Trade-offs in insect disease resistance

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Abstract

The ability to mount an efficient immune response should be an important life-history trait as parasitism can impact upon an individual's fecundity and survival prospects, and hence its fitness. However, immune function is likely to be costly as resources must be divided between many important traits. Whilst many studies have examined host resistance to particular parasite types, fewer have considered general immune responses. Studies that have considered general immune responses tend to do so in vertebrate models. However, the complexity of the vertebrate immune system makes the examination of evolutionary aspects of immune function difficult.

Using larvae of the genus *Spodoptera* (Lepidoptera: Noctuidae) as a model system, this study examines genetic and phenotypic aspects of innate immunity. The aims were to assess the levels of additive genetic variation maintained in immune traits, to consider possible costs that could maintain this variation, and to assess the role of phenotypic plasticity in ameliorating those costs.

A key finding of this study was that high levels of additive genetic variation were maintained in all of the measured immune traits. Analysis of the genetic correlations between traits revealed potential trade-offs within the immune system and between immune components and body condition. In addition, it was shown that larvae living at high densities invest more in immune function than those living in solitary conditions, suggesting that larvae can minimise the costs of immune function by employing them only when the risk of pathogenesis is high.

Declaration

This thesis is the result of my own research, and no part of this thesis has been submitted in application for a higher degree at this, or any other institution. All collaborative involvement has been duly acknowledged.

Signed · **Date** 19.8.02.

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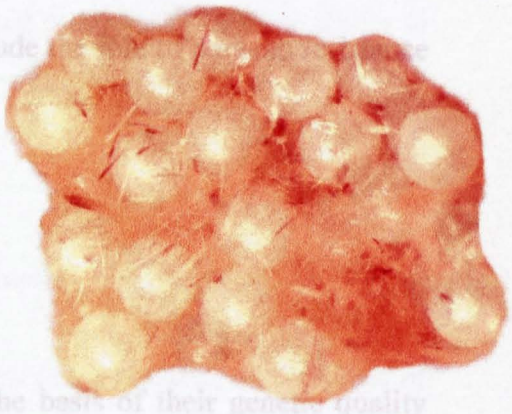
Figure caption: newly laid *S. littoralis* eggs camouflaged with hairs from the tip of the female's abdomen.

Chapter 1

General Introduction



Ecological immunology

Parasites are ubiquitous; the effects they have on their hosts range from mild fitness costs to complete inhibition of reproduction, or death. In the face of this onslaught, hosts have evolved a complex array of behavioural and physiological mechanisms to either prevent infection, or minimise the costs once an infection has taken hold. Despite the obvious impact of parasitism on host fitness, interest in the ecological and evolutionary aspects of immune function is a relatively recent development (Sheldon & Verhulst 1996).

Parasites and the evolution of their hosts

Haldane (1949) was the first to suggest that parasites could exert a significant selective pressure on their hosts and so play a key role in evolutionary biology. However, it is only in the last two decades that interest in this idea has become widespread. In 1980, Hamilton proposed the hypothesis that parasites could play a central role in the maintenance of genetic diversity in populations, and provide an explanation for the prevalence of sexual reproduction despite its apparent costs (Hamilton 1980). This idea was expanded to include the role of parasite resistance in sexual selection (Hamilton & Zuk 1982).

Parasite-mediated sexual selection

The suggestion that mates could be chosen on the basis of their genetic quality (Trivers 1972; Zahavi 1975; Andersson 1982) ran counter to evolutionary theory, which states that alleles under strong directional selection should quickly become

fixed in the population, thus leaving no variation in genetic quality on which a choice could be based. However, co-evolution between parasite and host could maintain variation in resistance. Simply, selection will favour hosts that are resistant to the most common parasites. As these increase in frequency, the parasites will be selected to overcome that resistance. The cycles of co-adaptation that ensue prevent any one host genotype from becoming fixed and so variation is maintained. This allows mate choice on the basis of genetic quality; by selecting males that are resistant to the most common parasites, females will produce resistant offspring.

However, for mate choice to occur, females must be able to discriminate between males on the basis of their genetic quality. Zahavi's handicap hypothesis provides the means for males to signal their quality to females (Zahavi 1975). This hypothesis states that the signal must be costly in order to provide reliable information about the signaller and thus prevent cheating. According to the Hamilton and Zuk hypothesis, as parasites are expected to have a negative effect on host condition, only the most resistant individuals should be able to invest strongly in such signals (Hamilton & Zuk 1982). Therefore, by choosing a bright, highly ornamented mate, an individual is in fact choosing a mate based on their ability to resist infection.

The immunocompetence handicap hypothesis

The original theory linking parasites and "good genes" was based on the costs of parasitism. The focus has since shifted to the costs of maintaining the immune system and mounting an immune response. The immunocompetence handicap

hypothesis (ICHH) proposed that the dual role of androgens could provide a mechanistic link between the immune system and sexual signals (Folstad & Karter 1992). Testosterone simultaneously increases the expression of sexually selected traits and suppresses immune function. Therefore, males with the most extravagent sexual traits would be the ones most able to cope with immunosuppression. An alternative version of the ICHH omits hormones entirely and proposes that, based on the rule of allocation, an individual must divide finite resources between immune function and the ornament, i.e. there is a trade-off between the two traits. Individuals in better condition will have more resources to allocate to each trait and so will suffer less from the costs of increased ornament size (Sheldon & Verhulst 1996).

Costs of resistance

The idea of costs or trade-offs between life-history traits is central to the study of evolutionary ecology. Stearns (1989) defines trade-offs as "the costs paid in the currency of fitness when a beneficial change in one trait is linked to a detrimental change in another". Trade-offs can occur between any life-history traits, the most commonly studied being trade-offs between current and future reproduction and between number and fitness of offspring.

Phenotypic studies

More recently, the costs of immune function have been incorporated into life-history analysis. In addition to their role in sexual selection, costs to immune

function could provide the link between reproductive effort and future reproduction (Sheldon & Verhulst 1996). Evidence that reproductive individuals have higher parasite loads suggests that resources are channelled into reproduction at the expense of the immune system (Festa-Bianchet 1989; Gustafsson *et al.* 1994; Sorci *et al.* 1996; Allander 1997; Nordling *et al.* 1998). As parasites have a negative effect on body condition, this could impact on future reproduction (Sheldon & Verhulst 1996). Direct measurements of immune parameters, and manipulation of the immune system during reproduction has provided further evidence of the trade-off between reproductive effort and immunity (Siva-Jothy *et al.* 1998; Williams *et al.* 1999; Ilmonen *et al.* 2000; Hosken 2001; McKean & Nunney 2001; Rolff & Siva-Jothy 2002).

The experimental manipulations of traits such as reproductive effort and immune function, and the examination of the phenotypic correlations between them are essential for understanding the rules governing resource allocation and their consequences in the individual. Natural selection acts on phenotypes but the response to selection depends on the genotype. Therefore, for phenotypic trade-offs to have evolutionary significance, the traits must negatively covary at the genetic level (Reznick 1985; Stearns 1992).

Genetic studies

There are two methods by which the genetic basis of trade-offs can be measured – pedigree analysis and selection experiments. First, by using pedigree analysis the heritability of traits (the proportion of the phenotypic variance that is accounted for by additive genetic variance), and the genetic correlation between them, can be

estimated (Falconer & Mackay 1996). Second, by artificially selecting for one trait, the correlated responses in other traits can be measured. The heritability of the selected trait can be calculated from the response to selection; the response is equal to the heritability of the trait multiplied by the selection differential (the strength of selection).

Specific resistance

Many studies have used these methods to examine the genetic basis of trade-offs with immunity. Several studies have examined the costs of resistance to specific parasites or pathogens. Fuxa and Richter (1988, 1998) artificially selected lines of the noctuid moths, *Spodoptera frugiperda* and *Anticarsia gemmitalis,* that were resistant to their nuclear polyhedroviruses (NPV). Resistant *Spodoptera frugiperda* showed a shorter adult lifespan, lower fecundity and lower egg viability than controls, and resistant *Anticarsia gemmitalis* had lower fecundity and pupal weights with an increased lifespan.

Populations of the Indian mealmoth, *Plodia interpunctella,* were reared with and without a granulosis virus for two years (Boots & Begon 1993). After this time, it was found that the population exposed to the virus became 1.96 times more resistant than the control. Correlated with this increase in resistance was a lengthening of development time, a reduction in egg viability and an increase in pupal weight. It was calculated that the resistant population suffered a 15% reduction in fitness.

Groeters *et al* (1994) found that a strain of the diamondback moth, *Plutella xylostella*, resistant to the bacterium, *Bacillus thuringiensis*, had lower survival, fecundity and percentage egg hatch than the susceptible strain. Milks (1997) looked

at the susceptibility of the cabbage looper, *Trichoplusia ni,* and found that females with the highest LD50 for TnSNPV laid fewer eggs. *Aedes aegypti* mosquitoes, refractory to the avian malaria parasite, *Plasmodium gallinaceum*, had a shorter development time than the susceptible line, but the resulting adults were smaller, took smaller blood meals and laid fewer eggs (Yan *et al.* 1997).

Work on the fruit fly, *Drosophila melanogaster* and its parasitoids, *Asobara tabida*, *Leptopilina boulardii* and *Pachycrepoideus vindemia,* has examined the costs of both maintaining an effective immune system and of using it (Kraaijeveld & Godfray 1997; Fellowes *et al.* 1998a). The studies looked at the ability of *Drosophila* larvae to encapsulate parasitoid eggs. This is easy to measure in this species, as the encapsulated egg is visible in the abdomen of the surviving adults. It was found that in lines selected for enhanced parasitoid resistance the encapsulation rate increased from 5% to 60% in five generations. Trade-offs were apparent only in stressful conditions such that larval competitive ability suffered if there was a lack of food (Kraaijeveld & Godfray 1997). The actual costs of surviving attack by *Leptopilina boulardii* were reduced adult fecundity and size, and an increased probability of attack by the pupal parasitoid, *Pachycrepoideus vindemiae* (Fellowes *et al.* 1998b).

Webster and Woolhouse (1999) artificially selected lines of the snail, *Biomphalaria glabrata,* resistant and susceptible to infection with the schistosome, *Schistosoma mansoni,* and found resistant lines to have lower fecundity than susceptibles in the absence of parasites.

Selection for resistance to parasites in vertebrates is mainly confined to murine models or domesticated animals, and their nematodes. The costs of resistance seem to be less clear than in the invertebrate studies, but seem to involve a dichotomy

between different arms of the immune system. Resistant lines show increased inflammatory, Th-2 type responses, effective against multicellular parasites, but reduced Th-1 type responses which control microparasitic infection, suggestive of a possible trade-off within the immune system (Grencis 1997; Shaw *et al.* 1999; Gill *et al.* 2000).

Non-specific resistance

Many studies have measured the costs of non-specific resistance by examining components of the immune system; this technique is more commonly used in vertebrate than in invertebrate studies. A technique frequently used in chickens is to select for high and low antibody production after inoculation with sheep red blood cells. One such study showed high resistance to three pathogens and feather mites, but low resistance to two bacterial pathogens in the high responder lines compared to the low responder lines (Gross 1980). In addition, chickens from the low responder lines gained weight more rapidly. A more recent study on chickens using the same selection criteria found that high responder lines had lower lymphocyte mitogenic activity compared to lines selected for low antibody production (Gehad *et al.* 1999). This suggests that selection for a greater humoral response may be at the expense of cellular responses. A further study examined the relationship between the antibody response and comb size, which is known to be important in inter- and intrasexual selection (Verhulst *et al.* 1999). Comb size was smaller in the high responder line relative to the low responder line suggesting a trade-off between ornamentation and immune function.

Several invertebrate studies have measured components of the immune system, but evidence for costs is rare. Ryder and Siva-Jothy (2001) found that haemocyte density and the volume of cells covering an artificial parasite were positively genetically correlated with each other, and with body size in the house cricket, *Acheta domesticus*. Selection for increased resistance to the parasitoid, *Asobara tabida*, in the fruit fly, *Drosophila melanogaster* resulted in a doubling of the haemocyte density relative to control flies (Kraaijeveld *et al.* 2001). As selected flies had reduced larval competitive ability, it is possible that there is a trade-off between haemocyte density and feeding rate. Twelve generations of forced polyandry in the dung fly, *Sarcophaga stercoraria*, resulted in increased testis size and a corresponding decrease in phenoloxidase activity in males (Hosken 2001). This suggests that there is a trade-off between one form of immunity and sperm production in this species.

Resistance to multiple parasites

The majority of invertebrate studies measure the costs associated with resistance to a specific parasite. However, resistance to one parasite may not confer resistance to others. Ham *et al* (1996) selected lines of the yellow fever mosquito, *Aedes aegypti*, that displayed high or low inducible antibacterial responses to a gram-negative bacterium. Though high lines showed a significant increase in resistance to microfilariae they did not show increased antibacterial activity to a gram-positive bacterium. Resistance in *Drosophila melanogaster* to one parasitoid, *Asobara tabida*, resulted in complete susceptibility to another, *Leptopilina boulardii* and vice-versa (Benassi *et al.* 1998). Mosquito-malaria models have provided similar

results, with lines refractory to one species of *Plasmodium* being susceptible to other species (Graves & Curtis 1982; Somboon & Takagi 1999). Vertebrate studies have shown that there may, in fact, be trade-offs within the immune system, as up-regulation of certain components seems to result in down-regulation of others (Grencis 1997; Gehad *et al.* 1999; Shaw *et al.* 1999; Gill *et al.* 2000), but comparable studies in invertebrates are lacking.

Assessing immunocompetence

In order to assess "immunocompetence", its relationship with fitness and any associated costs, it is important to measure a range of non-specific immune parameters in order to quantify the costs of maintaining an immune system capable of responding to multiple parasite types (Siva-Jothy 1995; Owens & Wilson 1999; Norris & Evans 2000). However, the importance of the environment in which traits are measured must not be overlooked. Many life-history traits are phenotypically plastic, that is, a single genotype can display a range of phenotypes depending on the environment in which it is raised. Assuming that at least some phenotypically plastic responses are adaptive, costs associated with increased trait expression can then be minimised by employing them only in the environments where they are most likely to be needed. For example, *Daphnia pulex*, grown in the presence of a predator, *Chaoborus americanus,* develop "neck teeth" which reduce the risk of predation. By allocating resources to such a structure, only when it is required, the phenotypically plastic genotype can maximise its fitness in both environments (Lessells 1991).

Phenotypic plasticity in response to parasitism

There is some evidence that responses to parasitism, or the risk of parasitism, display phenotypic plasticity. The snail, *Biomphalaria glabrata*, increased its egg production when exposed to cercariae of the schistosome, *Schistosoma mansoni*, thereby increasing allocation to current reproduction, when the risk of death was high (Minchella & Loverde 1981). Similar results have been found in crickets exposed to bacteria; though infection by a parasitoid did not elicit the same response (Adamo 1999). Many invertebrates display a "behavioural fever" in response to parasitism, whereby the preferred body temperature is increased by basking, either to improve the efficiency of the immune response or to retard the development of the parasite (Louis *et al.* 1986; Magnuson *et al.* 1986; Boorstein & Ewald 1987; Ramos *et al.* 1993; Inglis *et al.* 1996; Adamo 1998; Blanford *et al.* 1998; Wilson *et al.* 2002). In addition, there is evidence from the bumblebee, *Bombus terrestris*, that infected individuals reduce their body temperature in order to retard parasite development (Müller & Schmid-Hempel 1993).

Phenotypic plasticity in immune function

There is also evidence for plasticity in resistance to pathogens, with some species showing reduced susceptibility to infection when reared at high densities (Kunimi & Yamada 1990; Goulson *et al.* 1995; Reeson *et al* 1998; Wilson *et al.* 2002). Assuming that the maintenance of an effective immune system is costly, by increasing the allocation of resources to immune function only when the risk of parasitism is high, i.e. at high densities, costs can be minimised (Wilson & Reeson 1998). However, direct evidence for plasticity in immune function is scarce and

warrants further investigation (Reeson *et al* 1998; Barnes & Siva-Jothy 2000; Wilson *et al.* 2002).

Insects as a model system

Although there is substantial evidence for trade-offs with resistance to specific parasites, few studies have examined the underlying genetic variation in components of the immune system. The possibility that phenotypic plasticity in immune function could minimise its costs underlines the importance of understanding how these components vary across environments. The majority of the work on immune system components comes from vertebrate studies but the complexity of the vertebrate system makes it a less than ideal model with which to address fundamental questions in evolutionary ecology. However, insects can provide an excellent model system with which to examine the rules by which individuals allocate resources to immune function. The insect immune system is significantly simpler than the vertebrate system. There is no acquired immunity; insects do not possess lymphocytes or immunoglobulins (Gillespie *et al.* 1997). Instead, a combination of cellular and humoral factors work in concert to recognise the invader and remove it, either by killing it, or simply by isolating it from the rest of the body. This simple but effective system shares many fundamental characteristics with the innate immune system of vertebrates, with many of the basic factors showing remarkable homology across many species (Vilmos & Kurucz 1998). Therefore, a thorough investigation of both phenotypic and genetic aspects of immunity in insects may give insights into the role of immune function in life-history evolution in a wide range of taxa.

The insect immune system

From the point of view of a parasite or pathogen, gaining access to an insect is made considerably more difficult due to their possession of an exoskeleton. The cuticle covers the outside of the body as well as the foregut, the hindgut and the trachea. The most exposed part of the insect body is the midgut and this is often protected by the possession of a peritrophic membrane (Chapman 1998). Therefore, invaders have to overcome this first line of defence before the haemocoel, and its suite of immune responses, are even reached.

The cuticle

The cuticle is a non-living matrix of protein and carbohydrate, secreted by the epitheliar cells (Wolfgang & Riddiford 1986). In addition to providing physical protection for the insect, it is covered in a variety of medium-chain fatty acids and hydrocarbons, and contains enzymes that can provide protection against parasites and pathogens (Briese 1981; Bidochka & Khatchatourians 1992; St Leger 1993; Ashida & Brey 1995).

Penetration by fungi and parasitoids

Parasitoids and entomopathogenic fungi gain entry to the insect body via the cuticle. Entomopathogenic fungi have wide host ranges, infect different stages of host and often cause natural epizootics (Varela & Moreles 1996; Hajek 1997). Infection of a host starts with the passive contact of spores with the cuticle. The spores need to

maintain contact long enough for germination to occur. Some spores secrete a mucilaginous coat whilst others maintain attachment via hydrophobic forces. Specific recognition systems then cause the growth of germ tubes or appressoria. Secreted enzymes from the appressoria can alter the cuticular surface, (St Leger *et al.* 1986) aiding attachment and growth through the cuticle (Bidochka & Khatchatourians 1990). Narrow infection pegs develop and the growth of penetrant hyphae is via the route of least resistance. The fungus develops, eventually killing the host and new spores are produced. For parasitoids that lay their eggs inside the host, gaining access to the insect's body is less of a problem than for fungi as the female merely punctures the cuticle with her ovipositor. However, for ectoparasitoids, the eggs and larvae must be able to resist both cuticular and haemolymph defences in order to survive.

Physical and chemical properties of the cuticle

The fatty acids and hydrocarbons present on the cuticle form the insect's first line of defence. If a fungal pathogen is unable to utilise these compounds it will be unable to grow through the cuticle. The fungus, *Beauvaria bassiana* (Hyphomycetes) has been shown to use few of the available fatty acids; two other fungi in the same class *Metarhizium anisopliae* and *Nomuraea rileyi,* are able to utilise some of the hydrocarbons. This allows these species to penetrate cuticles refractory to most other microorganisms (Briese 1981; Bidochka & Khatchatourians 1992; St Leger 1993).

The second defence attribute of the cuticle is its mechanical strength. 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 *et al.* 1988; Hajek & St. Leger 1994). Verhoog *et al.* (1996) found that the ovipositor of the parasitoid wasp *Venturia canescens,* appeared to become stuck in the cuticle of a melanic form of the Mediterranean mealmoth *Ephestia kuehniella,* suggesting that melanin toughened the cuticle, making it harder to pierce.

Melanin also has chemical properties that may inhibit fungal growth. When the crayfish-parasitic fungus *Aphanomyces astaci* was grown on agar containing melanin or any of its constituent quinones there was significant inhibition of fungal growth (Söderhäll & Ajaxon 1982). In addition, melanized cuticles of the tobacco hornworm, *Manduca sexta* were significantly more resistant to fungal penetration than unmelanized cuticles (St. Leger *et al.* 1988).

Cuticular phenoloxidase

Melanin is the end-product of the prophenoloxidase enzyme cascade, present in the cuticle, midgut and haemolymph. Phenoloxidase (PO) is a copper containing enzyme that catalyses oxygenation of mono-phenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-quinones (Ashida & Brey 1995). Three known forms of PO occur in the insect cuticle: granular PO, responsible for the darkening of the cuticle at ecdysis, laccase-type PO, which is thought to be involved in sclerotization and hardening of the newly formed cuticle, and injury or wounding PO (Ashida & Brey 1997). It is injury or wounding PO that may play a role in immune function. In the silkworm *Bombyx mori*, PO is synthesised in the haemocytes before being transported to the cuticle via the integumental epithelia (Asano & Ashida 2001).

