions of mating system and SSD to the observed pattern of SBP in insects (in her data set, there was no significant covariation between mating system and the direction of size dimorphism). She found that variation in the degree of SBP was explained by a significant interaction between mating system and sexual size dimorphism (Fig. 3.13A). This was a consequence of a significant relationship between SBP and SSD for polygynous species, but a non-significant relationship for nonpolygynous species. Thus, it appears that the parasitism cost associated with sexual size dimorphism is paid only by polygynous insect species.

In mammals, male-biased parasitism is associated with male-biased mortality (Moore & Wilson, 2002). In a separate analysis using a different dataset, Moore (2003) found that insects also exhibit a small but significant male-bias in mortality (Fig. 3.13b). Moreover, following exposure to parasites, polygynous insect species exhibited a significant male-bias in mortality, whereas nonpolygynous species exhibited a significant female-bias in mortality (mean effect sizes = +0.117 and -0.191, respectively); there was also a positive relationship between sex-biased mortality and SSD. These results are consistent with parasites having a greater impact on the survival of males than females, especially in polygynous species and those in which males are larger than females.



In their study of SBP in mammals, Moore and Wilson (2002) focused on sexual selection as the *ultimate* mechanism generating male-biased parasitism and mortality and the exact *proximate* mechanisms were not explored. However, they did argue that there could be multiple mechanisms that might generate increased male *exposure* to parasites (including higher food consumption rates, increased mobility, increased body surface area, increased fighting propensity, etc.) and/or increased male *susceptibility* to infection (including reduced immune function, possibly due to immune-depressing testosterone). Building on the idea that the two sexes have evolved different strategies for maximizing their long-term fitness, Rolff (2002) argued that “Bateman’s principle” (Bateman, 1948) could be applied to explain sex differences in immunity (and hence susceptibility to infection). He suggested that because males gain fitness by increasing their mating success, whereas females increase theirs through longevity because their reproductive effort is much higher, females should invest more in immunity than males. If this is true, then the extent of this immune dimorphism is predicted to covary with mating system and the genetic correlation between males and females in immune traits. In a comparative analysis of immune measures across nearly 200 studies of mammals, Nunn et al. (2009) found that females tended to have higher total white blood cell (WBC) counts than males, with significantly higher counts of eosinophils and lymphocytes, but not neutrophils, monocytes, or basophils. They found no statistical association between SSD and sexual dimorphism in any of the immune cell counts tested or between SSD and variation in WBC. However, there were significant positive relationships between cell counts and longevity for some immune cell types in some analyses, but not in others, providing partial support for Bateman’s principle. Also consistent with this hypothesis, there was a positive correlation between the sex-bias in duration of breeding and the sex-bias in basophil and lymphocyte counts.



Figure 3.13 Summary of meta-analyses in insects which sex-biases in (a) parasitism, (b) parasite-induced mortality, and (c) immune function are categorized according to mating system (polygynous, P, or nonpolygynous, N) and degree of sexual size dimorphism (SSD) (male-biased, ♂, or female-biased, ♀). Open circles and error bars in shaded areas represent mean effect sizes and 95% confidence intervals for all data combined. Solid circles and error bars in unshaded area represent mean effect sizes and 95% confidence intervals for the four categories: P♂: polygynous/male-biased SSD; P♀: polygynous/female-biased SSD; N♂: nonpolygynous/male-biased SSD; N♀: nonpolygynous/female-biased SSD. Asterisks indicate significant differences from zero. Only those comparisons that involve multiple species are included in the plot. Numbers attached to the vertical axis represent number of species/comparisons in the analysis. (Data from (Moore 2003)).

Sex-biases in immune measures have also been reported in invertebrates. Nunn et al. (2009) conducted a meta-analysis of 43 studies reporting sex differences in two immune traits: PO activity and THCs. They found that the mean effect sizes for PO and THC were -0.187 and -0.0136 , respectively, showing strong sexual dimorphism for PO but only weak dimorphism for THC, both in favor of higher levels in females than males. Moore (2003) also conducted a meta-analysis of immunity including 11 species of insects from six orders and four immune measures: THC, PO, encapsulation response, and antibacterial activity. She found an overall female-bias in immune function (mean effect size = -0.347), and a significant female-bias for PO activity, hemocyte count, and antibacterial activity, but not for encapsulation response. This bias differed between polygynous and nonpolygynous species in some analyses but not others (Fig. 3.13c), but the extent of the sex-bias in immune function varied positively and significantly with the degree of male-biased sexual size dimorphism.

Thus, in both vertebrates and invertebrates, there are consistent sex differences in immune function that mirror the observed sex differences in the parasitism rates. There appears to be little direct support that these are driven by testosterone or related mechanisms consistent with the ICHH. However, there is some support for the notion that sex differences in parasitism are driven by sex-specific life histories that, in one sex (usually females), prioritize traits that enhance long-term survival, including immunity and, in the other sex (usually males), prioritizes traits that enhance shorter-term reproductive benefits (such as large body size and increased aggression in males), even if these reduce longevity and increase the risk of exposure or susceptibility to parasites. As the number of studies on invertebrate immunity increase, there is the opportunity to determine whether vertebrates and invertebrates exhibit common patterns in terms of sex differences in immunity and whether these are sufficient to explain the observed sex-biases in parasitism rates or whether sex differences in exposure need also to be invoked. Future studies also need to address further the impact of SBP for patterns of sex-biased mortality and the implications of this for parasite epidemiology and the ecoevolutionary dynamics of the host-parasite interaction (Miller et al. 2007; Restif & Amos 2010; Bacelar et al. 2011).