Cuticular PO is subsequently activated when it is punctured or stimulated by microbial cell wall components such as peptidoglycan, β-1,3 glucan and lipopolysaccharide, resulting in the production of toxic quinones and melanin (Chapman 1998). In addition to sealing wounds and preventing bacterial infection, PO can be involved in the melanization of fungal hyphae before they reach the haemocoel (Tanada & Kaya 1993), and the melanization of eggs and larvae of ectoparasitic wasps.

The midgut

The midgut is the only part of the insect not covered by the cuticle and so is an important route of entry for many pathogens. A thin envelope called the peritrophic membrane separates food and gut flora from the midgut epithelia. The peritrophic membrane is comprised of chitinous microfibrils embedded in an extra-cellular matrix of proteins and glycoproteins. Although the membrane must be permeable in order to digest food, the pores are too small for most bacteria providing some protection against infection (Chapman 1998).

Penetration by viruses

Viruses typically enter the host via this route after ingestion. The occlusion bodies of nuclear polyhedroviruses (NPV's) are ingested by the larvae, they dissolve in the midgut and the virions are released (Federici 1993). With the help of a viral encoded protein the virions pass through the peritrophic membrane and invade the midgut epithelial cells (Begon *et al.* 1993; Wang & Granados 1998). The infected midgut cells produce budded virus, which is equipped with GP64, an envelope

fusion protein that is necessary for the infection of neighbouring cells (Monsma *et al.* 1996). The tracheoblasts and tracheal epidermal cells associated with the midgut are the next to be infected, the tracheal system giving the virus access to the rest of the insect's tissues (Engelhard *et al.* 1994; Washburn *et al.* 1995). Once the virus has infected most of the body cells, occluded virions are produced, the cells lyse and the body of the host liquefies, releasing billions of polyhedra into the environment to be ingested by fresh hosts (Federici 1993).

Physical and chemical properties of the midgut

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 *et al.* 1989). The virus can beat this defence by replicating more quickly than the cells are sloughed, or by infecting the regenerative cells (Keddie *et al.* 1989). PO activity has been reported from the midgut and in *Helicoverpa zea,* refractory to AcMNPV infection, virally infected midgut cells were found to be melanized, presumably via the action of midgut PO (Washburn *et al.* 1996).

Insect susceptibility to viruses decreases with larval age, with later instars, in some species, showing total immunity. A study on NPV infection in the noctuid moth *Heliothis viriscens,* suggested that the changing proportions of midgut cell types prior to pupation in fifth instar larvae may play a role in decreasing susceptibility to infection (Wang & Granados 1998). Furthermore, in the noctuids *Helicoverpa punctigera* and *Trichoplusia ni*, developmental resistance was observed in larvae infected orally but not intra-haemoceolically, suggesting that the increase in

resistance is linked to changes in the midgut (Teakle *et al.* 1986; Engelhard & Volkman 1995).

The haemocoel and haemolymph

Once in the haemocoel, small invaders, such as bacteria or fungal protoplasts, may be phagocytosed by circulating haemocytes. Larger organisms, such as parasitoid eggs, are encapsulated, a process by which the parasite is covered by a capsule of haemocytes, which then hardens and melanizes (Götz 1986). As insects rely on the tracheal system for oxygen transport, there are no haemocytes comparable to vertebrate erythrocytes. Haemocytes are highly variable both within and across species making classification difficult. However, the main cell types are the plasmatocytes, granulocytes and prohaemocytes. The plasmatocytes are the most abundant cell type, often comprising 30% of the total haemocyte count. They are smooth, often tapered in shape and are involved in phagocytosis and encapsulation. The granulocytes are rounded and contain numerous granules, which are discharged onto the surface of invading organisms as part of the defence response. Prohaemocytes are stem cells from which other haemocyte types are formed. They are small and usually comprise less than 5% of the total cell count (Chapman 1998). The haemolymph also contains a variety of antibacterial, antiviral and antifungal proteins, some of which are rapidly synthesised in response to infection (Boman & Hultmark 1987).

Recognition of non-self

First, the host has to recognise a pathogen in the haemocoel as non-self. It has been shown that hydrophobicity and charge interactions are involved in recognition of non-self, as well as cell-bound and circulating lectins (Olafsen 1986). These are proteins with a high binding specificity to particular cell-bound sugar residues (Boman & Hultmark 1987). In addition, phenoloxidase is thought to play an important role in non-self recognition in many insect species (Ratcliffe *et al.* 1984; Hagen *et al.* 1994). It is activated by microbial cell wall compounds, such as β-1,3-glucan, peptidoglycan and lipopolysaccharide (Pye 1974; Ashida & Brey 1997). Products of the cascade, referred to as "sticky proteins," may have an opsonic role as they can attach to a foreign body, possibly immobilising it (Nigam *et al.* 1997), and either stimulate phagocytosis or encapsulation directly, or cause the degranulation of granulocytes which in turn stimulate the plasmatocytes (Söderhäll & Smith 1986). How the haemocytes find the invader is unclear, although chemotaxis may play a part. It has been shown *in vitro* that plasmatocytes are attracted to bacteria and fungi (Ratcliffe *et al.* 1985). Another theory is that haemocytes are not attracted to an invader but are stimulated upon accidental contact (Götz 1986).

Cellular responses - phagocytosis and encapsulation

Once the haemocytes have come into contact with a small pathogen, such as bacteria, they attach to it. This may be facilitated by opsonic factors present in the haemolymph, by the release of an enzyme by the granulocytes, or by cell-bound

recognition factors (Yoshino 1986). Following attachment, the microorganism is ingested. Either the cell membrane invaginates or pseudopodia are formed, enclosing the organism in a phagocytic vacuole. Lysosomes are released into the vacuole, digesting the organism, following which undigested particles are exocytosed (Tanada & Kaya 1993).

Encapsulation is the formation of an envelope around an invading organism, biotic or abiotic, that is too large to be phagocytosed. Encapsulation can be cellular or humoral. Cellular encapsulation involves the formation of a three-layered envelope. The first is the provoking agent surrounded by disintegrated and melanized blood cells, the second consists of flattened haemocytes and the third of normally shaped haemocytes in loose connection (Götz 1986).

The first stages of non-self recognition and attachment are the same as for phagocytosis. The granular cells disintegrate, releasing material that sticks to the foreign surface. This is either an enzyme that then attracts components of the prophenoloxidase cascade, or phenoloxidase itself. The granulocytes then attach to the foreign surface and in turn attract plasmatocytes which form a layer of flattened cells. The plasmatocytes also form microtubules and microfilaments giving the envelope strength. Melanization commonly occurs soon after the onset of encapsulation, it does not always occur, however, and the level of melanization is variable and dependant on the foreign body; biotic factors provoke a greater level of melanization than abiotic factors. Finally, the outermost plasmatocytes leave the envelope. It could be that they are attracted by a signal from the disintegrated granulocytes and once these are covered the signal weakens and the last plasmatocytes leave (Ratcliffe 1993).

Humoral responses – antimicrobial proteins

Many different types of antimicrobial proteins have been isolated from insect haemolymph, though most research has focussed on phenoloxidase (PO) and antibacterial proteins (Briese 1981; Olafsen 1986; Boman & Hultmark 1987; Funakoshi & Aizawa 1989; Jones *et al.* 1989; Bidochka *et al.* 1997). The most important antibacterial proteins are lysozymes, cecropins and attacins. These immune-proteins are rapidly synthesised in response to infection, primarily in the fat body (Boman & Hultmark 1987), although there have been reports of antibacterial protein synthesis in haemocytes and cuticular epithelial cells (Brey *et al.* 1993).

Haemolymph phenoloxidase

Phenoloxidase is a copper-containing protein closely related to invertebrate haemocyanins. Indeed, the haemocyanin present in tarantula haemolymph has been shown to have phenoloxidase activity (Decker 1998). PO's are highly reactive and are stored as an inactive precursor prophenoloxidase (pro-PO). Several isoforms of haemolymph PO have been isolated from a single species, two from the silkworm (Asano & Ashida 2001), two from the tobacco hornworm (Jiang *et al.* 1997) and six from the yellow fever mosquito (Müller *et al.* 1999). Pro-PO has been reported from the plasma as well as certain haemocytes. Oenocytoids, large cells found only in the Lepidoptera, have been shown to contain large amounts of pro-PO, (Ashida & Brey 1997). Granulocytes have also been reported to contain PO (Söderhäll & Smith 1986), however, more recent methods of detection found PO in the coagula of degranulated granulocytes and spherulocytes but not in whole cells (Ashida &

Yoshida 1988). However, as PO lacks a signal sequence required for secretion, the mechanism by which PO is secreted into the haemolymph is unclear (Ashida & Brey 1997). PO is activated by microbial cell wall components, such as peptidoglycan and zymosan, in the presence of Ca^{2+} via a serine protease cascade, though the role of Ca^{2+} is unclear. PO is thought to be involved in non-self recognition, as well as the encapsulation of larger organisms, and so plays a crucial role in the insect immune response (Ashida & Brey 1997).

Lysozyme, cecropins and attacins

Lysozyme was the first antibacterial factor purified from insect haemolymph and its structure is remarkably similar to lysozymes found in chickens (Boman & Hultmark 1987). Lysozyme is bactericidal to some gram-positive bacteria, but it is likely that its main function is to work in concert with cecropins and attacins. Cecropins were first discovered in the giant silkworm moth, *Hyalaphora cecropia*, and several have been isolated from this species alone (Boman & Hultmark 1987). Since their discovery, cecropins have been isolated from several Lepidopteran species, some Diptera and even some mammals (Vilmos & Kurucz 1998). Cecropins have activity against gram positive and gram negative bacteria (Boman & Hultmark 1987). Attacins, larger proteins than cecropins, have been shown to have good activity against relatively few bacteria. As they act on the outer membrane of the bacterium, it is likely that they facilitate the action of cecropins and lysozymes (Boman & Hultmark 1987).

Summary

In summary, the insect immune system has two main components, the cellular component comprising phagocytic blood cells and cells involved in the encapsulation response, and the humoral component, comprising a suite of antimicrobial proteins. The humoral component can be further subdivided into antibacterial proteins, which are rapidly produced in the fat body in response to infection, and phenoloxidase, an enzyme produced in the haemocytes and then transported to the cuticle and midgut. PO is involved in the recognition of non-self, encapsulation of parasites in the haemocoel, midgut and cuticle, and melanization of the cuticle.

Study aims

Although many recent studies have focussed on the role of parasites in the evolution of life-histories (Hamilton & Zuk 1982; Folstad & Karter 1992; Sheldon & Verhulst 1996), general mechanisms of host resistance, in the form of non-specific immune responses, have received less attention. Using larvae of the genus *Spodoptera* (Lepidoptera: Noctuidae) as a model system, this study examines genetic and phenotypic aspects of innate immunity. The aims are threefold: to assess the levels of additive genetic variation maintained in immune traits, to consider possible costs that could maintain this variation, and to assess the role of phenotypic plasticity in ameliorating those costs.

The Armyworms *Spodoptera littoralis* and *Spodoptera exempta*

The Egyptian cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae; Figure 1.1a,b), is a polyphagous species found in Africa and the Near East. It is a serious crop pest, feeding on strawberry, artichoke, fodder crops, maize and cotton. The African armyworm, *Spodoptera exempta* (Lepidoptera: Noctuidae; Figure 1.1c,d), mainly feeds on monocotyledonous plants such as maize and wheat. It is widespread throughout Southern Africa, but is also found in parts of Asia and Australia. Females of both species lay their eggs in clusters on the host plant and camouflage them with hairs from the tip of the abdomen. After hatching, *S. exempta* larvae disperse on fine, silken threads; *S. littoralis* larvae tend not to disperse unless food is unavailable. Both species can occur at very high densities, though outbreaks are much more extreme in the migratory *S. exempta,* which can exceed densities of 1000 larvae per square metre.

Both species display density-dependent phase polyphenism; a number of the life-history traits are phenotypically plastic with respect to population density, such as growth rate, pupal weight and larval activity (Hodjat 1970). The most striking difference between solitary and crowd-reared larvae, however, is the level of cuticular melanization. At low densities, *S. littoralis* larvae tend to be pale brown/grey but when exposed to high densities, most larvae develop a highly melanized cuticle (Figure 1.1b).

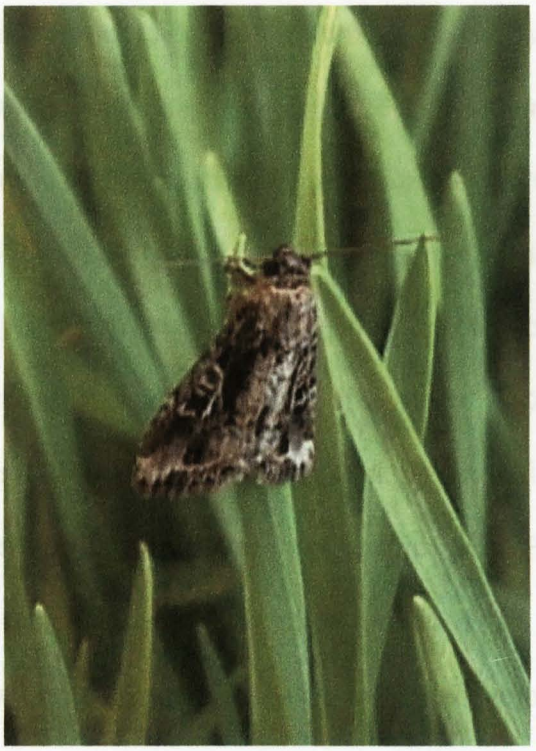
The response to crowding in *S. exempta* is similar, but more marked; solitary larvae are green or brown and crowded larvae black (Figure 1.1d). The adaptive significance of cuticular melanization is unclear though a number of benefits have been proposed such as increased infra-red absorbance, aposematism and increased disease resistance (Iwao 1968; Johnson *et al.* 1985; Wilson & Reeson 1998; Wilson 2000). The possibility that these species may have phenotypically plastic immune responses (Reeson *et al.* 1998) make them ideal models with which to investigate the costs of immunity.

Figure 1.1 – Phase polyphenism in *S. littoralis and S. exempta*

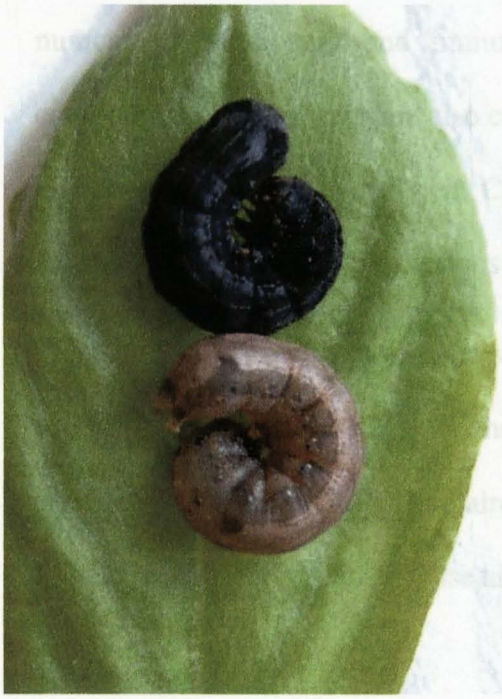
Spodoptera littoralis (a,b) and *Spodoptera exempta* (c,d) are phase polyphenic species; the density experienced during the larval development period, in part, determines an individual's phenotype. At low densities, *S. littoralis* larvae tend to remain pale grey/brown but as the rearing density increases individuals develop into the typical "crowded" or "gregarious" form, characterised by a dark, highly melanized cuticle (b). Solitary *S. exempta* are green or brown but when reared in crowds they become jet-black (d).

(a)

(c)



(b)



(d)

Thesis outline

Chapter 2 details the methods by which investment in immune function was assessed, and considers the accuracy and repeatability of each method. Chapter 3 investigates the genetic basis of variation in haemolymph phenoloxidase (PO) activity, its relationship with encapsulation ability and with PO activity in the cuticle and midgut. In Chapter 4, prophylactic investment in immune function, measured as PO activity in the haemolymph, midgut and cuticle, is assessed across colour phenotypes and rearing densities. Resistance to a fungal pathogen and a parasitoid is compared across treatment groups and its relationship with PO considered. The basis of prophylactic resistance is evaluated in greater detail in Chapter 5, as investment in a range of immune parameters is compared across colour phenotypes and rearing densities. The possibility of trade-offs within the immune system is also discussed. In Chapter 6 the genetic basis of variation in a number of life-history and immune parameters is determined. The genetic correlations between traits are also calculated in order to examine any possible costs of immune function. Finally, in Chapter 7, the findings and implications of this study are summarised and directions for further research are suggested.

Chapters 3, 4, 5 and 6 have been written as manuscripts for submission to peer-reviewed journals and as such there has been necessary duplication of some material. Chapters 3 and 4 have already been published (in **Heredity** and **Ecology Letters**, respectively) and so these are presented in their published form.

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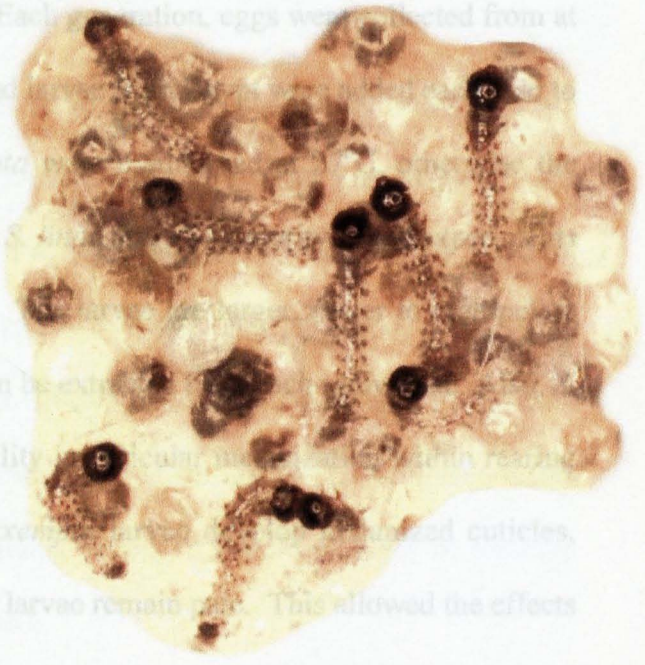
Figure caption: 1st instar *S. littoralis* larvae hatching from eggs.

Chapter 2

Background methodology



The *Spodoptera littoralis* and *S. exempta* stock cultures

The *Spodoptera littoralis* culture was established from eggs collected near Alexandria in Egypt in 1998. Stocks were kept in a CT room at 25°C on a 12:12 light:dark cycle. Larvae were reared at a density of 10-15 per dish (10 cm diameter by 5 cm) from the second instar, and fed on semi-artificial wheatgerm diet (Appendix 1). Fresh diet was supplied three times per week. Larvae were allowed to pupate in the pots but were transferred, soon after pupating, to clean pots containing filter paper. This reduced the levels of cannibalism in the stocks. After emergence, adults were transferred to moth rearing chambers (12 cm diameter by 24 cm) at a density of approximately 30 per chamber. Moths were provided with water, filter paper and nappy liner on which the females could lay eggs. Eggs were collected daily and placed in pots with fresh diet. When larvae reached the second instar, a large number of larvae were randomly selected and placed in dishes at a density of 10-15 larvae per container. Each generation, eggs were collected from at least 100 adults to reduce inbreeding and larvae from Egypt were added each year to maintain genetic variation. *S. exempta* were maintained at 27°C, otherwise the rearing conditions were the same as for *S. littoralis*. The majority of the experiments used *S. littoralis* for practical reasons. The larvae are larger, easier to manipulate and a larger volume of haemolymph can be extracted from each individual. Also, *S. littoralis* larvae show a greater variability in cuticular melanization within rearing densities. At high densities, all *S. exempta* larvae develop melanized cuticles, whereas a proportion of the *S. littoralis* larvae remain pale. This allowed the effects

of colour and density on immune function to be considered separately for *S. littoralis.*

Measuring immune parameters

Measuring variation in components of the immune system can provide an insight into how individuals allocate resources to immunity. Components most commonly measured in invertebrates are phenoloxidase activity levels, haemocyte count (either differential haemocyte density per mm^3 of haemolymph, total haemocyte density per mm^3 of haemolymph, or total number of haemocytes in the haemocoel), antibacterial activity (growth inhibition or lysis) and encapsulation ability. Many of these components are inter-related, for example encapsulation is a process by which haemocytes accumulate around a parasite in the haemocoel and are then hardened and melanized through the action of phenoloxidase. Antibacterial peptides may require components of the phenoloxidase system to recognise the invader and opsonise it before they can work.

Choosing which components to measure depend upon the ease with which they can be measured and whether or not the individual has to survive the measuring process. For example, it is possible to extract up to 50 µl of haemolymph from a fourth or fifth instar *Spodoptera littoralis* larvae without any apparent ill effects. Haemolymph was sampled from larvae on two consecutive days and the survival rates and adult fecundity were comparable to control larvae (Unpublished data). Measuring encapsulation or phenoloxidase levels in tissues (midgut or cuticle), however, requires that the larvae be killed.

Haemocyte density

Haemocytes play three main roles in the insect immune system. Firstly they are responsible for phagocytosing small microorganisms in the haemocoel such as bacteria. Secondly, they isolate larger organisms by forming a capsule around them in a process called encapsulation. Thirdly, they are involved in the wound-healing response as they move to the site of a break in the cuticle and lyse, forming a sticky barrier that melanizes, reducing the chance of infection. Haemocytes may also store antibacterial or antifungal proteins, prophenoloxidase or its activators.

Haemocytes can easily be counted in a haemolymph sample using a bright line haemocytometer with improved Neubauer ruling. Firstly, the haemocytometer is cleaned with a lens tissue and the cover slip pressed firmly on top until "Newton's rings" are visible. This ensures that the cover slip is firmly attached and that only a layer of haemolymph one cell thick can lie underneath. Ten microlitres of haemolymph are pipetted onto each side of the haemocytometer and left to settle for 20 minutes. Cells are counted in five, non-adjacent squares on each side under a light microscope at x25 magnification. The counts from each side of the haemocytometer are summed to give two counts per sample. This gives a measure of haemocyte density in the haemolymph; counts can then be multiplied by 5000 to give the number of cells per ml of haemolymph.

Total haemocyte number can also be assessed by flushing out the haemocoel with a known quantity of buffer, and performing counts in the same way (Barnes & Siva-Jothy 2000). This procedure was not undertaken in the present study.

Although differential haemocyte counts may be informative due the different roles played by the haemocytes in the defence reaction, distinguishing the cell types

visually is difficult and time consuming. In *Drosophila melanogaster*, selected for increased resistance to the parasitoid *Asobara tabida*, haemocyte density was found to be significantly higher in resistant lines than in control lines (Kraaijeveld *et al.* 2001). Haemocyte density has also been shown to positively correlate with encapsulation ability across species (Eslin & Prévost 1998). Therefore, as undifferentiated haemocyte counts provide an indication of encapsulation ability, differential cell counts were not performed in this study.

The effects of buffer and freezing on haemocyte counts

Haemocyte density can be very high within a haemolymph sample and so dilution is necessary to allow an accurate count to be made. For the haemocytes to remain intact during counting it is necessary to use a buffer that is iso-osmotic with the haemolymph. Many samples may need to be counted in any one experiment and haemocytes are not very stable once they have been removed from the body. Within a short period of time, cells begin to lyse, thus samples need to be stored before counting. Freezing samples is the quickest and easiest method of storage, but cells can be disrupted by the formation of ice crystals during the freezing process. It is therefore necessary to find a suitable buffer and storage method if haemocyte counts are to be used in large-scale experiments. Previous attempts at fixing haemocytes with formaldehyde and glutaraldehyde in varying concentrations were unsuccessful, as cells tended to clump together. The addition of glycerol is meant to protect cells during freezing so this method was tested.

Osmolality

Method

Haemolymph was sampled from 10 larvae and kept on ice. The osmolality of each sample was measured three times using a *Wescor Vapor Pressure Osmometer*. The osmolality of two buffers was also measured three times, phosphate buffered saline (PBS, pH 7.4, Appendix 2) and of sodium cacodylate buffer (Na-Cac, pH 6.5, Appendix 2). Both buffers are commonly used in insect physiology.

Results

There was a significant difference between the osmolality of the Na-Cac buffer and fresh haemolymph ($t = 11.2$, $df = 11$, $p < 0.001$). However, the osmolality of the PBS and haemolymph were not significantly different from each other ($t = -0.03$, $df = 11$, $p = 0.97$; Table 2.1).

Discussion

As PBS was iso-osmotic with fresh *S. littoralis* haemolymph, this buffer was used in all subsequent experiments. Storage of haemolymph in Na-Cac buffer could cause disruption of the cells, which would reduce the accuracy of the counts.

Table 2.1 – Comparison of buffer osmolality

Osmolality +/- SE for two buffers, commonly used in insect physiology, and fresh, undiluted *S. littoralis* haemolymph.

Substance	Osmolality
Fresh haemolymph	479.2 ± 8.1 mosmol/kg
Phosphate Buffered Saline	479.5 ± 4.1 mosmol/kg
Sodium Cacodylate Buffer	387.3 ± 1.3 mosmol/kg

Table 2.2 – Within sample and between treatment repeatabilities of haemocyte counts

Repeatabilities calculated according to the methods of Lessells and Boag (1987) for haemocyte counts +/- SE. Values on the diagonal represent within-sample repeatabilities, a comparison of the two counts made on each haemolymph sample. The other values represent a comparison of the mean counts per sample between the different treatments.

	Fresh	Frozen 1 day	Frozen 1 week
Fresh	0.885 ± 0.032		
Frozen 1 day	0.770 ± 0.048	0.933 ± 0.019	
Frozen 1 week	0.718 ± 0.057	0.727 ± 0.055	0.845 ± 0.043

Freezing

Method

Haemolymph was sampled from 42 final instar larvae as described before. Each 30 µl sample of haemolymph was then diluted with 15 µl glycerol and 15 µl EDTA anticoagulant in PBS (Appendix 2). Two counts were then performed on each fresh sample using a haemocytometer to assess the within-sample repeatability of the counting method. The remaining 40 µl samples were then separated into two Eppendorf tubes. One set of samples was frozen for one day and the other set for one week at –20°C to assess the effect of both short-term and long-term freezing on the haemocytes.

Results

The repeatability of the haemocyte counts was calculated using the method of Lessells and Boag (Lessells & Boag 1987). The within-sample repeatability of the counting method was high for all measures, ranging from 0.85-0.93. The repeatability of the counts after differing periods of time in the freezer was also high, with an estimate of $r = 0.718 \pm 0.057$ after one week (Table 2.2).

Discussion

The results show that degradation during freezing is low, as samples are highly repeatable after one week in the freezer. Therefore, the storage of haemocytes for up to one week, is feasible using this method.

Phenoloxidase activity

Phenoloxidase (PO) is a copper containing enzyme responsible for catalysing key steps in the synthesis of melanin, a pigment found in the cuticle (Ashida & Brey 1995) and around encapsulated foreign bodies. Phenoloxidase is thought to be a particularly important component of the immune response and has been implicated in resistance to a range of pathogens. Reeson et al (1998) showed that haemolymph PO levels correlated with resistance to NPV infection in *Spodoptera exempta* (Reeson *et al.* 1998). Washburn *et al* (1996) showed that in refractory *Helicoverpa zea,* infected midgut cells were encapsulated and melanized, suggesting that this process halted the spread of the virus. In *Anopheles gambiae*, it has been shown that individuals refractory to *Plasmodium cynomolgi* infection have higher midgut PO levels than susceptibles after an infective blood meal, suggesting that their refractoriness may, in part, be due to phenoloxidase activity (Paskewitz *et al.* 1989). Active PO in the cuticle may play a role in halting pathogens that enter the haemocoel via that route, such as fungi, by the production of harmful quinones (Chapman 1998) or by encapsulation of hyphae in the cuticle (Tanada & Kaya 1993).

Products of the prophenoloxidase cascade rapidly become visible to the naked eye and so the level of melanization can be used as an indicator of the levels of phenoloxidase in the haemolymph. Using a fine needle and a micropipette, haemolymph can be extracted from larvae and added to an appropriate buffer. A substrate (e.g. L-Dopa) is then added and the solution is incubated. The level of melanization is then measured spectrophotometrically at an absorbance of light

between 475 and 496 nm (Pye 1974; Nigam *et al.* 1997). This gives a measure of the phenoloxidase levels in the larval haemolymph.

Enzyme kinetics

Phenoloxidase is an enzyme and as such its kinetics need to be investigated. Without this information, the assumption cannot be made, that appearance of the end-product, melanin, is a good measure of the quantity of the active enzyme. Three parameters were assessed. Firstly, the portion of the reaction over which the rate of formation of end-product is linear with respect to time. It is important to measure the absorbance during this period as this is when the rate of the reaction is maximal. Secondly, the linearity of the relationship between enzyme concentration and the formation of the end-product was measured. This ensures that the formation of product accurately reflects the levels of enzyme present. It is also important to ensure that the only limiting factor in the speed of the reaction is the quantity of enzyme present. The substrate can limit the reaction if its concentration is too low. It is therefore necessary to quantify V_{max}, which is the maximum velocity of the reaction at infinite substrate concentration, and K_m, or the Michaelis constant, which is equal to the substrate concentration at half V_{max}. From these two values the minimum substrate concentration for V_{max} can be calculated. The substrate concentration used in any experiments must be higher than this value to ensure that it is only the amount of enzyme present that limits the rate of the reaction.

Method

Linearity with time

Ten final instar larvae were randomly selected and weighed. The cuticle between the last pair of prolegs was pierced with a fine needle and the haemolymph pooled onto parafilm. Twelve microlitres of haemolymph were added to 150 µl of ice-cold PBS (Appendix 2) in an Eppendorf and vortexed. Triplicate 50 µl samples of the haemolymph/PBS mixture from each larva were added to 50 µl of 20 mM L-Dopa (Sigma) in a microtitre plate. The plate was read at 490 nm every minute for 10 minutes and every 5 minutes thereafter up to 65 minutes.

Linearity with enzyme concentration

To determine the effect of enzyme concentration on the rate of change of absorbance, a series of dilutions of haemolymph, pooled from 10 larvae were made up. They ranged from 5 to 65 µl/ml of PBS at 5 µl intervals. Triplicate 100 µl samples of each dilution were pipetted into a microtitre plate and 100 µl of 20 mM L-Dopa added. The absorbance was read on a plate reader after 20 minutes incubation at 25°C.

Assessing V_{max}

Haemolymph was sampled from 10 final instar larvae as before and pooled. Eighty µl of haemolymph was then added to 4 ml of ice-cold PBS and vortexed. Serial dilutions of L-Dopa were then made from 20 mM down to 2 mM at 2 mM intervals. Triplicate 100 µl samples of each dilution were then added to 100 µl of the haemolymph/PBS mixture and incubated at 25°C before being read on a plate reader at 490 nm.

Results

Linearity with time

The mean absorbance for each larva was plotted for each time point. The rate of the reaction remained linear for up to 65 minutes (Figure 2.1a). It is also apparent that the rankings of each larva remain the same after approximately 10 minutes.

Linearity with enzyme concentration

The mean rate of change in optical density (y) was plotted against haemolymph concentration (x). There was a significant positive relationship between the quantity of enzyme present and the rate of change in optical density, i.e. the rate of appearance of the end-product, melanin ($y = 0.00048x$, $F_{1,12} = 95.07$, $p < 0.001$) the squared term was not significant indicating that the relationship was linear ($F_{1,11} = 0.81$, $p = 0.39$; Figure 2.1b).

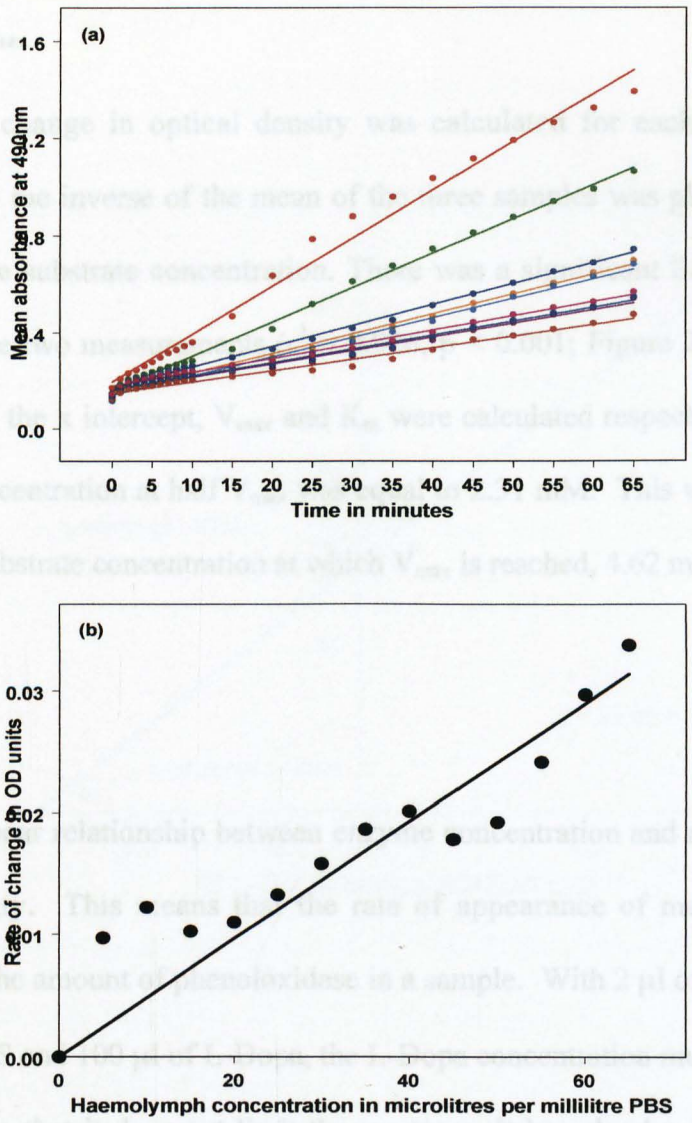


Figure 2.1 – The rate of production of melanin over time and with varying PO concentration

Reaction curves for haemolymph samples taken from ten individuals (a). The enzyme reaction is in the linear phase up to 65 minutes after the addition of the substrate. Figure (b) shows the relationship between the concentration of PO and the rate of change appearance of the end-product melanin

Assessing V_{max}

The rate of change in optical density was calculated for each dilution of the substrate and the inverse of the mean of the three samples was plotted against the inverse of the substrate concentration. There was a significant linear relationship between these two measurements ($r^2 = 0.926$, $p < 0.001$; Figure 2.2). From the y intercept and the x intercept, V_{max} and K_m were calculated respectively. K_m, or the substrate concentration at half V_{max} was equal to 2.31 mM. This was then doubled to give the substrate concentration at which V_{max} is reached, 4.62 mM.

Discussion

There is a linear relationship between enzyme concentration and rate of change of optical density. This means that the rate of appearance of melanin is a good indicator of the amount of phenoloxidase in a sample. With 2 µl of haemolymph in 100 µl of PBS and 100 µl of L-Dopa, the L-Dopa concentration must be above 4.62 mM to ensure that it does not limit the reaction. It has also been shown that the absorbance can be read at any time up to 65 minutes, during which time the reaction is still in its linear phase.

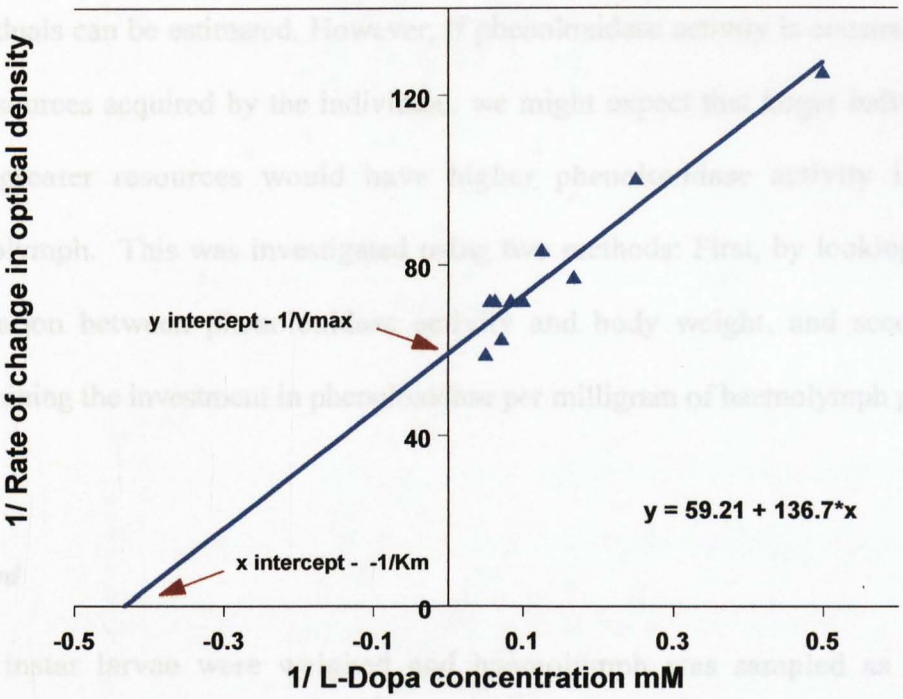


Figure 2.2 – The Lineweaver-Burk plot for PO activity

V_{max} and K_m, the Michaelis constant can be calculated from the Lineweaver-Burk plot. The y-intercept is equal to V_{max}, the maximum velocity of the reaction at infinite substrate concentration. The x-intercept is equal to $-1/K_m$, K_m is the substrate concentration at which the reaction proceeds at half V_{max}.

y = 59.21 + 136.7*x (**y** = 1/Rate of reaction and **x** = 1/L-Dopa conc. mM)

Variation in phenoloxidase activity levels

If phenoloxidase is to be used as an indicator of an individual's level of investment in immunity, there must be significant variation between individuals. It is also necessary that the measurement method is repeatable so that variation between individuals can be estimated. However, if phenoloxidase activity is constrained by the resources acquired by the individual, we might expect that larger individuals, with greater resources would have higher phenoloxidase activity in their haemolymph. This was investigated using two methods: First, by looking at the correlation between phenoloxidase activity and body weight, and second, by determining the investment in phenoloxidase per milligram of haemolymph protein.

Method

Final instar larvae were weighed and haemolymph was sampled as before. Triplicate measurements were taken from each haemolymph sample to give an estimate of the repeatability of the measurement technique. In addition to measuring phenoloxidase activity levels, protein was measured using the *BioRad* protein assay kit with BSA as the protein standard (the standard curve was calculated from a serial dilution of BSA ranging from 0 - 1mg/ml at 0.1 mg/ml intervals). Triplicate 10 µl samples of the haemolymph/PBS mixtures were used to measure the protein in each sample. Absorption was measured on a plate reader at 600 nm. The amount of PO in each sample was calculated as PO units, where one unit is the amount of enzyme required to increase the absorbance by 0.001 per minute. PO was also expressed as PO units per mg of protein (Hung & Boucias 1996).

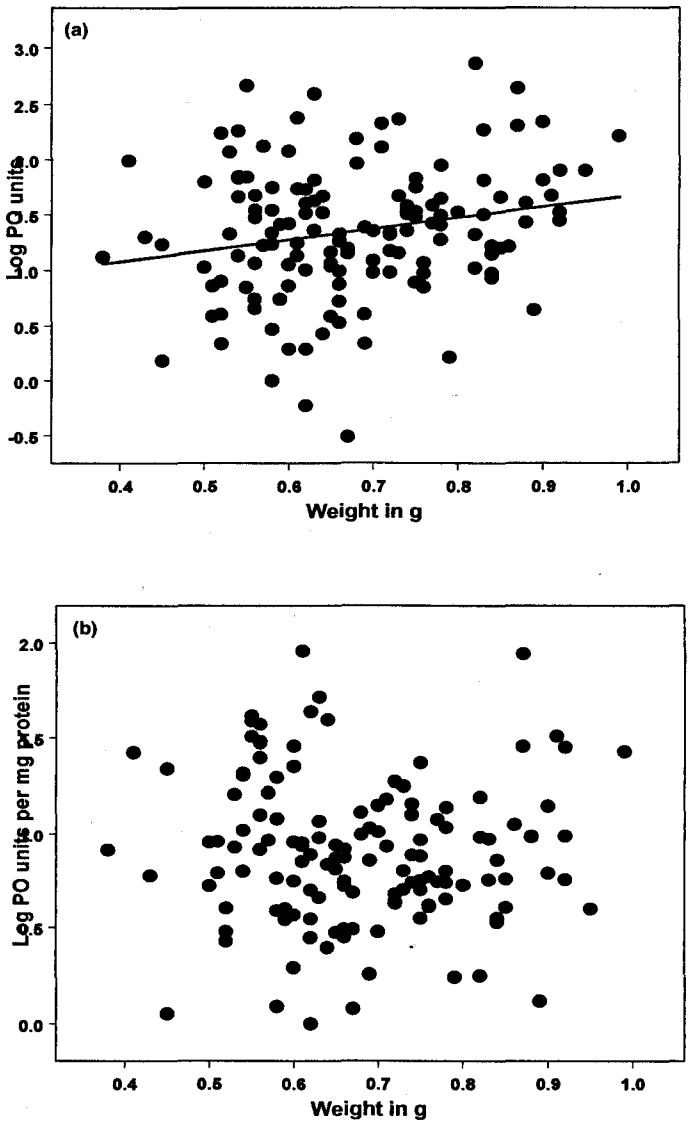


Figure 2.3 – The relationship between larval body weight and haemolymph PO activity.

Haemolymph PO activity (a) and haemolymph PO per mg of protein (b) are plotted against body weight. There is a positive correlation between haemolymph PO and body weight but this relationship disappears once PO is corrected for haemolymph protein.

Results

There was significant variation between individuals in PO activity in the samples (ANOVA: $F_{139,279} = 11.11$, $p < 0.001$). The repeatability, r, of the measurement technique was found to be high, ($r = 0.932 \pm 0.026$). There was a positive relationship between haemolymph PO levels and larval body weight ($r = 0.\ 0.207$, $df = 138$, $p = 0.014$; Figure 2.3a). However, there was no relationship between PO activity corrected for protein content of the haemolymph and larval body weight ($r = -0.045$, $df = 138$, $p = 0.597$; Figure 2.3b). There was also significant variation in PO activity levels per mg of protein between individuals (ANOVA: $F_{139,279} = 18.74$, $p < 0.001$). Haemolymph protein levels were highly repeatable within samples ($r = 0.921 \pm 0.014$). However, the additional error associated with the measurement of protein in each sample meant that the repeatability of the measurement of PO per mg of protein was slightly lower than for PO alone ($r = 0.848 \pm 0.020$).