5.2. Reproductive Restraint versus Terminal Investment

Across species, variation in breeding systems and the size differences between males and females seems to explain some of the patterns we can see in immune investment, but within species, how do parents balance

the costs of reproduction and immunity? As indicated previously (Section 2.7.2), a common response to becoming infected with a parasite or activating the immune system is to reduce reproductive effort. For example, pied flycatcher females immunized with a nonpathogenic antigen reduced their feeding effort to their current brood, reducing the quality and number of fledglings produced (Ilmonen, Taarna, & Hasselquist, 2000). Similarly, female *Euoniticellus intermedius* dung beetles reduced the number of brood balls they produced directly after wounding or immune challenge (Reaney & Knell, 2010). However, whilst under certain circumstances “reproductive restraint” following an immune challenge may be beneficial or necessary, classical life-history theory predicts that reproductive investment should increase if the risk of death is suddenly increased, often referred to as “terminal investment” (Clutton-Brock, 1984; Hirschfield & Tinkle, 1975; Williams, 1966). In many cases, an immune-challenge could be considered a reliable cue to an imminent risk of death and so we might expect it to trigger a terminal investment in the reproduction (Velando, Drummond, & Torres, 2006; Cotter, Ward, et al., 2011). For example, female house crickets, *A. domesticus*, challenged with either a bacterial injection, or LPS, increased their egg-laying immediately after the challenge. However, infection with a parasitoid, or injection with beads to mimic a parasitoid attack, did not elicit the same response (Adamo, 1999). In contrast, when male mealworm beetles were challenged with a nylon monofilament inserted into the body cavity, to mimic a parasitoid attack, there was not only an upregulation of PO activity, but also an upregulation of female-attracting pheromones, such that challenged males became more attractive to females (Sadd et al., 2006). Whether or not this increased production of pheromones would have resulted in increased reproductive success was unfortunately not tested. However, in both cases the response of challenged individuals was indicative of terminal investment in reproduction given that they might not survive to breed again.

So should immune-challenged organisms terminally invest or show reproductive restraint to recoup energetic or nutritional losses that could be used in future breeding attempts? The many studies addressing this question produce conflicting results (see Table 1 in Reaney & Knell, 2010), and this may be due to the age or state of the individuals tested, since whether or not to terminally invest should depend on many intrinsic and extrinsic factors (McNamara & Houston, 1996; McNamara, Houston, Barta, Scheuerlein, & Fromhage, 2009). This idea was tested with the burying beetle, *N. vespilloides*, where the age and state of beetles were independently

manipulated to determine which factors affected reproductive investment (Cotter, Ward, et al., 2011).

Burying beetles (Fig. 3.14) rely on small vertebrate carrion for breeding, an ephemeral resource (Eggert & Muller, 1997; Scott, 1998). As such, breeding is opportunistic and so at each reproductive attempt beetles may have widely varying levels of damage accumulation, due to aging, infections, or prior reproductive effort. This also means that the burying beetles cannot infer from their age alone whether or not their first breeding attempt will also be their last. Reproductive decisions are therefore likely to be sensitive to current state, making it ideal for this sort of investigation (see also Creighton, Heflin, & Belk, 2009; Lock, Smiseth, Moore, & Moore, 2007; Trumbo, 2009 for work on *Nicrophorus orbicollis*). Once a carcass has been located, beetles will fight viciously to secure it (Trumbo, 1991), making them vulnerable to opportunistic infections arising from fight wounds, because they live in a microbe-rich soil environment (Plaistow, Outreman, Moret, & Rigaud, 2003). Consequently, they might rely on the state of their immune system to infer the risk of death, and therefore on how much they should invest in reproduction.

Female burying beetles were bred for the first time at different ages, and then allowed to breed every week until death. Breeding is costly in this species (Ward, Cotter, & Kilner, 2009) and so females at different ages had different levels of prior reproductive investment, and thus different levels of physical deterioration (Cotter, Ward, et al., 2011). As expected,



Figure 3.14 A female burying beetle (*Nicrophorus vespilloides*) tends to her brood. (Photo courtesy of Oliver Krueger). (For color version of this figure, the reader is referred to the online version of this book.)

the current level of reproductive investment declined independently with both age and state. Next, young (3 weeks) and old (6 weeks) females had their immune systems challenged by injection with dead bacterial cells to create the illusion that the likelihood of breeding again was low for some individuals (e.g. [Bonneaud, Mazuc, Chastel, Westerdahl, & Sorci, 2004](#); [Velando et al., 2006](#)). All females, whether young or old, produced heavier broods in response to the immune challenge, as predicted by classic life-history theory ([Fig. 3.15](#)), which indicates that they are typically, strategically showing reproductive restraint ([McNamara et al., 2009](#); [McNamara & Houston, 1996](#)). However, whilst females in both age groups increased their investment to a similar degree when immune-challenged, absolute levels of investment were consistently lower in older females ([Fig. 3.15](#)), probably due to senescence limiting fecundity as females age ([Cotter, Ward, et al., 2011](#)).

This last study highlights the importance of considering intrinsic (age, condition, prior reproductive investment) and extrinsic (parasitism, predation) factors when trying to unravel the interaction between reproduction and investment in antiparasite defenses, and may go some way to explaining the inconsistencies in the findings from different studies.

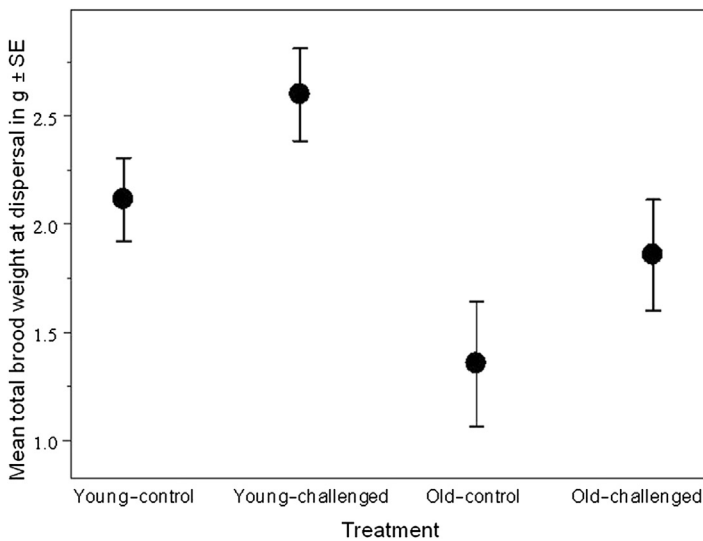


Figure 3.15 The effect of age and immune challenge treatment on investment in the current brood in *Nicrophorus vespilloides*. Beetles were bred for the first time at 3 weeks (young) or 6 weeks (old) of age. (Reproduced from [Cotter, Ward, et al. \(2011\)](#)).