Discussion

PO activity varies between individuals and the method for measuring the activity is highly repeatable. However, as there is a positive relationship between PO activity and body weight, this could mean that larger individuals have greater resources to divert to the production of this enzyme and so absolute levels may not accurately represent "investment" in immunity. PO per mg of haemolymph protein was independent of body weight. This measure identifies individuals that divert proportionately more of their resources to PO production. There is still significant variation between individuals, which means that individual variation in resources did not account for all the variation in PO levels.

The effects of buffer and freezing on PO activity

As with haemocyte counts, if phenoloxidase measurements are to be used in large-scale experiments, it will be necessary to store samples until they can be measured. It was therefore necessary to determine the effect of freezing on PO activity in the samples. The buffer in which the samples are stored could also affect PO activity and so the effects of freezing were tested with two buffers, commonly used in insect physiology, PBS and Na-Cac (Appendix 2).

Method

Haemolymph was sampled from 30 final instar larvae as described previously. From each sample, 8 µl were added to three Eppendorfs containing 400 µl of PBS and three Eppendorfs containing 400 µl of Na-Cac buffer. The samples were then randomly allocated to each treatment; freezing for 1 hour (fresh), 1 day or 1 week. Samples in the "fresh" treatment group were frozen for 1 hour to disrupt the haemocyte membranes and release the PO into solution.

Measurement of PO activity was the same for each group, triplicate 100 µl samples of the buffered haemolymph were pipetted into a microtitre plate and 100 µl of 20 mM L-Dopa was added to each as the substrate for the reaction. The absorbance was read at 492 nm after 20 minutes at 25°C on a temperature-controlled *Versamax tuneable microplate reader* (Molecular Devices Corporation, Sunnyvale, CA).

Results

There was no significant effect of the duration of freezing on PO activity but there was a significant effect of buffer (ANOVA: freezing, $F_{2,176} = 2.11$, $p = 0.124$; buffer, $F_{2,176} = 30.07$, $p < 0.001$; Figure 2.4). The within sample repeatability was high for both buffers for all treatments. The lowest repeatabilities for both buffers were between samples frozen for 1 hour and those frozen for either 1 day, or 1 week. However, high repeatabilities were obtained in both buffers between samples frozen for 1 day and 1 week (Table 2.3).

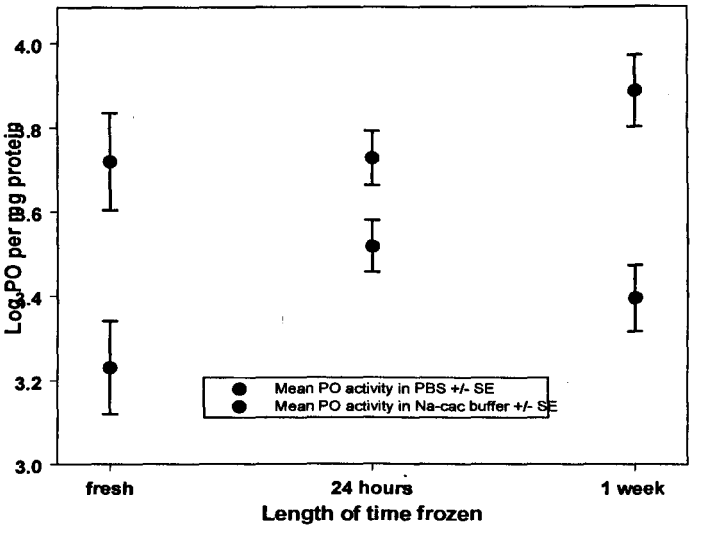


Figure 2.4 – Mean PO activity levels +/- SE in each buffer for each storage treatment

Mean PO activity is plotted for each buffer and storage treatment. PO activity differed in the two buffers, being higher in the Na-cac buffer, but there was no significant effect of freezing on the samples.

Table 2.3 – Within sample and between treatment repeatabilities of PO per mg protein

Repeatabilities calculated according to the methods of Lessells and Boag (1987) for PO per mg protein +/- SE, in (a) sodium cacodylate buffer and (b) PBS. Values on the diagonal represent within-sample repeatabilities, a comparison of the three measurements taken for each haemolymph sample.

(a)

	Fresh	**Frozen 1 day**	**Frozen 1 week**
Fresh	0.798 ± 0.056		
Frozen 1 day	0.496 ± 0.139	0.827 ± 0.049	
Frozen 1 week	0.528 ± 0.133	0.716 ± 0.090	0.858 ± 0.053

(b)

	Fresh	**Frozen 1 day**	**Frozen 1 week**
Fresh	0.847 ± 0.044		
Frozen 1 day	0.444 ± 0.148	0.742 ± 0.068	
Frozen 1 week	0.453 ± 0.146	0.740 ± 0.083	0.868 ± 0.039

Comparison of buffers

There were significant positive correlations between the levels of PO activity in the PBS and the Na-Cac buffers in all of the freezing treatments. The weakest correlation was found in the samples frozen for 1 hour ($r = 0.572$, $t = 3.62$, $df = 27$, $p < 0.01$; Figure 2.5a). The intercept (_) was significantly different from 0, and the slope (_) of the line was significantly different from 1 (_ = 2.03 ± 0.491: $t = -4.14$, $df = 27$, $p < 0.001$; _ = 0.536 ± 0.148: $t = 3.14$, $df = 27$, $p = 0.004$).

There was a strong positive correlation between the levels of PO activity in the PBS and the Na-Cac buffer in samples frozen for 1 day ($r = 0.788$, $t = 6.77$, $df = 28$, $p < 0.001$; Figure 2.5b). The intercept (_) and the slope (_) of the line were not significantly different from a 1:1 line passing through the origin (_ = 0.796 ± 0.438: $t = -1.82$, $df = 28$, $p = 0.08$; _ = 0.835 ± 0.124: $t = 1.33$, $df = 28$, $p = 0.19$). There was also a strong positive correlation between the levels of PO activity in samples frozen for 1 week ($r = 0.704$, $t = 5.24$, $df = 28$, $p < 0.001$; Figure 2.5c). The intercept (_) of the line was significantly different from zero but the slope (_) was not significantly different from one (_ = 1.306 ± 0.497: $t = -2.63$, $df = 28$, $p = 0.014$; _ = 0.760 ± 0.145: $t = 1.65$, $df = 28$, $p = 0.11$).

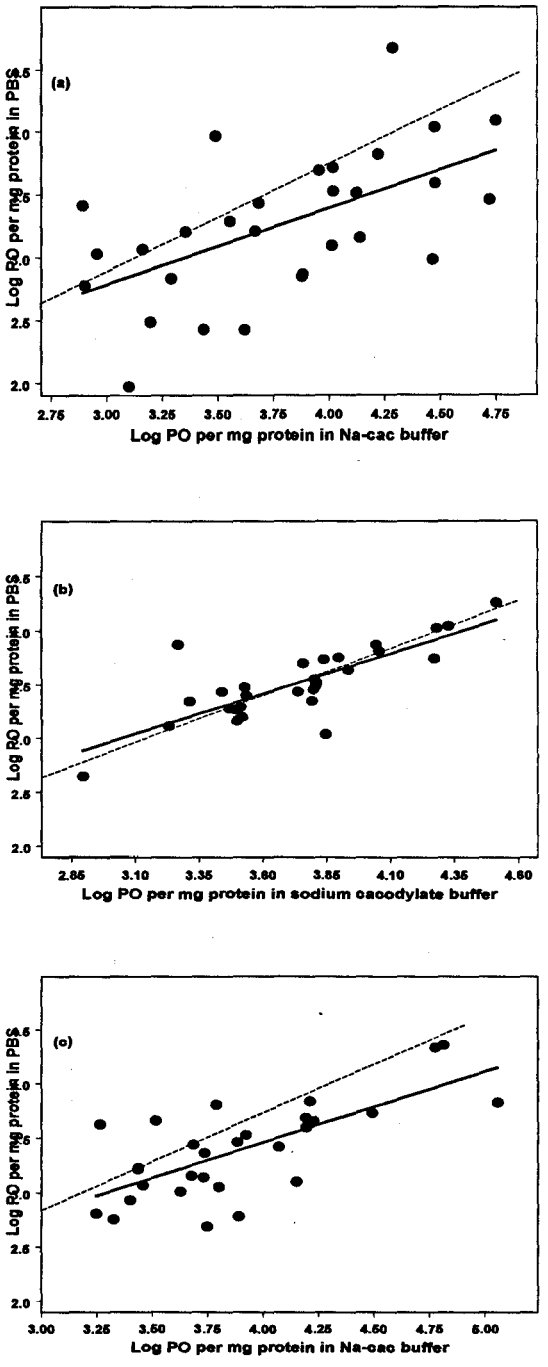


Figure 2.5 – The relationship between PO activity measured in either PBS or Na-cac buffer.

PO activity measured in samples that were stored in PBS is plotted against samples stored in Na-cac buffer. Samples were either fresh (a), frozen for 24 hours (b) or frozen for one week (c). The dotted line represents the 1:1 relationship.

Discussion

The results obtained for both buffers were very similar. In each case the repeatability of PO activity between samples frozen for 1 hour and those frozen for 1 day or 1 week was quite low. However, the repeatability between samples frozen for 1 day and 1 week were high in both cases. There was much greater variability in PO activity levels in the samples frozen for 1 hour. This suggests that freezing for such a short period may not lyse all the haemocytes in many of the samples. The high repeatability of samples frozen for 1 day and those frozen for 1 week show that there is very little degradation in the freezer over this time period.

PO activity levels are generally higher in Na-Cac buffer, probably due to the calcium present in the buffer, which is known to enhance the PO reaction for many species. However, the strong correlations between values obtained in either buffer suggest that variation in calcium levels in the haemolymph was not responsible for much variation between individuals. As PBS is iso-osmotic with *S. littoralis* haemolymph, it was used as the storage buffer for all subsequent phenoloxidase measurements.

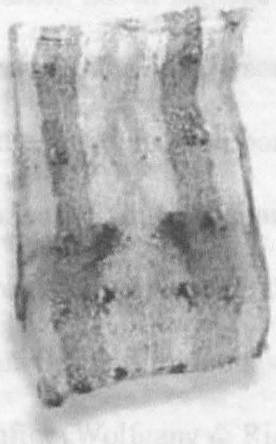
Phenoloxidase activity in other parts of the body

Phenoloxidase plays an important role in the haemolymph but it also occurs in other tissues, such as the midgut and cuticle, which form biologically active barriers to infection. As this is the case, it may be useful to be able to measure the level of investment in phenoloxidase in these areas. As the PO in the midgut and cuticle is attached to the tissues, an alternative method for assessing the levels of PO activity is required. In addition, as PO is embedded in the tissues, analysis of the enzyme kinetics would be very difficult. However, PO that has been extracted from the cuticle of the silkworm *Bombyx mori*, has been shown to have almost identical enzyme kinetics to haemolymph PO (Asano & Ashida 2001).

Method

Final instar larvae were frozen and once thawed, the head and the tip of the abdomen were removed with sharp scissors. The cuticle was then cut along the ventral surface between the legs and peeled back. This allowed the gut to be removed in one piece and retained for later use. The cuticle was then held with blunt forceps and the attached fat-body and trachea removed by carefully scraping the cuticle with a seeker (Watkins and Doncaster). The cleaned cuticle was then rinsed in de-ionised water to remove any remaining tissue or haemolymph. The unpigmented ventral tissue, including the legs and prolegs, was removed, leaving a rectangular piece of pigmented cuticle (Figure 2.6).

(a)



(b)

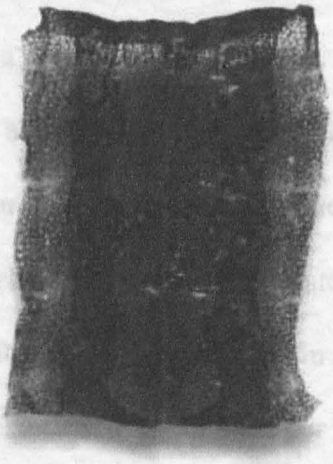


Figure 2.6 – Cuticles dissected from pale and dark larvae

Cuticle dissected from (a) pale and (b) dark larvae and mounted on slides.

The food was rinsed out of the gut by holding one end with blunt forceps and gently squeezing the contents out with a blunt instrument. This was performed under de-ionised water, and care was taken not to damage the tissue. Any attaching fat or trachea was removed with forceps. The midgut can be easily identified, as there are constrictions in the tissue at the junctions with both the foregut and hindgut. Both cuticle and midgut were then washed in potassium phosphate buffer (Appendix 2) and fixed in 2% formaldehyde and 0.5% glutaraldehyde in potassium phosphate buffer. After fixation, the cuticles and midguts were washed over three hours in three changes of phosphate buffer (Wolfgang & Riddiford 1981).

Each midgut and cuticle was then cut in half and each half weighed. One half of each midgut and cuticle was placed in 0.5 ml and 1 ml of 20 mM L-Dopa respectively, the other half in the same volume of 20 mM L-Dopa saturated with phenylthiourea (PTU) as a control (PTU acts as a PO inhibitor and none of these samples darkened). Any active phenoloxidase present in the tissue causes the L-Dopa to melanize as before. After 30 minutes incubation at 25°C, 200 µl of the darkened L-Dopa was pipetted into a microtitre plate and the absorbance read at 492 nm. As the PO is attached to the tissue, protein content cannot be determined, therefore PO activity was expressed as PO units per gram of cuticle/midgut.

Lysozyme assay

Overcoming bacterial infection relies on two modes of attack, the inhibition of bacterial growth and the killing of bacterial cells. Various antibacterial proteins have been found in the haemolymph responsible for bacterial inhibition. The chief protein responsible for killing cells is lysozyme. Two different assays are required to test these two types of antibacterial protein. The first is an inhibition zone assay, the second, a lytic zone assay. Both tests require agar plates to be prepared and haemolymph to be placed in small holes punched in the agar. The inhibition zone assay uses live bacteria and the clear zone around the hole is an indication of the amount of inhibitory proteins present in the haemolymph. The lytic zone assay uses dead bacterial cells and the clear zone indicates lysozyme-like activity. For practical reasons I decided to use the lytic zone assay as it does not require the production of large amounts of live bacteria and it is a more general response than growth inhibition. Moreover, preliminary experiments indicated that the clear zones were more defined and easier to measure using this technique.

Method

Test plates

To make 15 test plates 1.5 g of agar (Agar agar, Sigma) were dissolved in 100 ml distilled water, autoclaved and subsequently held at 48°C in a waterbath. The bacterial solution was prepared by dissolving 0.75 g of freeze-dried *Micrococcus luteus* (Sigma) in 50 ml of 0.2 M potassium phosphate buffer (Appendix 2). Streptomycin sulphate (Sigma) was dissolved in sterile distilled water at a

concentration of 100 mg/ml, 150 µl were added to the bacterial solution and this was also held at 48°C.

The agar solution and the *M. luteus* solution were then mixed and held at 48°C. 10 ml of this mixture were then pipetted into petri dishes (diameter 8.5 cm) which were left at room temperature until the agar set. The last plate of each batch was used as a test for contamination by incubating bottom-up at 33°C, over night. Contaminated batches were discarded, otherwise plates were stored in the fridge for up to 5 days.

Performing the lysozyme assay

Approximately 20 holes (diameter 3 mm) per plate were punched in the agar with a glass, pasteur pipette attached to a vacuum pump. Each hole was filled with 1 µl of ethanol saturated with PTU. The ethanol then evaporated leaving the PTU in the hole. This inhibits the melanization of the haemolymph samples, which would make the clear zones in the agar more difficult to detect. One µl of undiluted haemolymph from each sample was then pipetted into the holes, two replicates per sample. The plates were left for 20 minutes without moving to allow the haemolymph to diffuse into the agar. They were then incubated bottom up for 24 hours at 33°C.

Calibration

Hen egg white lysozyme (Sigma) was diluted to the following concentrations, 0.01, 0.05, 0.1, 0.5, 1 and 2 μg/ml of distilled water. Previous experiments found that the levels of lysozyme present in the haemolymph samples fell into this range (unpublished data). For each assay, five replicates of this series were pipetted into the holes and left overnight as with the haemolymph.

Measuring the clear zones and calculating the concentration of the lysozyme

The clear zones around each hole were photographed using a *Polaroid DMC* digital camera (Figure 2.7) and their diameter calculated using *Image Pro Plus* software (Media Cybernetics 1999). There is a positive linear relationship between the logarithm of lysozyme concentration in the sample and the diameter of the lytic zone such that:

$$D = \_ + \_ \ln C$$

$$C = e^{(D - \_)/\_}$$

Where **C** = lysozyme concentration in the sample, **D** = diameter (mm), _ = intercept and _ = slope of the line. Using this equation, lysozyme activity in the haemolymph was calculated as μg of "hen egg white lysozyme equivalents" per ml of haemolymph.

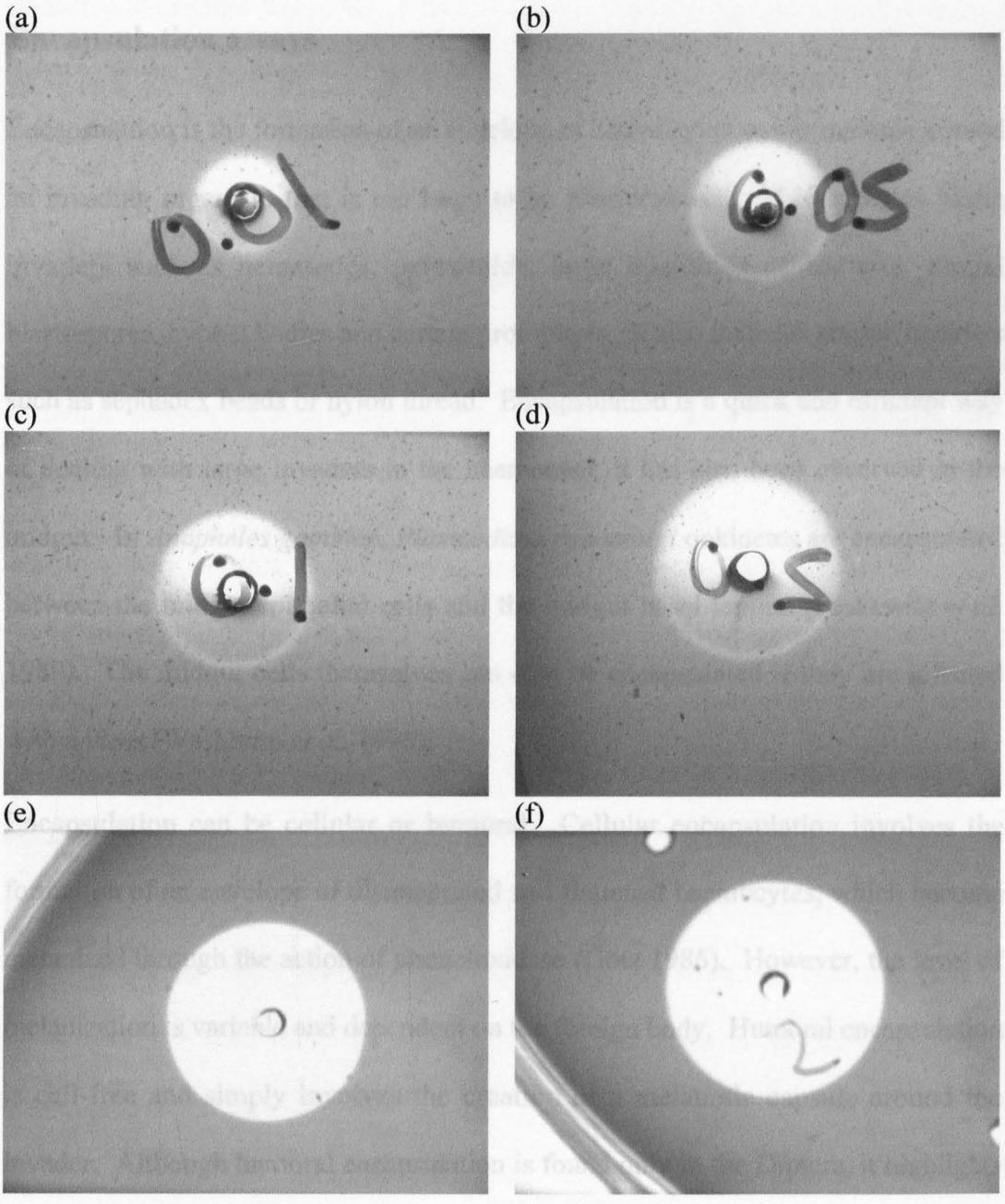


Figure 2.7 – *S. littoralis* haemolymph lytic activity against *Micrococcus luteus*

The activity of lysozyme in *S. littoralis* haemolymph, against the bacterium *Micrococcus luteus,* was calibrated against a standard curve obtained with a serial dilution of hen egg-white lysozyme (a) 0.01- (f) 2 μg/ml of distilled water.

Encapsulation assays

Encapsulation is the formation of an envelope of haemocytes and/or melanin around an invading organism that is too large to be phagocytosed. This includes biotic invaders such as nematodes, parasitoids, large quantities of bacteria, fungal blastospores, hyphal bodies and certain protoplasts. It also includes abiotic invaders such as sephadex beads or nylon thread. Encapsulation is a quick and efficient way of dealing with large invaders in the haemocoel; it has also been observed in the midgut. In *Anopheles gambiae*, *Plasmodium cynomolgi* ookinetes are encapsulated between the midgut epithelial cells and the midgut basal lamina (Paskewitz *et al.* 1989). The midgut cells themselves can also be encapsulated if they are infected with a virus (Washburn *et al.* 1996).

Encapsulation can be cellular or humoral. Cellular encapsulation involves the formation of an envelope of disintegrated and flattened haemocytes, which become melanized through the action of phenoloxidase (Götz 1986). However, the level of melanization is variable and dependent on the foreign body. Humoral encapsulation is cell-free and simply involves the creation of a melanotic capsule around the invader. Although humoral encapsulation is found only in the Diptera, it highlights the fact that encapsulation is made up of two parts: the haemocytic capsule and the melanization through the action of PO. Measuring encapsulation, therefore, quantifies the combined response of haemocytes and phenoloxidase, which may be very different from the levels of each individually.

Insects can be infected with the foreign bodies listed above and subsequently dissected to examine the levels of encapsulation. This can be done visually, using a light or electron microscope, by scoring the level of melanization (Zahedi *et al.* 1992; Paskewitz & Riehle 1994; Gorman *et al.* 1996) or alternatively, by photographing the capsule and calculating the level of melanization. The assumption is that higher levels of encapsulation equate to better immunity. There is some evidence for this as *Plasmodium*-refractory lines of *Anopheles gambiae* exhibit a stronger encapsulation response to sephadex beads than susceptible lines (Paskewitz & Riehle 1994).

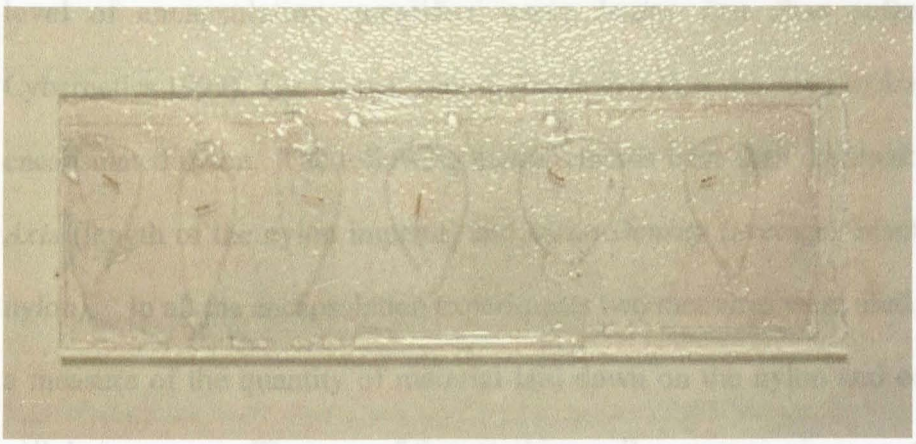
The advantage of using abiotic foreign bodies is that the host's natural defences can be tested without having to allow for any variation in virulence on the part of the parasite. It also avoids the problems of host choice on behalf of the parasite, other defences encountered upon entry of the host, i.e. the cuticular defences or behavioural mechanisms, and the uneven distribution of pathogens that would occur naturally. The disadvantage of this method is that abiotic bodies are less actively melanized than biotic invaders, although it has been shown that the genetic mechanism for both forms of encapsulation is the same in *Anopheles gambiae* (Gorman *et al.* 1996).

Method

Fine nylon monofilament, (*Orvis*, fishing line, diameter 0.5 mm) was dyed prior to use in Congo Red dye (Sigma). This ensures that the encapsulated piece of nylon can be easily detected during the dissection. The thread was cut into small pieces, about 3 mm long. The larval cuticle was then pierced between the final pair of prolegs with a fine needle, creating a hole large enough for the nylon to slide into the haemocoel. It is important not to pierce the gut with the nylon, as the bacteria entering the haemocoel would create an additional challenge for the immune system.

Larvae with the nylon implant were then left for 24 hours so that they could attempt to encapsulate the "parasite" after which time they were frozen. The encapsulated nylon pieces were dissected out and mounted on slides (encapsulated nylon implants can also be stored in 70% alcohol then rehydrated in decreasing dilutions of alcohol prior to use). Six small circles of nylon were glued onto each slide and an encapsulated piece of nylon placed in the centre of each, thus allowing coverslips to be placed on top and sealed with clear nail varnish (Figure 2.8a).

(a)



(b) (c)

Figure 2.8 – Encapsulation of an abiotic parasite implanted into the haemocoel.

A small piece of nylon was implanted into the haemocoel and left for 24 hours. The encapsulated nylon was then dissected out, mounted on slides (a) and digitally photographed. The level of encapsulation was scored using *Image Pro Plus* software, ranging from weak encapsulation (b), where very little of the surface of the nylon is covered, to strong encapsulation (c) where the majority of the nylon surface is covered with highly melanized cellular material.

The encapsulation of the nylon was scored visually after which each piece of nylon was photographed using a *Polaroid DMC* digital camera (Figure 2.8b,c) and the level of encapsulation quantified using *Image Pro Plus* software (Media Cybernetics 1999). The "wand" tool in *Image Pro Plus* was used to draw around the encapsulated nylon. The following measurements were then reported: *Area*, *Major Axis* (length of the nylon implant) and *Mean Density* (average "blackness" of the nylon). In all the encapsulation experiments two measures were used, the first was a measure of the quantity of material laid down on the nylon and equated to the cellular response. The area of the attaching cell mass was given as *Area*. As this measure was strongly correlated with implant length, the residuals of the regression of *Major Axis* on *Area* were used as a measure of capsule size. The second was a measure of the level of melanization and equated to the action of phenoloxidase. This was given as *Mean Density*, but as this score gave the highest values for the palest implants, the reciprocal was used. Both of these scores were correlated with each other ($r = 0.579 \pm 0.144$, $df = 15$, $P = 0.012$) and with a visual, qualitative score (capsule melanization: $r = 0.961 \pm 0.018$, $df = 15$, $p < 0.001$; capsule size: $r = 0.615 \pm 0.148$, $df = 15$, $p = 0.014$).

Cuticular melanization

Cuticular melanization occurs during the larval moult. The declining juvenile hormone titre and peak ecdysteroid titre during head cap slippage causes the synthesis of granular phenoloxidase (PO). As the ecdysteroid titre falls, PO is incorporated into the cuticle. The subsequent activation of PO in the cuticle results in melanization (Hiruma 1986; Hiruma & Riddiford 1988a; Hiruma & Riddiford

1988b, 1990). However, the degree to which the cuticle is melanized is dependent on both genetic and environmental factors. In particular, increased cuticular melanization can be triggered by a high population density. In order to consider the relationship between the degree of cuticular melanization and investment in immunity, both a quantitative and a qualitative scoring method were used.

Quantitative score of cuticular melanization

Larval cuticles were dissected and fixed as described for the cuticular PO assay. Fixed cuticles were then mounted and photographed using a *Polaroid DMC* digital camera and the images scored for their degree of melanization using *Image Pro Plus* software (Cybernetics 1999; Figure 2.6). Cuticular melanization was measured as the mean and maximum melanization scores (1/*mean density* and 1/*min density*, respectively, on the *Measurements* tab of *Image Pro Plus*).

Qualitative score of cuticular melanization

As the quantitative scoring method required that the larvae be killed, for some experiments it was necessary to estimate the degree of melanization visually. Larvae were scored shortly after the larval moult, as the cuticle appears lighter just prior to moulting due to the degree of stretching. For *S. littoralis*, larvae were classified as: *pale*, light grey/ brown with white/yellow markings, *medium*, mid-grey/brown with red/black markings, or *dark*, dark-grey with black markings to uniformly black. The phases are much more distinct for *S. exempta* and so larvae were scored as: *pale*, grey/green/brown, or *dark*, black.

Summary

In conclusion, all the measurement techniques for the haemolymph immune parameters are highly accurate, with repeatabilities for fresh samples all higher than $r = 0.80$. In addition, haemolymph samples in which haemocyte density or PO activity is to be determined can be frozen for up to a week without a significant reduction in the accuracy of the measurement (Tables 2.2 & 2.3).

Measuring encapsulation or phenoloxidase levels in tissues (midgut or cuticle), requires that the larvae be killed, therefore repeatabilities cannot be obtained for these measurements. PO activity, haemocyte density and lysozyme activity can all be measured in a single haemolymph sample as it is possible to extract up to 50 µl of haemolymph from *Spodoptera littoralis* larvae, from the fourth instar onwards, without any apparent ill effects. Furthermore, haemolymph can be sampled from larvae on two consecutive days without a significant reduction in fecundity or survival rates (Unpublished data).

Figure caption: pale and dark *S. littoralis* larvae. There is a clear difference between the phenotypes by the 3rd instar.

Chapter 3

Heritability of immune function in the caterpillar, *Spodoptera littoralis*

S.C. Cotter, & K. Wilson (2002)

Heredity, 88, 4: 229-234

Abstract

Phenoloxidase (PO) is believed to be a key mediator of immune function in insects and has been implicated both in non-self recognition and in resistance to a variety of parasites and pathogens, including baculoviruses and parasitoids. Using larvae of the Egyptian cotton leafworm, *Spodoptera littoralis*, we found that despite its apparent importance, haemolymph PO activity varied markedly between individuals, even amongst insects reared under apparently identical conditions. Sib-analysis methods were used to determine whether individuals varied genetically in their PO activity, and hence in one aspect of immune function. The heritability estimate of haemolymph PO activity was high ($h^2 = 0.690 \pm 0.069$), and PO activity in the haemolymph was strongly correlated with PO activity in both the cuticle and midgut; the sites of entry for most parasites and pathogens. Haemolymph PO activity was also strongly correlated with the degree to which a synthetic parasite (a small piece of nylon monofilament) was encapsulated and melanized ($r = 0.622 \pm 0.142$), suggesting that the encapsulation response is also heritable. The mechanism maintaining this genetic variation has yet to be elucidated.

Introduction

The invertebrate immune system is significantly simpler than our own in that it lacks an acquired immune response and yet it can cope with a variety of parasites and pathogens. Once recognised as non-self, small invaders may be phagocytosed by circulating haemocytes. Larger organisms are encapsulated, a process by which the parasite is covered by a capsule of haemocytes, which then hardens and melanizes (Götz 1986). The haemolymph also contains a variety of antibacterial, antiviral and antifungal proteins. One protein thought to be particularly important in the immune response of insects is phenoloxidase (PO). PO is a copper containing enzyme that catalyses the oxygenation of mono-phenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-quinones. These are key steps in the synthesis of melanin, a pigment found in the cuticle (Ashida & Brey 1995) and around encapsulated foreign bodies (Götz 1986). It has been shown in *Drosophila* that mutants lacking PO are unable to melanize and harden their capsules (Rizki & Rizki 1990). Other types of phenoloxidase are involved in wound healing and sclerotization of the cuticle (Ashida & Yamazaki 1990).

Haemolymph PO has been implicated in resistance to a range of pathogens, including nucleopolyhedroviruses (NPVs), fungi, nematodes and parasitoids (Rowley *et al.* 1990; Ourth & Renis 1993; Hagen *et al.* 1994; Hung & Boucias 1996; Washburn *et al.* 1996; Bidochka & Hajek 1998; Reeson *et al.* 1998). However, PO in other parts of the body may also play an important role in immunity. NPVs enter the body via the midgut and proceed by infecting the associated tracheal cells. Washburn et al (1996) showed that in refractory *Helicoverpa zea,* these infected cells

were encapsulated and melanized, halting the spread of the virus. This suggests a possible role for midgut PO in viral resistance. In *Anopheles gambiae*, *Plasmodium cynomolgi* ookinetes are encapsulated between the midgut epithelial cells and the midgut basal lamina. It has been shown that refractory individuals have higher midgut PO levels than susceptibles after an infective blood meal. This suggests that their refractoriness may, in part, be due to phenoloxidase activity (Paskewitz *et al.* 1989).

Active PO in the cuticle may play a role in halting pathogens that enter the haemocoel via that route (e.g. fungi), by the production of harmful quinones (Chapman 1998) or by encapsulation of hyphae in the cuticle (Tanada & Kaya 1993). Despite its apparent importance, there is considerable variation in haemolymph PO activity between individuals in, *A. gambiae* (Paskewitz *et al.* 1989), *Spodoptera exigua* (Hung & Boucias 1996) and *Lymantra dispar* (Bidochka & Hajek 1998).

PO has also been used as an indicator of immune function. For example, Reeson et al (1998) showed that larvae of the African armyworm, *Spodoptera exempta,* that had been reared at high densities had significantly higher haemolymph PO levels and higher NPV resistance than those reared solitarily. However, as far as we are aware, a direct link between PO activity and intra-specific variation in parasite resistance has yet to be conclusively demonstrated. Thus, as part of an ongoing study to examine the association between PO and pathogen resistance, and to determine any associated costs of pathogen resistance, we have addressed the following questions:

1) Are PO activity levels in the haemolymph repeatable and heritable?

2) Is haemolymph PO activity correlated with PO activity in different parts of the body, specifically the cuticle and midgut?

3) Is haemolymph PO activity correlated with the ability to encapsulate parasites in the haemocoel?

Methods

Spodoptera littoralis culture

Spodoptera littoralis larvae were collected in Egypt in April 1999 and reared in the laboratory for three generations before experiments were carried out. Larvae were reared at 25°C and fed on a semi-artificial wheatgerm-based diet (Appendix 1). Experimental larvae were separated into 25 ml polypots at the third instar and assays conducted on newly emerged final instar larvae.

Haemolymph phenoloxidase and protein assays

Final instar larvae were randomly selected and weighed. A proleg was pierced with a fine, sterile needle and the haemolymph pooled onto parafilm. Eight µl of haemolymph were added to 400 µl of ice-cold phosphate buffered saline (PBS, pH 7.4; Appendix 2) and vortexed. Samples were frozen at -20°C at this point and thawed when all the samples were ready to be measured. Samples were frozen for

less than a week in all cases; there is no significant decline in PO activity over this time period ($t = 1.24$, $df = 58$, $p = 0.22$). A 100 µl sample of the haemolymph/PBS mixture was added to 100 µl of 20 mM L-Dopa and incubated at 25°C. After thirty minutes, the absorbance was measured on a microplate reader at 492 nm. Previous results have shown the reaction to be in the linear phase during this time period (unpublished data). The amount of PO in the sample was calculated in PO units, where one unit is the amount of enzyme required to increase the absorbance by 0.001 per minute. Protein was measured using the *BioRad* protein assay kit with BSA as the protein standard. Ten µl of the haemolymph/PBS mixtures were used to measure the protein in each sample. Absorption was measured on a microplate reader at 600 nm. PO was then expressed as PO units per mg of protein (Hung & Boucias 1996).

Repeatability of phenoloxidase levels

Haemolymph was sampled twice from each larva, as described above; the second sample taken either 24 or 48 hours after the first. PO activity was measured in each haemolymph sample and its repeatability determined following the methods of Lessells & Boag (1987).

Midgut and cuticular phenoloxidase assays

The midgut and cuticle were dissected from each larva, fixed in 2% formaldehyde and 0.5% glutaraldehyde in phosphate buffer (Appendix 2). After fixation the cuticles and midguts were washed three times and stored in phosphate buffer (Wolfgang & Riddiford 1981), they were then cut in half and each piece weighed.

One half of each midgut and cuticle was then placed in 1 ml of 20 mM L-Dopa. The other half was placed in 20 mM L-Dopa saturated with PTU as a control. Samples of 20 mM L-Dopa were incubated along with the experimental samples to control for any spontaneous darkening of the substrate during the experiment. Samples were vortexed and 200 μl of the solution pipetted into microtitre plates, absorbance was then measured on a microplate reader at 492 nm during the linear phase of the reaction. PO activity was expressed as PO units per gram of cuticle/midgut.

Sib-analysis

Pupae were sexed and separated into males and females. Ten males were randomly selected and each mated to a virgin female. The females were removed after 24 hours and left in a pot to lay eggs. The males were given further virgin females every day for five days. Eggs were collected daily and the caterpillars reared until the third instar. Ten caterpillars were randomly selected from each brood and reared in individual polypots until the final instar. Each caterpillar was weighed and the haemolymph sampled as before and then frozen. When all the samples had been collected they were defrosted and PO and protein were measured as described above, taking three estimates per caterpillar. Variance components were estimated using restricted maximum likelihood (REML) methods in *Genstat 5*, in which the identity of the sire, dam and individual caterpillar were considered as nested random effects. Heritability estimates were then calculated using the methods of Falconer & Mackay (1996). To ensure robust parameter estimates and standard errors, the variance components procedure was bootstrapped 1000 times.

Encapsulation assay

Final instar larvae were selected and haemolymph was sampled as described above. A small piece of nylon monofilament (2.82 mm $\pm$ 0.3) was inserted into the haemocoel as an artificial parasite. Twenty four hours later the larvae were killed, the nylon dissected out and stored in 70% ethanol. The pieces of nylon were rehydrated, mounted on slides and digitally photographed. The level of encapsulation was scored visually by estimating the amount of cell cover and the level of melanization of each piece of nylon. The scores ranged from 1 to 9 with 1 indicating weak encapsulation and 9 indicating strong encapsulation. The level of melanization and the area of cell cover were also separately quantified using *Image Pro-Plus* software. Melanization was scored as the mean level of blackness of the nylon (1/*Mean Density* in *Image Pro-Plus*) and this was independent of the length of the implant (*Major Axis*). The area of the attaching cell mass (*Area*) was also quantified but as this score correlated with nylon length the residuals were used as a corrected score. In order to obtain robust estimates for the correlation coefficients and their standard errors, all correlations were bootstrapped 1000 times.

Results

Repeatability of haemolymph PO levels

PO activity levels were independent of the larval weight ($r = 0.042 \pm 0.088$, $df = 138$, $p = 0.575$). There was a significant positive correlation between haemolymph PO levels measured over 24 hours and 48 hours (Table 3.1). The slopes (_) of both lines were significantly different from one and the intercepts (_) were significantly different from zero (24 hours: _ = 0.456 ± 0.167, $t = 3.27$, $df = 18$, $p = 0.004$; _ = 1.944 ± 0.675, $t = -2.88$, $df = 18$, $p < 0.001$) (48 hours: _ = 0.345 ± 0.181, $t = 3.62$, $df = 34$, $p < 0.001$; _ = 2.812 ± 0.630, $t = -4.46$, $df = 34$, $p < 0.001$). However, they were not significantly different from each other (comparison of slopes: $t = 0.45$, $df = 54$, $p = 0.655$, comparison of intercepts, $t = -0.94$, $df = 54$, $p = 0.351$).

Heritability of haemolymph PO levels

There was significant heritable variation in PO activity (Table 3.2). The sire and dam heritability estimates were not significantly different (bootstrapped difference between sire and dam estimates $\pm$ SE = 0.160 ± 0.230), indicating that there is not much non-additive genetic variance or variance due to common environment. Thus, the heritability of PO activity is best estimated by the combined sire + dam estimate, based on the resemblance between full sibs ($h^2 = 0.690 \pm 0.069$).

(n.b. heritability estimates using the Animal Model gave the same results as the nested ANOVA)

Table 3.1 – Repeatability of phenoloxidase activity.

The within-sample repeatability is a measure of the accuracy of the sampling method. The 24 and 48 hour measures indicate the relationship between PO levels within each larvae from day to day.

Component	Repeatability ± SE	df	P
Within sample	0.983 ± 0.005	34	P = 0.005
Between samples – 24 hours	0.501 ± 0.120	18	P = 0.002
Between samples – 48 hours	0.295 ± 0.169	34	P = 0.032

Table 3.2 – Heritability of phenoloxidase activity.

Variance components were estimated using REML, and heritability estimates, standard errors and 95 percent confidence intervals are based on 1000 bootstrapped samples (see text).

Component	Heritability ± SE	95% confidence interval	P
sire	0.770 ± 0.103	0.577 – 0.989	P < 0.001
dam	0.610 ± 0.160	0.300 – 0.928	P < 0.001
sire + dam	0.690 ± 0.069	0.561 – 0.831	P < 0.001

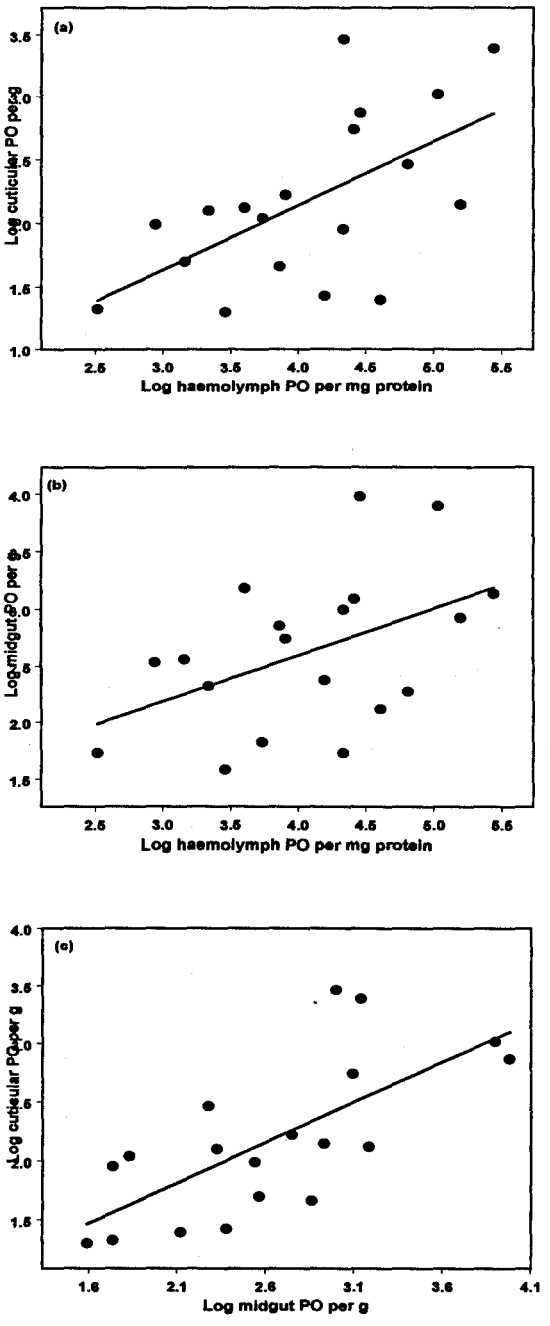


Figure 3.1 – The relationship between phenoloxidase activity in different parts of the body.