5.3. Social Immune Responses

Another interesting aspect of both invertebrate and vertebrate immune systems is that the benefits of investment in antiparasite defenses are not always restricted to the individual producing the response. The term “social immunity” was coined by Cremer, Armitage, and Schmid-Hempel (2007) to describe the group-level immune function exhibited by social insects and group-living primates. Specifically, it describes immune defenses that are mounted by a collective for the benefit of themselves and others (Cremer et al., 2007; Cremer & Sixt, 2009; Wilson-Rich et al., 2009). For example, as described above, (Section 2.4) termites coat the inside of their chambers with antifungal fecal pellets (Rosengaus, Guldin, et al., 1998), they also produce antimicrobial secretions from their sternal glands, (Rosengaus, Traniello, Lefebvre, & Maxmen, 2004) and they use grooming behavior to remove spores from nest-mates (Rosengaus, Maxmen, et al., 1998). Many similar behaviors have been described from honeybees and ants (see Cremer et al., 2007 and references therein).

However, this definition of social immunity has since been expanded to include any type of immune response that has been selected to increase the fitness of the challenged individual and one or more recipients. This brings the definition in line with the use of the word *social* in the evolutionary literature, which occurs when behaviors have fitness consequences for both actor and recipient (e.g. West, Gardner, & Griffin, 2006). According to this modified definition, therefore, social immunity includes the immune services provided for others in three different contexts: (1) herd immunity in kin-structured populations; (2) social microbes; and (3) animal families, which include socially breeding vertebrates, subsocial, and social insects, (Cotter & Kilner, 2010a). The collective social behaviors originally termed “social immunity” can then be considered a subset of the wider array of evolutionarily “social” immune responses (Figure 3.16).

5.3.1. Herd Immunity

An important epidemiological consequence of widespread immunity within the community, regardless of its “intended” beneficiaries, is that even susceptible individuals may effectively become protected from infection, due to the phenomenon known as “herd immunity” (Anderson & May, 1985). This is the mechanism by which an infectious agent may be eradicated from a population even though some susceptible hosts still remain, because the remainder of the community is immune and thus transmission is reduced (Watt et al., 1995). Indeed, this is the rationale behind population-level

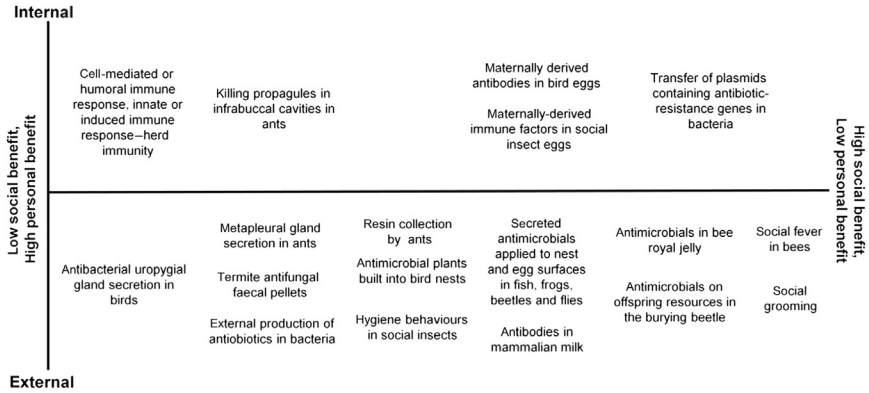


Figure 3.16 Examples of immune function are classified in two dimensions: according to whether they are internal or external, and according to the extent of cooperation by judging the magnitude of the benefits they bring to others, relative to personal gain. (Reproduced from *Cotter and Kilner (2010a)*).

vaccination programs, which aim to reduce the R_0 (reproductive rate) for the parasite below the critical threshold for persistence (*Anderson & May, 1985*). Just as with any investment for a public good, herd immunity is vulnerable to cheats who seek the benefits it confers without contributing to the costs involved (*Frank, 1998*). This is why we observe the periodic reemergence of childhood infections, such as whooping cough and measles, when the vaccine take-up rates drop below the threshold required to prevent the parasites' persistence and invasion via herd immunity. Theoretical analyses suggest that contributions to herd immunity are favored when individuals live in kin-structured populations (*Frank, 1998*). Indeed, it has been shown that cooperatively breeding adult African birds mount a stronger immune response than equivalently immune-challenged pair-breeding birds (*Spottiswoode, 2008*), perhaps because this reduces the levels of infection that their nestling kin then experience. Of course, herd immunity will also be observed in species that live in groups that are not kin-structured, due purely to the epidemiological consequences of the number of immune individuals in the population; in such cases, we would not refer to this as social immunity (see Section 5.4).

5.3.2. Social Microbes

If we consider immune defenses in their broadest sense, i.e. mechanisms that provide resistance to external agents that damage the body, and that have co-evolved with those agents specifically for this purpose (*Davies, 1994*), then

antibiotic resistance can be considered a microbial immune defense. Social microbes have been shown to mount these defenses on behalf of other individuals. For example, *Staphylococcus aureus* exhibit antibiotic resistance that is socially acquired. When grown in the presence of an antibiotic, some of the cells switch to an antibiotic-resistant phenotype that also confers protection to the remaining nonresistant cells (Massey, Buckling, & Peacock, 2001). By lowering the pH of the medium, the resistant cells render the antibiotic ineffective for the entire population, whether the cells themselves are resistant or not (Massey & Peacock, 2002). Once the proportion of resistant cells reaches 10% of the population, the growth of wild-type cells starts to increase (Massey et al., 2001). Like the social fever exhibited by bees (Starks, Blackie, & Seeley, 2000) several individuals must participate for the defense to be successful. This is not the only example of social immunity in microbes; for example, in many species, antibiotic-resistance genes are shared with susceptible neighbors (commonly kin) through the transfer of plasmids, thereby providing them with the means to defend themselves against an attack (Davies, 1994).