The correlation between haemolymph PO and cuticular PO (a), the correlation between haemolymph PO and midgut PO (b), the correlation between cuticular PO and midgut PO (c).

PO in the haemolymph, midgut and cuticle

There were strong positive correlations between the PO levels in the haemolymph and cuticle ($r = 0.590 \pm 0.142$, $df = 17$, $p = 0.005$; Figure 3.1a), the haemolymph and midgut ($r = 0.471 \pm 0.146$, $df = 17$, $p = 0.020$; Figure 3.1b) and the midgut and cuticle ($r = 0.701 \pm 0.085$, $df = 17$, $p < 0.001$; Figure 3.1c).

Encapsulation assay

There was a strong, positive correlation between the level of encapsulation of the nylon and the PO activity in the haemolymph using both a qualitative, visual scoring method ($r = 0.622 \pm 0.142$, $df = 15$, $p = 0.005$; Figure 3.2a) and two quantitative scores. The first, a melanization score, measured the mean level of blackness of the nylon and indicates the amount of melanin deposited on the cell capsule ($r = 0.589 \pm 0.149$, $df = 15$, $p = 0.006$; Figure 3.2b), the second scored the volume of the attaching cell mass (capsule area) ($r = 0.517 \pm 0.154$, $df = 15$, $p = 0.041$; Figure 3.2c). The visual score was strongly correlated with both the melanization score ($r = 0.961 \pm 0.018$, $df = 15$, $p < 0.001$) and the capsule area ($r = 0.615 \pm 0.148$, $df = 15$, $p = 0.014$). The two quantitative scores were also positively correlated with each other ($r = 0.579 \pm 0.144$, $df = 15$, $p = 0.012$).

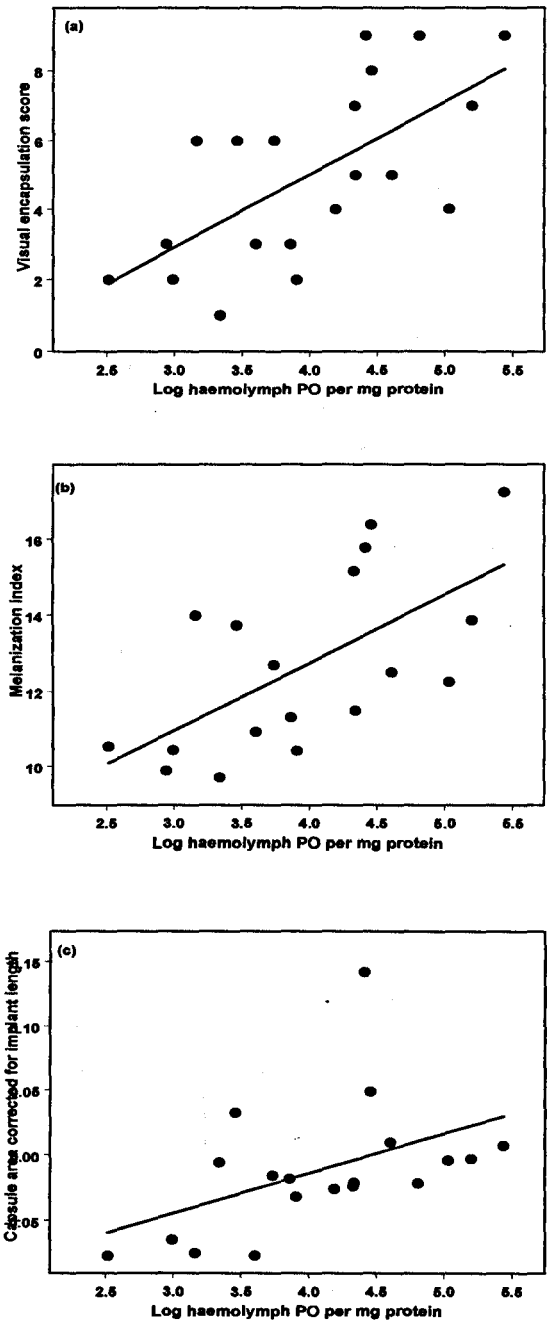


Figure 3.2 – The relationship between haemolymph phenoloxidase levels and the encapsulation of a nylon insert.

Encapsulation is measured by (a) visual encapsulation score, (b) degree of melanization of the nylon and (c) capsule area corrected for the length of the nylon

As the two quantitative encapsulation measurements were highly correlated, the relationship between encapsulation and haemolymph PO was determined using a MANOVA that included both the capsule area and the melanization score as dependent variables. There was a significant effect of haemolymph PO on the level of encapsulation (MANOVA: Wilks' lambda, _ = 0.602, approximate $F_{2,14} = 4.63$, p = 0.029; univariate tests: capsule area: $F_{1,15} = 5.75$, p = 0.03; melanization score: $F_{1,15} = 8.19$, p = 0.012). Thus, both the capsule area and the melanization score are affected independently by haemolymph PO levels.

Discussion

PO is not only an important component of the insect immune system but, as shown here, is a repeatable, heritable indicator of an individual's ability to cope with a novel parasite. Although there was significant repeatability of PO levels within individuals, the relationship was significantly different from unity indicating that PO levels were not the same each day. This is not particularly surprising as haemocyte number and the levels of certain proteins both increase with age and vary cyclically within instar, peaking just before ecdysis (Chapman 1998). The larvae used were all in their final instar and so the levels of variation could be due to small differences in the time since the last ecdysis, or the rate at which haemocytes are produced. As the repeatability of the measurement method was high (r = 0.983; Table 3.1) very little of the variation was due to within-sample measurement error.

It has been shown in a number of studies that the heritability of traits closely related to fitness tends to be low, as natural selection erodes genetic variation (Gustafsson

1986; Kruuk *et al.* 2000; Merila & Sheldon 2000). The high estimate of heritability of haemolymph PO activity could indicate that this trait is not closely linked to fitness. However, as an unsuccessful response to immune challenge could prove fatal, one would expect important components of the immune system to be strongly tied to individual fitness. So, what could be maintaining the additive genetic variance? One possibility is antagonistic pleiotropy, where a gene confers a positive effect on one component of fitness but a negative effect on another; this is the genetic basis for trade-offs (Roff 1992). Trade-offs have been shown to occur in a number of studies in which the resistance of a population to a parasite or pathogen has been increased by artificial selection. Boots & Begon (1993) showed that *Plodia interpunctella* resistant to its granulosis virus had lower egg viability and a longer development time. Kraaijeveld & Godfray (1997) found that *Drosophila melanogaster* resistant to the parasitoid *Asobara tabida* had lower larval competitive ability, and similar results have been obtained using the parasitoid *Leptopilina boulardi* (Fellowes *et al.* 1998a).

Trade-offs could occur between PO and life-history traits such as fecundity or longevity, or they could occur within the immune system. In other words, resistance to one parasite or pathogen may occur only at a detriment to another. Aso *et al.* (1985) suggested that haemolymph pro-PO was a precursor to both haemolymph and cuticular PO. Ashida & Brey (1995) have shown that in *Bombyx mori,* PO is synthesised in the haemocytes before being transported to the cuticle. The cuticle and midgut represent important, biologically-active barriers to infection (Paskewitz *et al.* 1989; Ashida & Brey 1995). However, with a common precursor, high PO levels in the cuticle could be bought at the expense of PO in the haemolymph and/or midgut. In this way, resistance to fungus and parasitoids that

attack via the cuticle could mean lowered levels of resistance to baculoviruses that enter via the midgut. In *S. littoralis* we have shown that this is not the case. We found no evidence for a trade-off between PO activity in different body tissues. In fact, there were strong, positive correlations between PO activity in the haemolymph, midgut and cuticle, indicating that individuals that invest in high levels of haemolymph PO have correspondingly high levels in the midgut and cuticle, suggesting that they could resist attack via either route. A similar pattern has recently been found in the closely related species, *Spodoptera exempta* (Wilson *et al.* 2001). Of course, this does not mean that a trade-off between PO levels in different parts of the body does not exist, simply that our experimental protocol was not able to reveal them. Only by manipulating insects so as to change their allocation rules are such trade-offs likely to be revealed (Lessells 1991). Trade-offs may also occur between functionally different aspects of the insect immune system. For example, it is possible that PO levels may be negatively correlated with the phagocytic activity of haemocytes or the levels of antibacterial proteins or agglutinins in the haemolymph, all of which are important in resistance against bacteria.

Despite all of its suggested roles in the co-ordinated response to immune challenge, there is little evidence linking PO activity in the haemolymph with an individual's ability to resist infection. It has been shown in *Anopheles* that the ability to encapsulate abiotic material is strongly related to the ability to encapsulate a parasite (Paskewitz & Riehle 1994; Gorman *et al.* 1996), and this was the motivation behind our experiment to determine the association between haemolymph PO and the ability to encapsulate a novel pathogen (nylon monofilament). We found that these two measures were strongly correlated,

suggesting that individuals that invest in PO activity in the haemolymph, midgut and cuticle have a greater capacity for encapsulating parasites. Future studies will examine any costs associated with increased levels of PO and the association between PO and resistance to entomopathogens.

Figure caption: pale and dark 4th instar *S. littoralis* larvae.

Chapter 4

Melanism and disease resistance in insects

K. Wilson, S.C. Cotter, A.F. Reeson & J.K. Pell (2001).

Ecology Letters, 4: 637-649.

Abstract

There is growing evidence that insects in high-density populations invest relatively more in pathogen resistance than those in low-density populations (i.e. density-dependent prophylaxis). Such increases in resistance are often accompanied by cuticular melanism, which is characteristic of the high-density form of many phase polyphenic insects. Both melanism and pathogen resistance involve the prophenoloxidase enzyme system. In this paper the link between resistance, melanism and phenoloxidase activity is examined in *Spodoptera* larvae. In *S. exempta*, cuticular melanism was positively correlated with phenoloxidase activity in the cuticle, haemolymph and midgut. Melanic *S. exempta* larvae were found to melanize a greater proportion of eggs of the ectoparasitoid *Euplectrus laphygmae* than non-melanic larvae, and melanic *S. littoralis* were more resistant to the entomopathogenic fungus *Beauvaria bassiana* (in *S. exempta* the association between melanism and fungal resistance was non-significant). These results strengthen the link between melanism and disease resistance and implicate the involvement of phenoloxidase.

Introduction

Density-dependent prophylaxis (DDP) is the phenomenon in which individuals invest more in immune function when at high population densities, as a counter-measure to density-dependent pathogen transmission rates (Wilson & Reeson 1998). Evidence in support of DDP is accruing from a number of insects that exhibit density-dependent phase polyphenism (Long 1953; Mitsui & Kunimi 1988; Kunimi & Yamada 1990; Goulson & Cory 1995; Reeson *et al.* 1998; Barnes & Siva-Jothy 2000; Reeson *et al.* 2000). In all of these cases, larval crowding is associated with cuticular melanization ('melanism'; Figure 1a). The co-occurrence of melanism and increased levels of disease resistance in the high-density form of phase polyphenic insects raises the possibility that the two phenomena are closely linked.

Five studies have examined the relationship between melanism and disease resistance whilst controlling for larval density. Melanic *Spodoptera exempta* larvae were up to four times more resistant to a nucleopolyhedrovirus (NPV) than non-melanic conspecifics (Reeson *et al.* 1998), melanic *Mythimna separata* larvae were twice as resistant to NPV (Kunimi & Yamada 1990) and five times more resistant to an entomopathogenic fungus (Mitsui & Kunimi 1988), and melanic *Tenebrio molitor* were up to three times more resistant to an entomopathogenic fungus (Barnes & Siva-Jothy 2000). In contrast, melanic *Mamestra brassicae* larvae were significantly more susceptible to NPV than non-melanics (Goulson & Cory 1995). Thus, in four out of five studies in which density-dependent effects have been controlled for, there was a positive association between melanism and pathogen resistance, and a number of authors have suggested that melanism may be a useful marker for high levels of

investment in immune defence in insects (Majerus 1998; Barnes & Siva-Jothy 2000; Reeson *et al.* 1998). If such an association exists, what is the mechanism generating it? In this paper, we argue that the cuticular melanin is functional in disease resistance and is one of a suite of prophylactic resistance traits exhibited by insects in response to the increased threat of disease associated with crowding.

The first, and probably best, lines of defence against most parasites and pathogens 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 favoured when the threat of disease is high (e.g. during periods of crowding). Melanin has at least two properties that are likely to increase immunocompetence (*sensu* Owens & Wilson 1999). First, because it is a polymer, melanin is likely to strengthen cuticle and so improve its ability to act as a *physical barrier* to the penetration of cutaneously-entering parasites and pathogens, such as fungi, bacteria and even parasitoids (St Leger *et al.* 1988; Hajek & St. Leger 1994). Second, and perhaps more importantly, melanin is *toxic* to microorganisms and has potent anti-microbial activity (e.g. Montefiori & Zhou 1991; Ourth & Renis 1993; Sidibe *et al.* 1996; Ishikawa *et al.* 2000). The mechanisms for this are unclear at present, but a number of studies have shown that melanin binds to a range of proteins (e.g. Doering *et al.* 1999) and inhibits many of the lytic enzymes produced by microorganisms, including proteases and chitinases (Kuo & Alexander 1967; Bull 1970). Söderhäll & Ajaxon (1982) showed that when the crayfish-parasitic fungus *Aphanomyces astaci* was grown on a PG-I agar medium, there was significant inhibition of fungal growth when the growth-medium contained melanin or any of its constituent quinones. Moreover, St. Leger *et al* (1988) showed that when larval cuticles of *Manduca sexta* were induced to melanize, they resisted fungal

penetration for 30 h longer than unmelanized cuticles. Thus, melanin may enhance disease resistance in insects not only by improving the physical properties of insect cuticle, but also by enhancing its chemical properties.

The prophenoloxidase cascade is a suite of enzymes that oxidize tyrosine derivatives to their corresponding quinones and their polymerization product, melanin (Mason 1955; Hiruma & Riddiford 1988b; Nappi & Vass 1993). These enzymes are involved not only in cuticular melanization, but also in the various immune responses directed against parasites and pathogens, including cellular encapsulation, humoral encapsulation and nodule formation (Poinar 1974; Götz 1986; Paskewitz *et al.* 1988; Hung & Boucias 1992; Beckage *et al.* 1993; Washburn *et al.* 1996; 2000). Phenoloxidase production is activated in the cuticle when it 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 *et al.* 1993). The midgut is an important site for resisting pathogens that enter the host orally, such as baculoviruses, protozoa and many bacteria, and melanin is also produced during this process, suggesting a role for phenoloxidase. For example, the spread of baculovirus in non-permissive hosts appears to be blocked by aggregations of haemocytes that form melanotic capsules around infected cells in the midgut trachea (Washburn *et al.* 1996; Washburn *et al.* 2000). Similarly, in a mosquito selected for resistance to malaria, parasite ookinetes are melanized between the midgut epithelial cells and the basal laminae (Collins *et al.* 1986). These resistant insects also show higher phenoloxidase activity in the midgut following exposure to the parasite (Paskewitz *et al.* 1989). Moreover, in tsetse flies (*Glossina* spp.), there was a significant

positive association between PO activity and refractoriness to the protozoan *Trypanosoma brucei rhodesiense*, both within species (male versus female *G. morsitans morsitans*) and among species (*G. m. morsitans* versus *G. palpalis palpalis*; Nigam *et al.* 1997).

If melanin enhances resistance to parasites and pathogens, both when it is a static component of the insect cuticle and when it is produced *de novo* in response to pathogen attack (via the action of the prophenoloxidase cascade), then both cuticular melanization and enhanced phenoloxidase activity might be expected to increase in response to cues predicting likely disease threat, such as increased population density. So far, the only studies to have examined the link between DDP and phenoloxidase have done so using phenoloxidase measured in the haemolymph. In *S. exempta*, haemolymph PO activity was greatest in melanic larvae and in individuals reared under crowded conditions (Reeson *et al.* 1998), whereas in *T. molitor*, there was no association between haemolymph PO and rearing density (the correlation between PO and melanism was not determined directly; Barnes & Siva-Jothy 2000) .

The main aim of the present study was, therefore, to quantify density-dependent production of PO in the key sites for pathogen resistance: the cuticle, haemolymph and midgut. We also set out to determine the relationships between PO levels across these tissues and how these relate to cuticular melanization; do individuals with melanic cuticles have higher levels of PO in the cuticle and are PO levels in the different tissues correlated? Finally, we tested whether density-dependent changes in the cuticle were associated with variation in resistance to parasites and pathogens that access their hosts percutaneously, using an ectoparasitoid and an entomopathogenic fungus. As our model system, we used Lepidopteran larvae from

the genus *Spodoptera* – the African armyworm, *S. exempta* and the Egyptian cotton leafworm, *S. littoralis*. Both of these species exhibit density-dependent phase polyphenism, developing melanized cuticles in response to larval crowding (Faure 1943; Hodjat 1970; Gunn 1998).

Materials and methods

Rearing of larvae

Except where stated, all experiments used fourth instar *S. exempta* larvae from laboratory stocks that had been maintained at 27°C and 12:12 light:dark photoperiod for at least ten generations. Larvae were reared from within 48 hours of hatching at either one (*solitary-reared*) or four (*crowd-reared*) larvae per 12 ml plastic pot. During this time they were fed on a wheatgerm-based artificial diet (Appendix 1) and their colour generally ranged from pale grey to jet black. For the parasitoid experiment, larval colour was scored by eye on a five point scale: -2 = very pale, -1 = pale, 0 = mid, 1 = dark, 2 = very dark, but in the other experiments, we categorised larvae as simply *pale* (score = –2 to 0) and *dark* (score = 1 or 2). Using image analysis software, we were subsequently able to quantify the difference between pale and dark cuticles (see below).

In phase polyphenic species, including *S. exempta*, development into the dark, high-density (*gregaria*) phenotype is triggered by the perception of (usually tactile) cues typical of high population densities (e.g. Kazimirova 1992). However, the threshold density at which this phenotypic change occurs is under genetic control (e.g. Goulson 1994), and so even when reared solitarily the development of some individuals is stimulated towards that of the high-density phenotype. Thus, we can

consider these 'dark-solitary' larvae to be at an intermediate position on the *gregarization* scale (i.e. they exhibit traits that are intermediate between the typical *solitaria* and *gregaria* phenotypes). Since 'pale-crowded' larvae are rare in *S. exempta*, we were generally able to assess the colour of only three phenotypes: the 'typical' pale-solitary form (Figure 1a, left), the 'typical' dark-crowded form (Figure 1a, right), and the dark-solitary form, which looks like the typical crowded form to the naked eye (in the statistical analyses below, these are referred to as phenotypes 1, 3 and 2, respectively).

S. littoralis had been in culture for eight generations at the start of this study. The rearing conditions were the same as for *S. exempta*, except that all larvae were reared at 25°C and crowded larvae were produced at a density of three larvae per 12 ml pot.

Phenoloxidase assays

Haemolymph was extracted from each larva between the last pair of prolegs and 8 µl placed in 400 µl of ice-cold phosphate-buffered saline (PBS, pH 7.4; Appendix 2) and mixed in a plastic Eppendorf tube. The sample was frozen to disrupt haemocyte membranes and PO activity in the defrosted sample was assayed spectrophotometrically using L-Dopa as a substrate (after Ashida & Söderhäll 1984). This involved pipetting triplicate 100 µl samples of the buffered haemolymph into a microtitre plate, adding 100 µl of 20 mM L-Dopa to each and incubating the mixture at 25°C. The absorbance was read at 492 nm on a temperature-controlled *Versamax tuneable microplate reader* after 20 minutes, which was during the linear phase of the reaction.