5.3.3. Animal Families

Examples of social immunity commonly occur in families with parental-care behaviors, and include many of the examples described in Section 2.4 above, such as the antimicrobial glue produced by many fish to protect their eggs (Giacomello et al., 2006; Knouft et al., 2003; Little et al., 2008). Even animals that show no direct parental care can provide immunity to their offspring; houseflies that lay their eggs in manure cover the surface of their eggs with bacteria that inhibit the growth of fungi that can affect larval development (Lam, Thu, Tsang, Moore, & Gries, 2009). Vertebrate parents can also provide immunity for their offspring internally by endowing the eggs or milk with immune factors (Grindstaff, Brodie, & Ketterson, 2003). Similarly, insects seem to be able to provide immune protection to their offspring, though the mechanisms behind this transgenerational transfer of immunity are currently unknown. Mealworm beetles, *T. molitor*, whose parents had been immune challenged, showed a stronger antimicrobial response to immune challenge themselves than did beetles whose parents had not been challenged (Moret, 2006). In bumblebees, *B. terrestris*, sexual offspring from challenged colonies had higher PO activity, but as it was the workers in the colony that were challenged, and not the queen, this cannot have been a direct transfer of immunity in the egg (Moret & Schmid-Hempel, 2001). Using the same species, Sadd, Kleinlogel, Schmid-Hempel, and Schmid-Hempel (2005) showed

that challenging the queen with heat-killed bacteria resulted in an increased antibacterial response in her offspring, which may indeed have been due to a transfer of immune components in the egg. This was later confirmed in a subsequent study in which eggs from challenged and control queens were raised by challenged or control surrogates. Only the challenge-status of the queen affected the antibacterial activity of the offspring, showing that the effects were mediated by the egg (Sadd & Schmid-Hempel, 2007). Indeed, eggs from challenged queens were shown to have higher levels of antibacterial activity themselves (Sadd & Schmid-Hempel, 2007). There is also evidence for paternally derived immune-priming, but again, precisely how this is mediated is unknown (Roth et al., 2010; Zanchi, Troussard, Martinaud, Moreau, & Moret, 2011).

A further aspect of social immunity (*sensu* Cotter & Kilner, 2010a) is the protection of a breeding resource for offspring. Leafcutter ants use secretions from their metapleural glands to combat the bacteria and fungi that threaten their fungus gardens (Nascimento, Schoeters, Morgan, Billen, & Stradling, 1996). This protection of resources is not limited to the social insects; a similar behavior has been found in several species of the burying beetles (Cotter & Kilner, 2010b; Hall et al., 2011; Hoback, Bishop, Kroemer, Scalzitti, & Shaffer, 2004). As described above, the burying beetles breed on carrion and so must compete with microorganisms for the resource (Janzen, 1977). Many species have therefore evolved antimicrobial strategies to overcome this threat (Cotter & Kilner, 2010b; Hall et al., 2011; Hoback et al., 2004; Rozen, Engelmoer, & Smiseth, 2008). Hoback et al. (2004) found protein-based antibacterial activity in the saliva of five of the seven *Nicrophorus* species they tested, and in the anal exudates of two species. This activity could be due to inducible AMPs or lysozymes (Arce et al., 2012)—key humoral immune factors that directly kill or inhibit bacterial growth (Boman & Hultmark, 1987; Gillespie, Kanost, & Trenczek, 1997; Rowley, Brookman, & Ratcliffe, 1990), or POs, which have been shown to have antimicrobial activity in insect hemolymph (Boman & Hultmark, 1987; Gillespie et al., 1997; Rowley et al., 1990). However, it was not clear if this activity was constitutive or induced, or if there was individual variation in activity, as has been shown for other personal immune responses.

These ideas have been addressed using the burying beetle, *N. vespilloides*. Cotter and Kilner (2010b) collected anal exudates from male and female beetles prior to breeding, after mating in the absence of a carcass, and after mating in the presence of a carcass. They then measured both PO activity and lytic activity in individual samples. Lytic activity was absent in the

exudates of nonbreeding beetles, but increased after mating, and increased more sharply if the beetles had access to a carcass. In contrast, PO activity was present in the exudates of nonbreeding beetles, but declined after mating, as has been shown with hemolymph PO levels in other species (e.g. Rolff & Siva-Jothy, 2002). It appears therefore that lysozyme activity is induced when required, similar to the upregulation of constitutively present lysozymes in the hemolymph following infection (e.g. Anderson & Cook, 1979; Boman & Hultmark, 1987; Haine, Pollitt, et al., 2008; Korner & Schmid-Hempel, 2004). A more detailed analysis of the change in exudate antibacterial activity during the breeding cycle has found that activity is rapidly upregulated, peaking at around day 4 of the breeding-bout, levels then decline again as the larvae grow and consume the carcass, and finally reduce back almost to nothing by the time the larvae disperse (Fig. 3.17; Cotter, Littlefair, Grantham, & Kilner, 2013).

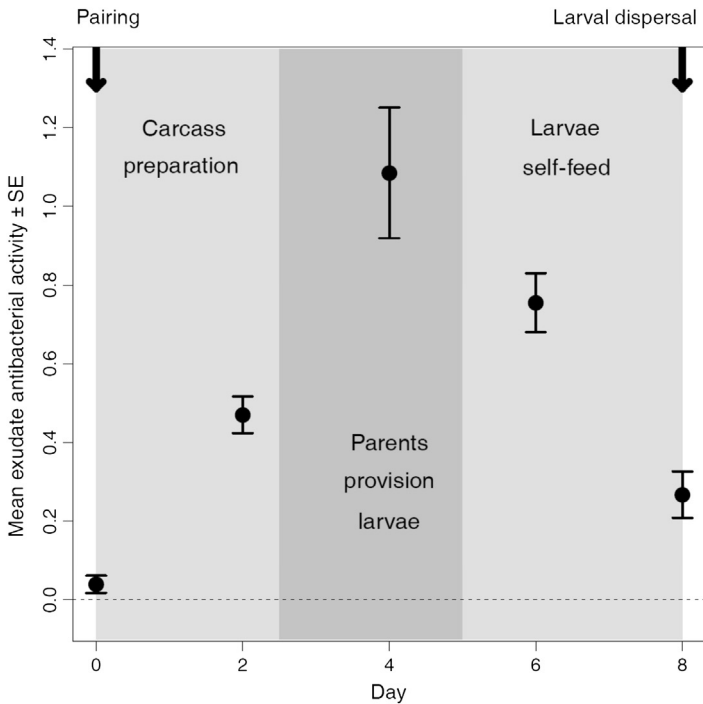
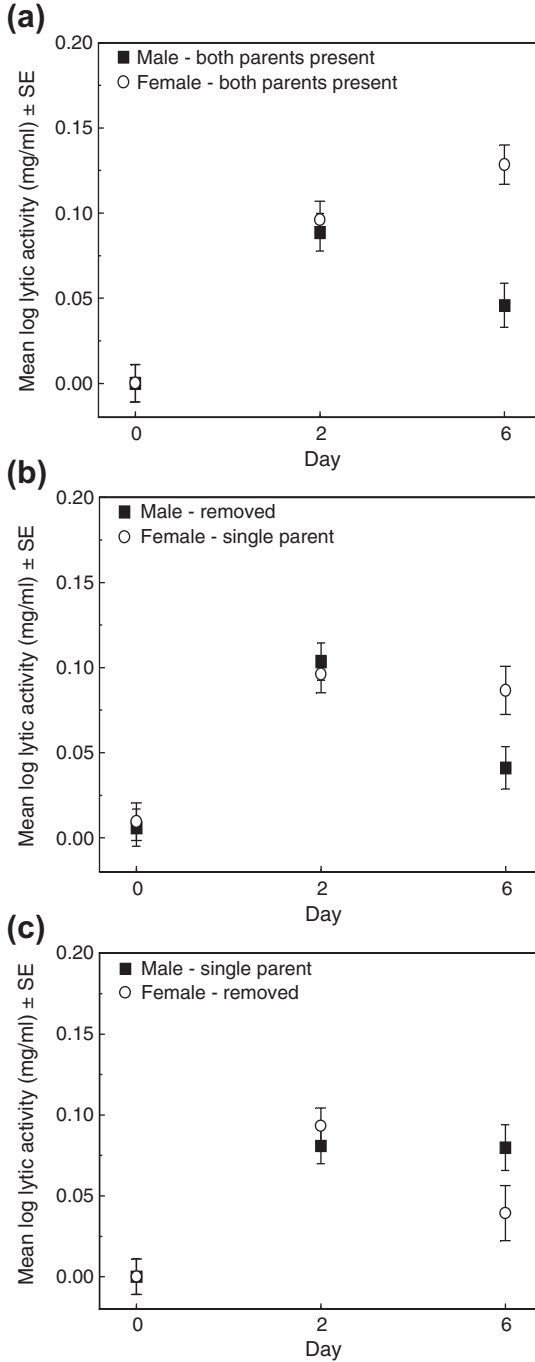


Figure 3.17 Antibacterial activity of female *Nicrophorus vespilloides*' anal exudates over the course of the first reproductive bout in unmanipulated, virgin females. Means and SEs are predicted values from a REML model controlling for female identity. (Reproduced from Cotter et al. (2013)).

Both sexes gain by mounting this social immune defense (Rozen et al., 2008), but it is interesting to ask how this task is divided between them. To address this, Cotter and Kilner (2010b) simulated the situation whereby one of a breeding pair was abandoned or widowed, leaving either the father or mother to bear the brunt of the care. In this situation, males and females responded differently. Whilst there was no effect of widowhood on PO activity, lysozyme-like lytic activity did appear to be more flexible. First, once removed from a carcass, the lytic activity in the exudates of both male and female beetles rapidly declined. For the beetles on a carcass, paired males tended to produce lower levels of antibacterial activity than females, but when widowed, they increased this activity to compensate for the loss of the female (Cotter & Kilner, 2010b). Females, on the other hand, reduced their antibacterial activity when widowed, such that they produced similar levels of activity to single males (Figure 3.18).

One possible explanation for this behavior is a division of labor, as seen in other brood-caring species where the female takes the primary role in specific jobs, such as an alarm calling in yellow warblers (Gill & Sealy, 2004), egg rejection in bush robins (Palomino, Martin-Vivaldi, Soler, & Soler, 1998), and burrow guarding in the tenebrionid beetle, *Parastizopus armaticeps* (Rasa & Heg, 2004). Perhaps females take primary responsibility for protecting the carcass from bacteria, whilst males put more effort into other caring roles (Scott, 1990). In the absence of the female, the male partially compensates by upregulating his activity to the minimum required for carcass maintenance, but perhaps he cannot fully compensate due to the requirement to fulfill other duties. Similarly, in the absence of the male, the female may have to take on a larger share of these other tasks, the extra cost of which reduces the investment she can put into lytic activity (Cotter & Kilner, 2010b). Partial compensation to the reduced partner effort has been predicted to be the optimal response when parents cooperate to rear young (Ratnieks, 1996), and indeed partial (Sasvari, 1986) and even full compensation (Saino & Møller, 1995) for reduced partner effort has been shown in birds with biparental care. However, differential responses by males and females have also been shown in great tits, where females fully compensated for reduced partner effort while males reduced their effort when their partners were handicapped (Sanz, Kranenbarg, & Tinbergen, 2000).

So is the burying beetle's social immune response costly to produce in the same way as the personal immune responses described above? The evidence so far suggests that it is: activity is induced only when required, during breeding; it declines once breeding is completed, and declines



rapidly if the beetle is removed from the carcass. This level of plasticity is likely to have evolved to reduce the usage costs of this antibacterial response. To test this idea in the burying beetle study, it was necessary to force beetles to increase their levels of antibacterial activity above the levels normally required to prepare and maintain a carcass, and then to measure any corresponding decrease in fitness. This was achieved by presenting beetles with carcasses that had been dipped in a bacterial solution, thus reducing the perceived quality of the carcass by mimicking decomposition (Cotter, Topham, Price, & Kilner, 2010). Beetles were then allowed to breed on the carcass, and subsequently allowed to reproduce on clean, nonmanipulated carcasses weekly for the remainder of their lives. As expected, beetles on the bacteria-dipped carcasses produced stronger antimicrobial activity in their exudates and, as a consequence reared, on average, 14 fewer offspring during their lives than control females, representing a 16% decrease in lifetime reproductive output (Fig. 3.19). This is the first evidence that mounting an immune response of any sort (i.e. social or personal) bears associated costs that reduce lifetime reproductive success (LRS), independent of any costs imposed by a parasite (Cotter et al., 2010).