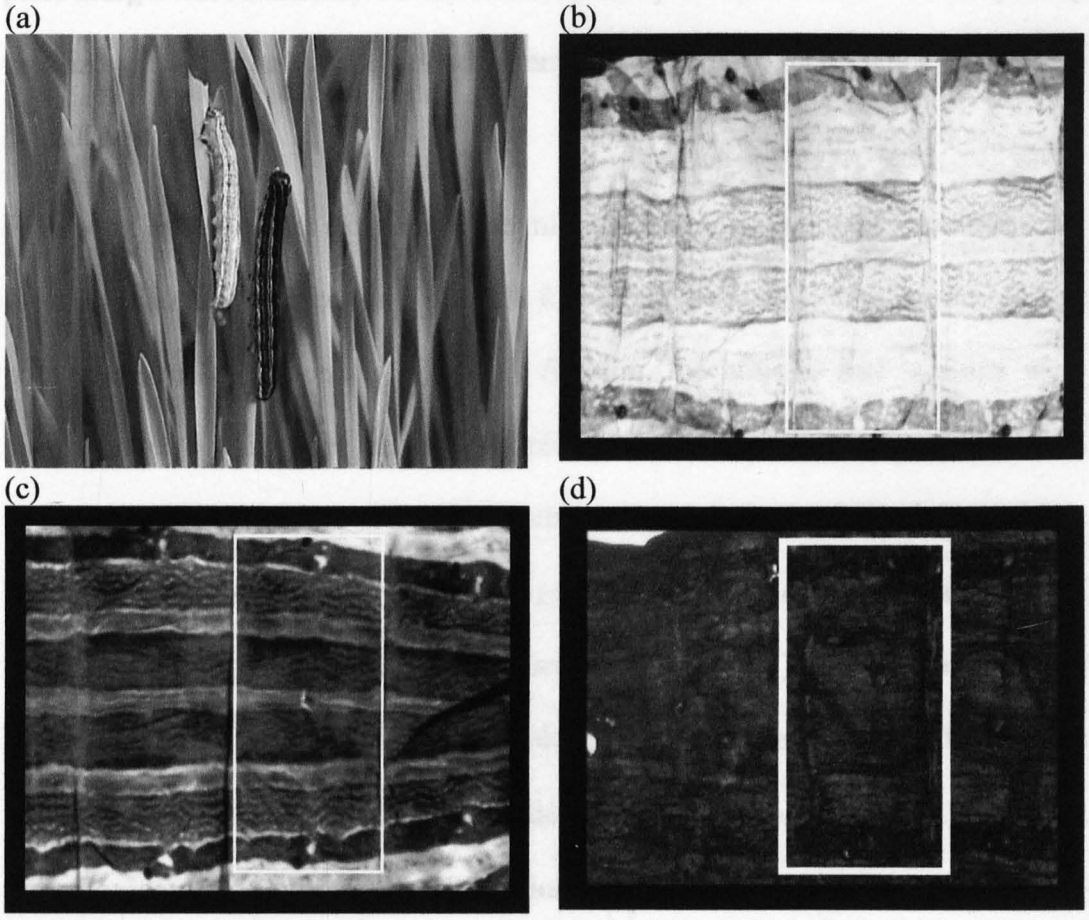


Figure 4.1 – Density-dependent cuticular melanization in *Spodoptera exempta.*

(a) Live larvae, showing the pale, low-density phenotype on the left and the dark, high-density phenotype on the right; (b) the dorsal cuticle of the pale phenotype; (c) the dorsal cuticle of the dark phenotype; (d) the dorsal cuticle of the pale phenotype following incubation in L-Dopa. Cuticular melanization was measured as the mean and maximum density score in the area shown by the white boxes in (b) - (d).

Using 10 µl of the haemolymph/PBS mixture, the amount of protein in the sample was also measured (calibrated using a standard curve created on the same microtitre plate using a BSA standard). Phenoloxidase activity is expressed as PO units per mg protein, where one unit is the amount of enzyme required to increase the absorbance by 0.001 per minute.

Following haemolymph extraction, the midguts and dorsal cuticles were dissected from each larva and fixed for 1 h in 2% formaldehyde and 0.5% glutaraldehyde in phosphate buffer (Appendix 2). After fixation, the cuticles and midguts were washed over 3 h in three changes of phosphate buffer (Wolfgang & Riddiford 1981). They were then cut in half and each piece weighed. One half of each midgut and cuticle was then placed in 1 ml of 20 mM L-Dopa. The other half was placed in 1 ml of 20 mM L-Dopa saturated with phenylthiourea (PTU) as a control (PTU acts as a PO inhibitor and none of these samples darkened). After 40 min, 200 µl of the mixture was pipetted into a microtitre plate and the absorbance measured at 492 nm. Phenoloxidase was expressed as PO units per g of tissue. Samples of 20 mM L-Dopa alone were incubated along with the experimental samples to act as a control for any natural darkening of the substrate during the experiment. None of these samples darkened, indicating that melanization of all samples was due to endogenous PO.

Cuticular melanization scoring

Fixed cuticles and midguts were mounted and photographed using a *Polaroid DMC* digital camera and the images scored for their degree of melanization using *Image Pro Plus* software (Cybernetics 1999). Cuticular melanization was measured as the

mean and maximum melanization scores (*1/mean density* and *1/min density*, respectively, on the *Measurements* tab of *Image Pro Plus*). The midgut is fairly homogeneous in colour and so a central area was chosen for scoring. The dorsal cuticle, however, comprises a series of longitudinal stripes varying in their degree of melanization. Thus, mean and maximum melanization was scored for an area that spanned these different degrees of melanization (shown by the boxes in Figures 1b-1d). We also measured melanization in each of the longitudinal stripes. However, since none of these measures distinguished between pale and dark caterpillars any better than mean density and maximum density, they will not be discussed further.

Resistance to ectoparasitoids

Six days post-hatch, the colour of solitary-reared and crowd-reared larvae was scored and they were left singly overnight with a mated, honeywater-fed *Euplectrus laphygmae* female in a Petri dish. The following day, the number of eggs laid on the larva was counted. Larvae were maintained singly in labelled pots containing artificial diet. Most eggs hatched within 2-3 days, but a significant percentage of eggs (34.7 $\pm$ 4.2% eggs; mean $\pm$ SE, n = 59 larvae) became melanized and failed to develop further. Most of these eggs subsequently appeared to shrink in size and in some cases disappeared, to be replaced by a patch of melanin on the surface of the cuticle. The proportion of parasitoid eggs that became melanized was used as our measure of host resistance to ectoparasitoids. The parasitoids and the larvae used in this experiment were collected in Arusha, Tanzania in April 1999 and had been in culture for just two generations at the start of the experiment.

Resistance to entomopathogenic fungi

We assessed resistance to entomopathogenic fungi using an isolate of *Beauveria bassiana* (re-isolation of *Mycotrol*® strain *GHA*). The fungus was grown on Sabouraud dextrose agar (SDA) in 9cm Petri dishes for 21 days at 23°C in darkness. Conidia were harvested from the dishes by scraping into 0.03% sterile Tween 80 using a sterile scalpel. After filtration through several layers of nylon netting, this provided a stock suspension ($1.31x10^9$ conidia per ml) that was diluted to provide suspensions of $1x10^8$ and $1x10^9$ conidia per ml for the bioassay. Control insects were bioassayed with the carrier surfactant (0.03% Tween 80). Prior to the bioassay, a sample of conidia from suspension was plated onto SDA and viability was assessed after 24 h at 23°C in darkness. This showed that the percentage germination was 98.3%.

Fungal resistance was assessed in two species of *Spodoptera* that differed in their susceptibilities to *B. bassiana*: *S. littoralis* (the more resistant species, at the doses used) and *S. exempta* (the more susceptible). Solitary-reared and crowd-reared larvae were scored as either pale or dark and dipped, in groups of 12, into 8 ml of conidial suspension (or Tween control). Inoculated larvae were then placed into a Buchner funnel and the residual suspension removed by vacuum filtration. After inoculation, all individuals were transferred to pots of fresh artificial diet and maintained singly in an incubator at 23° C (L:D 12:12). Mortality was recorded daily from four days post inoculation and the cause of mortality was verified by checking for sporulation in cadavers placed in square Petri dishes lined with 1% agar.

Statistical analysis

All analyses were conducted using either the *S-Plus 2000* statistical package (Statistical Solutions, Inc.) or *Minitab*, version 13.1 (Minitab, Inc.). Since many of the analyses involved multiple, correlated measurements taken from the same insects (e.g. several measurements of melanism or PO activity), where appropriate multivariate analysis of variance (MANOVA) was used to analyse the data. This allows one to consider multiple responses as a single multivariate response, rather than a collection of univariate responses, so allowing the covariation between multiple measurements to be explicitly modelled and the probability of Type I errors to be minimised. The statistical significance of the MANOVAs reported here was determined by using Wilks' Lambda (_), though the alternative tests (e.g. Pillai's trace) always gave similar results. We also report an approximate F and its associated p-value, based on a transformation of _. When the MANOVA was statistically significant (H_0 was rejected), univariate ANOVAs were performed for each response variable using sums of squares adjusted for the other dependent variables in the model. Partial correlation coefficients were calculated using the *VassarStats* website (http://departments.vassar.edu/~lowry/par.html).

Results

Larval density, colour and melanization of the cuticle and midgut

Mean and maximum melanization scores were highly correlated (Table 4.1). Therefore, the relationship between cuticular melanization and other larval attributes was determined using MANOVAs that included mean and maximum melanization scores as dependent variables. The intensity of cuticular melanization was significantly greater in crowd-reared larvae than in larvae reared solitarily (MANOVA: Wilks' lambda, _ = 0.477, approximate $F_{2,57} = 31.28$, $p < 0.001$; univariate tests: mean: $F_{1,58} = 60.50$, $p < 0.001$; maximum: $F_{1,58} = 47.67$, $p < 0.001$). Thus, both mean and maximum cuticular melanization scores responded independently to changes in larval rearing density, though the mean value appears marginally more responsive. Not surprisingly, within the solitary-reared larvae, dark individuals had significantly higher cuticular melanization scores than pale ones (Wilks' _ = 0.764, $F_{2,37} = 5.70$, $p = 0.007$; univariate tests: mean: $F_{1,38} = 10.81$, $p = 0.002$; maximum: $F_{1,38} = 8.04$, $p = 0.007$). As a consequence of the above patterns, there was a significant positive relationship between larval phenotype (scored 1-3) and the degree of cuticular melanization (MANOVA: Wilks' _ = 0.394, $F_{2,57} = 43.91$, $p < 0.001$; univariate tests: mean: $F_{1,58} = 83.66$, $p < 0.001$; maximum: $F_{1,58} = 64.22$, $p < 0.001$; Figure 4.2a).

Table 4.1 – Correlations between phenoloxidase activity and the degree of cuticular melanization.

Pearson's correlation coefficients are shown (n = 60). Mean and maximum melanization scores were determined by digital image analysis (see text for details).

	Haemolymph PO	Cuticle PO	Midgut PO	Mean Cuticle Melanization Score	Maximum Cuticle Melanization Score
Haemolymph PO		r = 0.480 p < 0.001 ***	r = 0.421 p < 0.001 ***	r = 0.408 p = 0.002 **	r = 0.386 p = 0.003 **
Cuticle PO			r = 0.465 p < 0.001 ***	r = 0.336 p = 0.009 **	r = 0.230 p = 0.077 +
Midgut PO				r = 0.220 p = 0.091 +	r = 0.305 p = 0.018 *
Mean Cuticle Melanization Score					r = 0.847 p < 0.001 ***

Midgut melanization also varied significantly with rearing density (Wilks' Λ = 0.864, $F_{2,49}$ = 3.86, p = 0.028; univariate tests: mean: $F_{1,50}$ = 0.01, p > 0.9; maximum: $F_{1,58}$ = 6.42, p = 0.014), but the response was largely restricted to changes in the maximum melanization score. Within solitary-reared larvae, pale and dark individuals did not differ in their degree of midgut melanization (Wilks' Λ = 0.919, $F_{2,32}$ = 1.41, p > 0.2). Thus, the significant positive relationship between larval phenotype (1-3) and degree of midgut melanization (Wilks' Λ = 0.862, $F_{2,49}$ = 3.90, p = 0.027; univariate tests: mean: $F_{1,50}$ = 0.37, p > 0.5; maximum: $F_{1,50}$ = 4.64, P = 0.036; Figure 4.2b) was largely a consequence of density-dependent differences in the maximum melanization score.

Analyses of whole mounts of cuticles under a light microscope showed that variation in the darkness of 'pale' and 'dark' caterpillars was due to variation in the density of small melanin granules (approx. 1 μm diameter) in the dorso-lateral stripes (cf. Figures 4.1b,c). Following incubation with L-Dopa, all cuticles became much darker due to the uniform deposition of melanin (cf. Figures 4.1b,d).

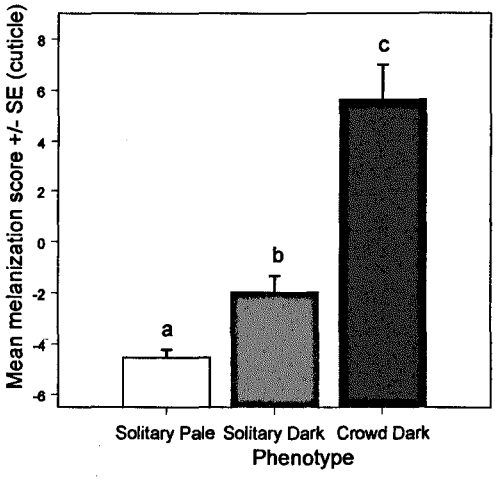
Larval density, colour and phenoloxidase activity

Haemolymph PO activity increased with both rearing density (ANOVA using all larvae: $F_{1,58}$ = 7.69, p = 0.007) and cuticle colour (ANOVA using solitary-reared larvae only: $F_{1,38}$ = 10.86, p = 0.002), resulting in haemolymph PO activity being greatest in 'typical' (dark) crowded larvae, lowest in 'typical' (pale) solitary larvae and intermediate in dark-solitary larvae (r_s = 0.651, p < 0.001; Figure 4.2c).

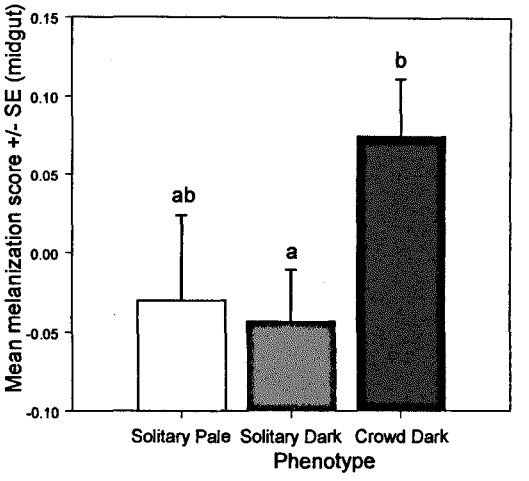
Figure 4.2 – Relationship between larval phenotype and (a,b) melanization scores and (c,d) phenoloxidase activity.

In the top panel, the maximum melanization score (mean ± standard error) is plotted against larval phenotype (solitary-pale, solitary-dark, crowded-dark) for (a) the cuticle and (b) the midgut. In the bottom panel, PO activity in (c) the haemolymph and (d) the cuticle and midgut is plotted against mean melanization score for each larval phenotype. In (c), PO activity (mean ± standard error) is expressed as PO units per mg protein. Symbols sharing a common superscript are not significantly different from each other, based on Fisher's LSD multiple comparisons following one-factor ANOVAs with larval phenotype as the factor. In (d), this applies only *within* tissue-type; comparisons *across* tissue types are not valid. Correlations across phenotypes and melanization scores are shown in Table 1.

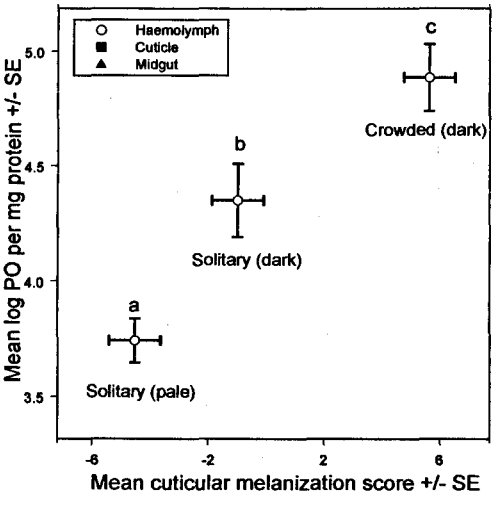
(a)


Mean melanization score +/- SE (cuticle)
8
6
4
2
0
-2
-4
-6
a
b
c
Solitary Pale
Solitary Dark
Crowd Dark
Phenotype

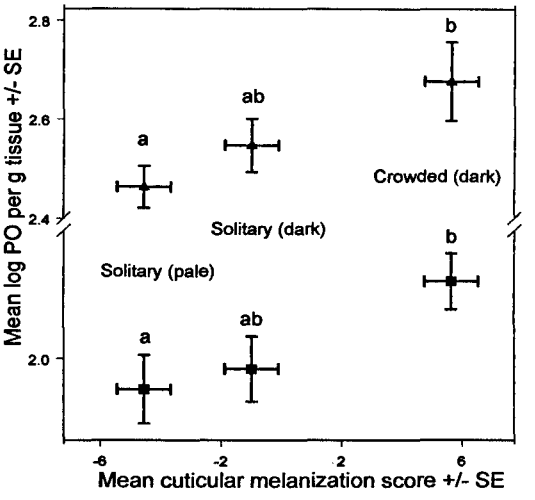
(b)


Mean melanization score +/- SE (midgut)
0.15
0.10
0.05
0.00
-0.05
-0.10
ab
a
b
Solitary Pale
Solitary Dark
Crowd Dark
Phenotype

(c)


Haemolymph
Cuticle
Midgut
Mean log PO per mg protein +/- SE
5.0
4.5
4.0
3.5
a
b
c
Solitary (pale)
Solitary (dark)
Crowded (dark)
-6
-2
2
6
Mean cuticular melanization score +/- SE

(d)


Mean log PO per g tissue +/- SE
2.8
2.6
2.4
2.0
a
ab
b
a
ab
b
Solitary (pale)
Solitary (dark)
Crowded (dark)
-6
-2
2
6
Mean cuticular melanization score +/- SE

Phenoloxidase activity in both the cuticle and midgut also increased with increasing 'gregarization' from pale-solitary to dark-crowded larvae (cuticle: $r_s = 0.312$, $p = 0.016$; midgut: $r_s = 0.295$, $p = 0.022$; Figure 4.2d), due mainly to a significant difference between solitary and crowded larvae (cuticle: $F_{1,58} = 6.38$, $p = 0.014$; midgut: $F_{1,58} = 4.71$, $p = 0.039$), rather than between pale and dark solitary larvae (cuticle: $F_{1,38} = 0.21$, $p > 0.6$; midgut: $F_{1,38} = 0.84$, $p > 0.3$).

All three PO measurements were significantly positively correlated with each other; the average correlation across all three phenotypes was $r \geq 0.416$, $p \leq 0.001$ (Table 4.1). The partial correlation coefficients were also high and generally significantly different from zero (haemolymph PO – cuticle PO: $r_{partial} = 0.354$, $p = 0.006$; haemolymph PO – midgut PO: $r_{partial} = 0.255$, $p = 0.051$; cuticle PO – midgut PO: $r_{partial} = 0.330$, $p = 0.010$). Thus, it is appropriate to examine the relationship between phenoloxidase activity and other larval attributes using a multivariate approach.

There was a highly significant relationship between overall phenoloxidase activity (in the haemolymph, cuticle and midgut) and larval rearing density (MANOVA: Wilks' _ = 0.719, $F_{3,56} = 7.29$, $p < 0.001$), with all three of these measurements making significant, independent contributions to the strength of the relationship (univariate tests: haemolymph PO: $F_{1,58} = 21.91$, $p < 0.001$; cuticle PO: $F_{1,58} = 6.39$, $p = 0.014$; midgut PO: $F_{1,58} = 4.72$, $p = 0.034$). Similar results were obtained when larval phenotype (1 – 3) was the predictor variable (Wilks' _ = 0.616, $F_{3,56} = 11.63$, $p < 0.001$; univariate tests: haemolymph PO: $F_{1,58} = 35.90$, $p < 0.001$; cuticle PO: $F_{1,58} = 5.81$, $p = 0.019$; midgut PO: $F_{1,58} = 5.55$, $p = 0.022$). When colour (pale or dark) was the predictor variable (for solitary larvae), the MANOVA remained statistically significant, but this was due primarily to the association between colour

and haemolymph PO (Wilks' _ = 0.767, $F_{3,36}$ = 3.63, p = 0.022; univariate tests: haemolymph PO: $F_{1,38}$ = 10.86, p = 0.002; cuticle PO: $F_{1,38}$ = 0.19, p > 0.6; midgut PO: $F_{1,38}$ = 1.54, p > 0.2). It is unclear, at this stage, whether the non-significance of the two other PO measures is due to genuine biological reasons if whether it is simply a consequence of the relatively small number of solitary larvae examined.

Phenoloxidase activity and melanization of the cuticle and midgut

Phenoloxidase activity in all three tissues (haemolymph, midgut and cuticle) was significantly positively correlated with one or both of the cuticular melanization measurements (Table 4.1). When all three phenoloxidase measurements were included as dependent variables in a multivariate ANOVA, there was a significant positive relationship between the cuticular melanization score and overall phenoloxidase activity (MANOVA: Wilks' _ = 0.839, $F_{3,56}$ = 3.59, p = 0.019; univariate tests: haemolymph PO: $F_{1,58}$ = 9.27, p = 0.004; cuticle PO: $F_{1,58}$ = 5.38, p = 0.024; midgut PO: $F_{1,58}$ = 4.39, p = 0.040). The fact that all three of the univariate tests were independently statistically significant indicates that cuticular melanization is a good indicator of relative investment in PO activity in all three tissues (Figures 4.2c,d).