A study on a type of digger wasp called a beewolf, *Philanthus triangulum*, which provisions brood cells with bees upon which their offspring feed, found evidence of similar behavior (Herzner, Engl, & Strohm, 2011). Beewolves embalm the bee corpses in much the same way as the burying beetles, using a cocktail of hydrocarbons (Herzner, Schmitt, Peschke, Hilpert, & Strohm, 2007; Herzner & Strohm, 2007). However, rather than having specific antimicrobial activity (Herzner et al., 2007), the embalming fluid instead prevents water from collecting on the surface of the bee, thus rendering it unsuitable for the fungal growth (Herzner & Strohm, 2007). This social immune response has been shown to be costly. Beewolves were forced to upregulate their production of hydrocarbons by being provided with extra bees to prepare (Herzner et al., 2011). As this species shows no postoviposition parental care, the majority of the cost of reproduction is determined by the hunting and preservation of bees, the first of which was eliminated by providing females with paralyzed bees at the entrance to their nests. Females accepted the extra



Figure 3.18 Mean (\pm SE) lytic activity in anal exudates of male and female beetles at pairing, with a carcass present (Day 0) and 2 and 6 days later where (a) both parents were present to rear the brood; (b) males were removed 2 days after pairing; (c) females were removed 2 days after pairing. (Reproduced from Cotter and Kilner (2010b)).

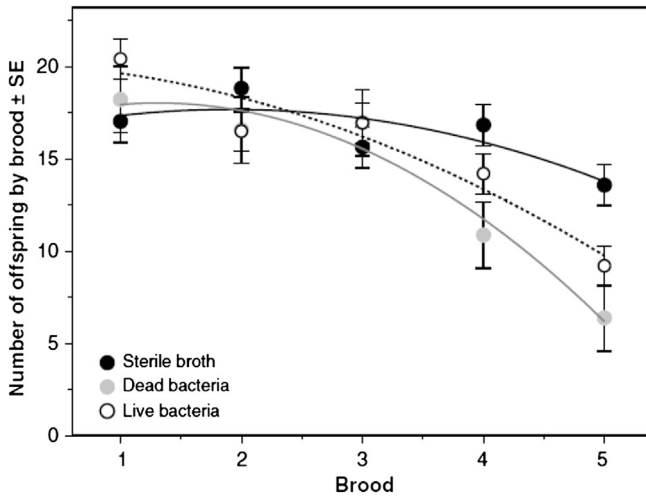


Figure 3.19 The number of offsprings produced by females that had been bred continuously throughout their lives. Females were bred twice on a carcass that had either been dipped in nutrient broth (broth) or dipped in a solution of heat-killed (dead bacteria) or live *Micrococcus lysodeikticus* cells in nutrient broth (live bacteria), subsequent carcasses were untreated. (Reproduced from Cotter et al. (2010)).

bees and embalmed them with hydrocarbons, but this upregulation of embalming activity reduced the amount of hydrocarbons that females were able to provide for the larva in the next breeding attempt (Herzner et al., 2011). As the level of hydrocarbons reduces the risk of fungal infection, and fungal infection compromises larval survival (Herzner et al., 2011), this reduction in hydrocarbons for the next brood cell to be provisioned would likely result in reduced reproductive success. It would be interesting to see if overproduction of hydrocarbons actually results in reduced LRS as has been shown for *N. vespilloides* (Cotter et al., 2010).

This broader definition of social immunity (Cotter & Kilner, 2010a) opens up the opportunity to examine the costs and benefits of immune function in both personal and social contexts in a diversity of species. Whilst examples of social immunity have been identified from a number of insect, amphibian, and fish species, as described above (Section 2.4), the potential costs of these defenses, and how organisms divide resources between social immunity and other traits, are currently lacking.

5.4. Personal versus Social Immunity

If social immunity is costly, then is there a conflict between personal and social immune responses? Whereas personal immune function brings survival

benefits, social immune function serves to improve the likelihood that individuals will successfully propagate their genes—for example by protecting offspring, or reproductive kin, or potentially even nonkin that can offer direct benefits to the focal individual. Personal immune function essentially protects the contribution of lifespan to fitness, while social immune function effectively defends the fecundity component of fitness (Cotter & Kilner, 2010a). A major goal of ecological immunology is to understand why individuals vary in the investment they devote to immune defense. If mounting a social immune response is costly (Cotter et al., 2010; Herzner et al., 2011), then investment in personal immunity and social immunity could well tradeoff against each other. There is circumstantial evidence for exactly this sort of relationship in the honeybee, a species that now possesses many fewer genes for personal immunity than any nonsocial insects, and instead carry genes for a colony-level immune function (Evans et al., 2006; Wilson-Rich et al., 2009).

There are similar examples with antimicrobial resins that are collected by ants and bees for use in the nest. In wood ants, the presence of the resin decreases the bacterial and fungal load in nest material, resulting in lower lytic and AMP activity in worker hemolymph (Castella, Chapuisat, & Christe, 2008). In honeybees, the presence of this resin also decreases bacterial load and so decreases expression of genes connected with personal immunity (Castella et al., 2008; Simone, Evans, & Spivak, 2009). In both of these cases, it is likely to be the reduction in pathogens that leads to the decrease in the immune response, rather than a direct physiological tradeoff between personal and social immune responses.

In contrast, immune-challenged workers of the carpenter ant, *Camponotus pennsylvanicus*, showed *increased* antimicrobial activity in the regurgitates they passed to nest-mates (Hamilton, Lejeune, & Rosengaus, 2011). However, as workers tend to be sterile, a personal immune challenge potentially represents a threat to the colony as a whole and defending inclusive fitness far outweighs the benefits of defending personal lifespan. Under these conditions, it is not surprising that a personal immune challenge triggers the upregulation of social immune defenses (Hamilton et al., 2011). For nonsocial insects, any tradeoff is less likely to be fixed and we might instead see phenotypic plasticity in the way that the tradeoff is balanced. For example, females investing in the social immune defense of their young might temporarily be unable to mount an effective personal immune defense (Cotter & Kilner, 2010a). Similarly, the tradeoff between social and personal immunity might vary with age, or the two forms of immune function may senesce at different rates (DeVeale, Brummel, & Seroude, 2004).

This has been addressed with the personal and social immune responses of the burying beetles (Cotter et al., 2013) by asking if there is a direct tradeoff between the two strategies of immune response, or, in the face of an immune challenge, whether both personal and social immune responses are maintained at a cost to current or future reproduction. To test this, the personal immune system of the female burying beetles was challenged during reproduction and their subsequent investment in the social immune response measured. The LRS was also quantified to assess whether females choose to maintain investment in both forms of immunity at a cost to future reproduction, or whether the costs of a personal immune response are paid by a reduction in the social immune response (Cotter et al., 2013).

Prior to breeding, females were either pierced with a sterile or bacteria-dipped needle, or left unchallenged. They were then provided with a carcass and a mate and allowed to breed. Males were removed on day 2 and exudates were collected from all females on days 2, 4, and 6 of the breeding-bout and their antibacterial activity assessed (Cotter et al., in 2013). After this initial breeding-bout, females were then allowed to reproduce weekly for the remainder of their lives and their exudates collected and analyzed for each breeding-bout (Cotter et al., 2013). Both sterile-wounding and challenge with a nonpathogenic bacterium resulted in the upregulation of antibacterial activity in the hemolymph (Cotter et al., 2013). This translated to an immediate decrease in exudate antibacterial activity during the subsequent breeding-bout, revealing a tradeoff between one aspect of the burying beetle's personal immune defense and one component of its social immune response. Downregulation was only temporary, however, because by the next breeding-bout the social immune response was restored to control levels (Fig. 3.20). However, there was no evidence that mounting a personal immune response reduced LRS. It seems likely that by downregulating expression of the social immune response, challenged females were able to maintain their LRS (Cotter et al., 2013).

Experiments on the congeneric burying beetle, *N. orbicollis*, similarly sought evidence for a tradeoff between the social immune response and personal immunity, but found none (Steiger et al., 2011). However, in this study all beetles were challenged with an artificial macroparasite (a piece of nylon monofilament) and their investment in the social immune response (exudate antimicrobial activity) measured. As both social immune responses and encapsulation activity were upregulated, they concluded that the two traits did not tradeoff. However, it is possible that a tradeoff might have been detected had they included a nonchallenged treatment group with which

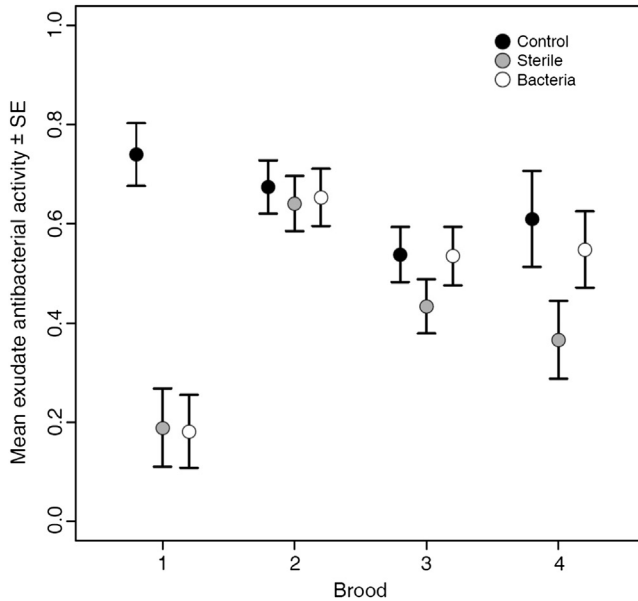


Figure 3.20 Mean exudate antibacterial activity for each brood in unmanipulated females and those whose immune systems had been challenged by wounding with a sterile or bacteria-dipped needle. Females experienced immune challenge prior to the first brood only and were bred repeatedly until death. (Reproduced from Cotter et al. (2013)).

to compare the social immune responses (Steiger et al., 2011). Another possibility is that the tradeoffs occur with certain components of the personal immune response (e.g. antibacterial response) but not others (e.g. encapsulation response). Understanding how organisms prioritize personal or social immune responses in different ecological contexts, and how they are affected by intrinsic factors such as age, state, and sex could be a fruitful area of research, and may shed light on some of the variation we see in the personal immune responses typically measured (Cotter & Kilner, 2010a).



6. CONCLUSIONS AND FUTURE DIRECTIONS

In this review, we have attempted to draw out the complex ramifications of evolving, maintaining, and deploying antiparasite defenses. We have emphasized that these defenses are not limited to the reasonably well understood innate and adaptive immune responses that modulate the host's susceptibility to primary and secondary parasite challenges, but also include a suite of additional behavioral, physical, and chemical defenses that

influence the probability that the immune system will be exposed to those parasites in the first place (Section 2). These defenses may affect not only an individual's chances of resisting or tolerating a parasitic infection, but also those of kin and nonkin in the social group or community (Section 5.3). As such, both individual and social immune responses (*sensu* Cotter & Kilner, 2010a), and their consequences, may be considered as part of the recipients' extended phenotype (Dawkins, 1982), and for eusocial animals in particular, the functional and epidemiological distinction between individual and social immunity may become blurred. We believe that this is an area of eco-immunology that will be particularly fruitful in going forward, especially if the same methodologies used on a limited number of insect species can be transposed to vertebrate species. An obvious candidate to explore in this regard is the immune system of eusocial mole rats. Indeed, a comparative study of different mole rat species varying in their degree of sociality would be particularly interesting. As seen in a comparative study of lepidopteran larvae, group-living animals may evolve very different levels of constitutive immunity from even closely related solitary species (Section 3.4). However, both modeling and meta-analyses indicate that the relationship between sociality and investment in antiparasite defenses may be nonlinear. Indeed, the risk of parasitism may be an important selective force in the evolution of sociality itself. Extending the definition of social immunity beyond its previous restriction to social and eusocial societies allows us to address fundamental questions about immune investment; an understanding of social immune responses may explain much of the variation we see in personal immune defenses, both within and among species, providing a fruitful area for future research. For example, in cases of sex-biased immune investment, "Bateman's principle" has been invoked (Bateman, 1948; Rolf, 2002) (see Section 5.1); however, in species with sex-biased dispersal, we might expect the dispersing sex to exhibit lower investment in personal immunity than the philopatric sex that remains near kin. More studies of this kind on kin-structured bird and mammal populations could provide key insights into sex-biased immune function (Cotter & Kilner, 2010a).

Great advances have been made in the study of antiparasite defenses in the last two decades. This is particularly true of our understanding of the immune system, due largely to the molecular revolution that has facilitated large-scale and detailed analyses of immune gene evolution and expression. A range of techniques such as microarrays, RT-qPCR, RNAi, and next-generation sequencing have revolutionized our thinking and, in conjunction with whole-genome sequencing, has allowed us to better understand how the

immune system has evolved and the mechanisms influencing its expression. The next decade promises further advances and the development of better and faster techniques, especially for nonmodel organisms. This is particularly true, perhaps, for invertebrates, which have already shed new insights into the evolution and plasticity of the innate immune system and phenomena such as immune-priming and immunological memory (Section 2.6), due to their ease of manipulation in the laboratory, short generation times, and ethical considerations. Understanding the epigenetic mechanisms underpinning immune system plasticity is likely to be an important growth area going forward (e.g. Swaminathan, Gajan, & Pile, 2012)—and one that may yield applied benefits in terms of developing new biopesticides that exploit these mechanisms.

One of the areas that is currently benefiting from the molecular revolution is our understanding of the human microbiome, i.e. the totality of microbial genomes resident in the host (e.g. Lederberg & McCray, 2001). The human body contains approximately 10 times more microbial cells than human cells (10^{14} versus 10^{13} ; Savage, 1977), and it has been argued by some that the genomes of these microorganisms should be included as part of the human genome (or at least as part of its “extended phenotype”). This is because of their influence on a whole suite of traits important to their hosts, including nutrition, metabolism, and development (Grice & Segre, 2012; Huttenhower et al., 2012; Yatsunenko et al., 2012). Importantly from the perspective of this review, there are also significant interactions with the immune system (Kau, Ahern, Griffin, Goodman, & Gordon, 2011; Maynard, Elson, Hatton, & Weaver, 2012) and diet (Wu et al., 2011). Our quantification and understanding of the microbiomes of nonhuman vertebrates are only slowly developing, especially outside the lab setting (Benskin, Rhodes, Pickup, Wilson, & Hartley, 2010; Ley et al., 2008; Muegge et al., 2011), and we are on a long way from establishing the functional significance of variation in microbiome communities and its implications for immune defense. Many insect species are also inhabited by large and diverse communities of microorganisms (Brooks, 1963; Dillon & Dillon, 2004; Douglas, 2010). However, studies have tended to focus on a single symbiont, such as *Wolbachia* or *Spiroplasma*. As with humans, insect studies clearly demonstrate that these microbes often play an important role in modulating immune function, parasite resistance, and nutrition. However, the interactions may be complex. For example, it has been shown that some *Wolbachia* strains may protect their hosts against viruses (e.g. Hedges, Brownlie, O’Neill, & Johnson, 2008) whilst others may make them more susceptible (Graham et al., 2012). Those studies that have examined the impact of the entire microbiome suggest that as

microbial diversity increases, so too does resistance to pathogenic microbes, possibly through interspecific competition (Dillon, Vennard, Buckling, & Charnley, 2005). Given that most studies of host–parasite interactions have so far (necessarily) ignored the potential interactions between the microbiome and nutrition, immunity and parasites, it seems likely that future studies that incorporate this information will reveal important new insights into how these complex interactions evolve and develop (Ponton *et al.*, 2013; Fig. 3.21). In particular, the competing nutritional requirements of the host, its immune system, its microbiome, and its parasites have never been considered within the same system. The geometric framework for nutritional ecology (Simpson & Raubenheimer, 2012) provides a robust approach for potentially dissecting apart these complex interactions and predicting a priori the outcome of parasitic infections.

Future studies also need to consider more the epidemiological and population dynamics impact of variation in antiparasite defenses (see Sections 3.2, 5.3 and Section 5.5). The next decade promises to be a growth area in the study of host–parasite interactions and the most exciting research is likely to involve collaborations between ecologists, behaviorists, molecular geneticists, mathematical modelers, and nutritional biologists.

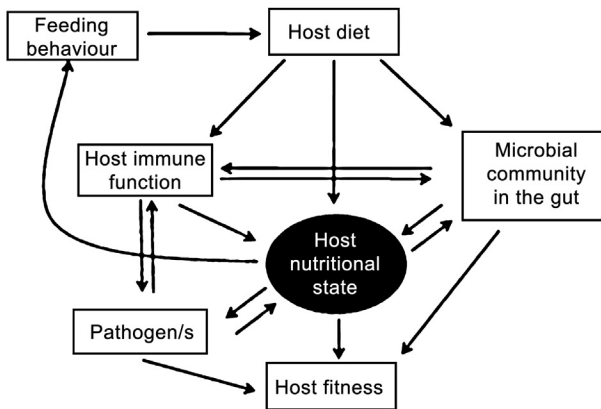


Figure 3.21 The network of interactions between nutrition and immunity. Diet affects host nutritional state and immune status, both of which interact with microbial symbionts, commensals, and pathogens to affect the fitness of all partners. Because nutrient feedbacks modulate the host-feeding behavior, the potential exists for the host to adjust its diet to optimize its microbial interactions and increase resistance to infection. Alternatively, parasites and pathogens might subvert host-feeding behavior to their nutritional advantage. (Reproduced from Ponton, Wilson, *et al.* (2011)).

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