The degree of midgut melanization was significantly positively correlated with haemolymph PO (r = 0.292, n = 60, p = 0.037), but not with midgut PO ($r \leq 0.156$, $p \geq 0.265$) or cuticular melanization score ($r \leq 0.214$, $p \geq 0.127$). When all three phenoloxidase measurements were included as dependent variables in a MANOVA, the association between PO activity and midgut melanization was non-significant (MANOVA: Wilks' _ = 0.888, $F_{3,48}$ = 2.01, p = 0.125).

Resistance to ectoparasitoids

The number of eggs laid by ovipositing female *E. laphygmae* ranged between 1 and 15, and was independent of larval colour and rearing density (linear regression: $F_{1,87} < 1.28$, $p > 0.25$). The proportion of eggs that became melanized was independent of the number of eggs laid on each host (logistic regression: $\chi^2_1 = 0.083$, $p > 0.77$) and the density at which the host was reared ($\chi^2_1 = 0.093$, $p > 0.77$). However, there was a strong positive relationship between larval colour and the proportion of eggs that became melanized ($\chi^2_1 = 9.003$, $p = 0.003$; Figure 4.3). In other words, the darker the cuticle, the greater the proportion of parasitoid eggs that became melanized.

Resistance to entomopathogenic fungi

Fungus-induced mortality in *S. littoralis* was significantly affected by dose (logistic regression: $\chi^2_1 = 7.86$, $p = 0.005$; Figure 4.4a,b), larval colour ($\chi^2_1 = 6.370$, $p = 0.012$; Figure 4.4a) and rearing density ($\chi^2_1 = 4.569$, $p = 0.033$; Figure 4.4b). None of the interaction terms were statistically significant. Thus, at both doses, fungus-induced mortality in *S. littoralis* was significantly greater in solitary-reared caterpillars and in caterpillars with pale cuticles, than in crowd-reared caterpillars and those with dark cuticles, and these effects were additive. There was no fungus-induced mortality in the control group.

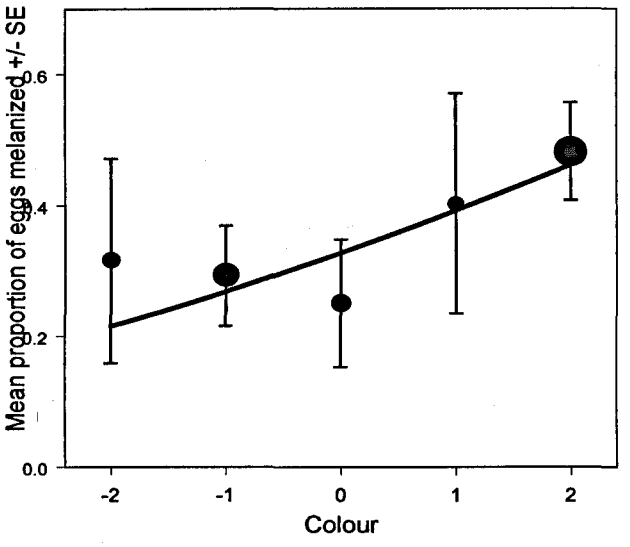


Figure 4.3 – Relationship between cuticular melanization and resistance to the ectoparasitoid *E. laphygmae*.

The vertical axis shows the proportion of melanized eggs (± standard error) 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.

Fungus-induced mortality was much greater for *S. exempta* than *S. littoralis* (Figure 4.4). There was a significant effect of dose on mortality in *S. exempta* ($\chi^2_1 = 24.81$, $p < 0.001$). Although rearing density ($\chi^2_1 = 0.001$, ns) and colour ($\chi^2_1 = 0.43$, ns) were non-significant as main effects, there were significant interactions between dose and rearing density ($\chi^2_1 = 13.72$, $p < 0.001$; Figure 4.4a) and between rearing density and colour ($\chi^2_1 = 5.47$, $p < 0.02$). When the low- and high-dose treatments were analysed separately, fungus-induced mortality was significantly greater in solitary larvae than crowded larvae in the low-dose treatment (64% vs 44%; $\chi^2_1 = 5.63$, $p = 0.017$), whereas the trend was significant and reversed in the high-dose treatment (73% vs 89%; $\chi^2_1 = 7.03$, $p = 0.008$; Figure 4.4a).

Discussion

Our results provide further support for an association between population density, melanism and disease resistance in insects. Image-analysis indicated that there are three quantifiable phenotypes of *S. exempta*, which can be considered to lie on a gregarization scale, with pale-solitary larvae the least 'gregarized' and dark-crowded larvae the most, while dark-solitary larvae are at an intermediate position. This is in accord with our earlier observation that dark-solitary larvae show levels of resistance to a baculovirus that are intermediate between pale-solitary and dark-crowded larvae (Reeson *et al.* 1998). An association between melanism and rearing density was also observed in the midgut, suggesting that melanin in the midgut might also be functional and aid in resisting pathogens, such as baculoviruses, that enter the host orally (see below).

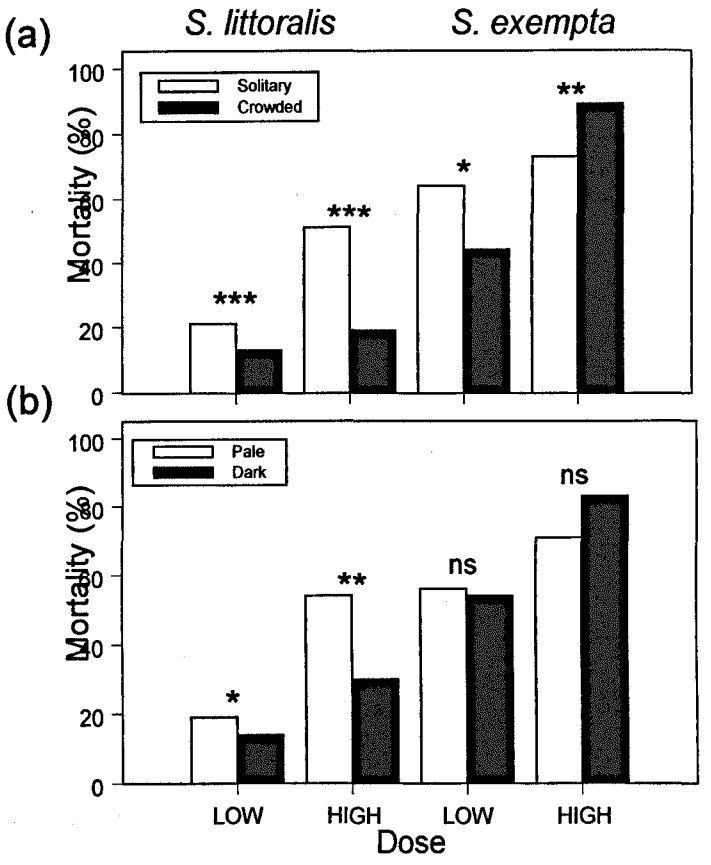


Figure 4.4 – Resistance to *B. bassiana* in relation to (a) rearing density and (b) cuticular melanization.

In (a), the comparison is between larvae reared solitarily and those in crowds (three or four larvae per pot); in (b) the comparison is between larvae with pale cuticles and those with dark cuticles. Low dose refers to 1 x 10^8 conidia/ml and 1 x 10^9 conidia/ml. Symbols above the bars refer to the statistical significance of the difference between treatments, as determined by logistic regression: ns $P > 0.05$, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$. See text for details of full analysis.

Overall, the midgut showed much lower levels of melanization than the cuticle, despite having higher levels of PO activity. This may reflect differences in the availability of PO substrates, since melanization in the midgut is likely to be constrained by functional requirements for the exchange of nutrients.

The density-dependent prophylaxis (DDP) hypothesis proposes that if the risk of being exposed to pathogens increases with density due to density-dependent pathogen transmission, then insects will use density as a cue to match their levels of investment in immune function to the perceived risk (Wilson & Reeson 1998). Consistent with this hypothesis, we found that phenoloxidase levels in the haemolymph, cuticle and midgut all, independently, increased with increasing 'gregarization'. Thus, the lowest PO levels were observed in pale, solitary-reared caterpillars and the highest were in dark, crowd-reared caterpillars. Within solitary-reared caterpillars, PO activity in all three tissues was greater in dark individuals than pale, though the difference was significant only for PO in the haemolymph. Thus, independent of rearing density, melanism tended to be associated with elevated phenoloxidase activity, particularly in the haemolymph.

The positive correlations (and partial correlations) between phenoloxidase activity in the haemolymph, cuticle and midgut indicate that individuals investing in pathogen resistance do so at all sites where pathogen defence might be required; there is no evidence from these data for a phenotypic trade-off between levels of PO expression at the different sites (Lessells 1991). Siva-Jothy *et al.* (2001) present evidence suggesting that maintaining high PO activity in both the haemolymph and midgut is costly; however, gregarized *S. exempta* larvae appear to be able to pay such costs, at least in the absence of an immune challenge.

The increase in midgut phenoloxidase levels with increasing gregarization is particularly interesting because the area around the midgut is one of the main sites of resistance to pathogens that infect orally. Therefore, we might expect that if phenoloxidase is involved in this process then elevated midgut phenoloxidase levels would be associated with increased resistance to such pathogens. Whilst it is not possible to test this hypothesis directly, it is suggestive that (across phenotypes) resistance to nucleopolyhedrovirus (NPV) in *S. exempta* was positively correlated with mean phenoloxidase activity in the midgut (from Reeson *et al.* 1998). However, this result should be viewed with some caution because resistance to baculoviruses occurs within the haemolymph as well as the midgut and it is possible that the correlation between phenotype and NPV resistance is associated with variation in haemolymph PO activity rather than PO activity in the midgut (or indeed that it is generated by an alternative, as yet unidentified, mechanism).

If dark cuticles have higher levels of phenoloxidase, then it is reasonable to assume that they will be better able to resist parasites and pathogens that invade their hosts via the cuticle. Results from the parasitoid experiment suggest that dark larvae are significantly better at melanizing ectoparasitoid eggs attached to the cuticle (Figure 4.3), so preventing them from developing further. High phenoloxidase activity in the haemolymph is associated with greater capacity to melanize and/or encapsulate foreign objects, including parasites and parasitoids, that enter the haemocoel (Rizki & Rizki 1990; Nappi *et al.* 1992; Shiao *et al.* 2001; Chapter 3). Due to the covariation between melanism and phenoloxidase activity in the cuticle and haemolymph, melanic larvae are likely to be more resistant to both ecto- and endo-parasitoids.

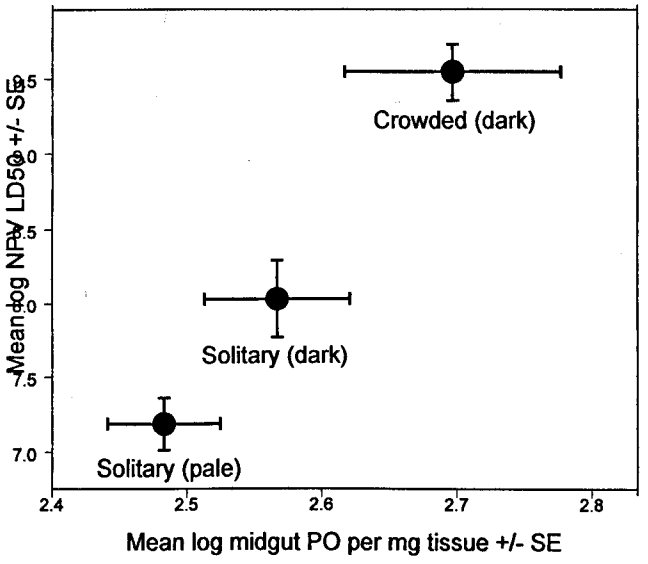


Figure 4.5 – Relationship between midgut phenoloxidase levels and resistance to nuclear polyhedrosis virus.

Mean LD_{50} data for *S. exempta* NPV come from Reeson et al. (1998), midgut phenoloxidase levels are from the present study.

This leads to the intriguing possibility that the melanin in the cuticle might act as an honest signal to parasitoids of an immunocompetent host that has high PO activity and should be avoided. There is some evidence that parasitoids can distinguish between different colour morphs of aphids and preferentially oviposit in the morph with the lower physiological resistance (Ankersmit *et al.* 1981; Michaud & Mackauer 1994; Battaglia *et al.* 1995; Michaud & Mackauer 1995), and Verhoog et al. (1996) found that the parasitoid wasp *Venturia canescens* was "not eager" to parasitize a melanic strain of the Mediterranean mealmoth, *Ephestia kuehniella* and its ovipositor "sometimes appeared to become stuck in the cuticle" (suggesting that the melanized cuticle may also provide a tougher physical barrier). We found no evidence that *E. laphygmae* altered its clutch size in relation to *S. exempta* melanism. However, our experimental design did not allow the parasitoid to choose between melanic and non-melanic larvae and so may not be an appropriate test of this hypothesis.

There is evidence from calopterygid damselflies that melanin might signal immunocompetence, not to parasitoids, but to potential mates. *Calopteryx splendens* is a sexually-dimorphic damselfly and males have prominent wing patches that contain melanin. Variation in the wing patch morphology affects the outcome of both intrasexual interactions and female reproductive decisions after courtship (Siva-Jothy 2000). Rantala et al. (2000) found that males with larger wing patches were better able to encapsulate an artificial parasite (a small piece of nylon monofilament), and Siva-Jothy (2000) found that males with wing patches that were more homogeneous (and presumably contained more melanin) had lower burdens of a eugregarine parasite. Given the apparent importance of wing patches in

inter- and intra-sexual interactions, it seems likely that melanin is involved in signalling some aspect of male condition or immunocompetence in this species.

Insect colour is more commonly associated with defence against predators, and it has been suggested that density-dependent colour change might have evolved in insects as a density-dependent anti-predator strategy: crypsis at low densities and aposematism at high densities (Sword 1999, 2000). Indeed, recently it has been speculated that this hypothesis might be applicable to those Lepidopteran larvae (including *Spodoptera* species) that exhibit density-dependent melanism (Wilson 2000). However, as yet, there is no evidence in support of this assertion, though experiments aimed at testing this hypothesis are underway (K. Wilson unpublished).

The results from the fungus experiments suggest different patterns for the two *Spodoptera* species. As predicted by the DDP hypothesis, crowded larvae were significantly more resistant to fungal infection than solitary larvae at high and low doses for *S. littoralis* and at low doses for *S. exempta* (Figure 4.4a). However, at the high dose, crowded *S. exempta* larvae were more susceptible to the fungus, suggesting that there might be a trade-off that results in greater resistance of crowded larvae when pathogen density is below some threshold, but lower resistance when pathogen density is above it. This might occur if, for example, crowded *S. exempta* larvae invest more in cuticular defences at the expense of haemolymph defences. Such a trade-off would be generated if a limiting resource for immune defence (e.g. PO substrate) is irreversibly allocated to one or other tissue (see Siva-Jothy et al. 2001), or if a limiting resource (e.g. an amino acid) could be used either to manufacture a biochemical that is important in cuticular defence (e.g. melanin) or to manufacture a different biochemical that aids in haemolymph defence (e.g. an anti-fungal protein). Either way, individuals

investing in cuticular defences would do so at a cost to haemolymph defences and could expose themselves to increased risk at high spore densities, when the cuticle is likely to be penetrated. This is analogous to the dilemma facing an army defending a fort: putting all of ones soldiers on the perimeter fences may work well if opposition numbers are low, but if they are high, then as soon as these defences are breached, the battle will be lost.

An alternative explanation is that, at high fungal doses, numerous fungal spores could penetrate the host simultaneously, causing premature insect death (e.g. due to water loss, etc) before the fungus could effectively exploit the resource. This would result in some larvae dying due to fungal invasion very rapidly after inoculation, but without subsequent sporulation on the cadaver. In support of this idea, we found that when we re-analysed the fungus-bioassay data for *S. exempta* and included all deaths, including those where there was no obvious mycosis, the interaction between fungal dose and rearing density became marginally non-significant ($\chi^2_1 = 3.66$, $p = 0.056$).

The relationship between colour and fungus-induced mortality was similar to that observed with respect to larval density. As predicted, dark larvae were better than pale larvae at resisting fungal infection at both low and high doses for *S. littoralis* and at low doses for *S. exempta.* But, again, this general trend was reversed for *S. exempta* at the high dose, though the difference between the colour morphs was non-significant at both doses (Figure 4.4b). Thus, it appears that the different patterns for the two species may be a consequence of their relative susceptibilities to this strain of fungus: larval mortality in *S. littoralis* was just 16% and 41% for the low and high doses, respectively, whereas they were 54% and 79% for *S. exempta.*

Similar patterns may have been produced for the two species had lower doses been used for *S. exempta.*

The results for *S. littoralis* are similar to those observed for the mealworm beetle *T. molitor* infected with the entomopathogenic fungus *Metarhizium anisopliae* (Barnes & Siva-Jothy 2000), and are also in accord with those for the armyworm, *M. separata*, percutaneously infected with the entomopathogenic fungus, *Nomuraea rileyi* (Mitsui & Kunimi 1988). Mitsui and Kunimi (1988) found that solitary-reared larvae were more susceptible to the fungus than crowd-reared larvae (LC_{50}s: solitary = 11.5 x 10^7, crowded = 25.1 x 10^7), and that when larvae were reared gregariously, pale individuals were substantially more susceptible than dark ones (LC_{50}s: pale = 4.9 x 10^7, dark = 27.2 x 10^7).

Our results suggest that there are several related properties of the DDP phenomenon that are likely to yield the observed positive association between melanism and disease resistance. First, because of the structural properties of melanin, a melanized cuticle is likely to provide a more secure *physical barrier* to the penetration of cutaneously-entering parasites and pathogens. Second, because of the *chemical properties* of melanin, and in particular its inhibitory effect on lytic enzymes (Kuo & Alexander 1967; Bull 1970), the ability of organisms to penetrate and grow on melanized cuticles may be reduced (St. Leger *et al.* 1988). Third, as shown in the present study, melanized cuticles have greater *phenoloxidase* activity, which can lead to the production of melanin in response to appropriate cues, including those produced by cuticle-penetrating fungal hyphae and by parasitoid eggs attached to the cuticle. Although our studies did not establish whether insects that resisted fungal attack had a stronger encapsulation response to invading fungi, they have shown that melanic larvae have a stronger melanization reaction to

attached parasitoid eggs, a reaction that is catalysed by enzymes of the prophenoloxidase cascade.

In conclusion, we have shown that an increase in larval density stimulates increased production of melanin in both the cuticle and, to a lesser extent, the midgut; an act that is likely to enhance larval immunocompetence (*sensu* Owens & Wilson 1999) as a consequence of the physical and chemical properties of melanin. We have also shown that melanism is associated with elevated phenoloxidase activity, not only in the haemolymph but also at two of the barriers to pathogen penetration – the cuticle and the midgut. High levels of cuticular phenoloxidase are associated with increased resistance to an ectoparasitic wasp and an entomopathogenic fungus; high levels of midgut phenoloxidase are correlated with increased resistance to a baculovirus. These results suggest that melanism is a reliable indicator of phenoloxidase production in the cuticle and midgut, and of resistance to parasites and pathogens that enter their host percutaneously or orally. Given recent studies indicating a potential role for melanin in the innate immune defences of vertebrates, including man (Mackintosh 2001), the present study suggests that the functional significance of melanism may have been underestimated.

Figure caption: pale and dark 5th instar *S. littoralis* larvae.

Chapter 5

Plasticity in immune function: Evidence for density-dependent prophylaxis?

S.C. Cotter & K. Wilson

Abstract

The risk of parasitism is expected to increase with population density. Therefore, species that encounter high fluctuations in density should exhibit plasticity in the immune response such that investment in costly immune defences is adjusted to match the environment (i.e. density dependent prophylaxis). Despite growing evidence that insects in high density populations show increased resistance to certain pathogens, few studies have examined any underlying alteration in immune function. The aim of this study was to quantify any variation in the allocation of resources to immunity, between colour phases (pale versus dark) and rearing densities (solitary versus gregarious) in a phase-polyphenic species, in order to determine the basis of this increased resistance.

Dark larvae exhibited higher cuticular phenoloxidase (PO) activity and haemolymph PO activity (corrected for larval weight) and produced a more strongly melanized nylon implant than pale larvae. Conversely, pale larvae had higher lysozyme activity than dark larvae. These results are examined in relation to pathogen resistance and the possibility of trade-offs within the immune system are discussed.

Introduction

Phase-polyphenism is a phenomenon exhibited by many insect species, in which an individual develops into one of two, or more, distinguishable phenotypes depending on extrinsic environmental cues. Often, it is the density of conspecifics that determines which of the phenotypes an individual will develop into. The phenotypic switch is typically triggered by tactile stimulation. In locusts, it is repeated stimulation of the face of the hind femur that initiates phase change (Simpson *et al.* 2001), though olfactory and visual stimuli have also been shown to be important (Pener 1991; Roessingh *et al.* 1998; Simpson *et al.* 1999). In many locust, phasmid and Lepidopteran species the "phase" induced by crowding, often referred to as the "gregaria" phase, is characterised by darkening or melanization of the cuticle, making these individuals darker and more conspicuous than the solitary-phase individuals.

There have been many hypotheses to explain the adaptive value of melanism in the high-density phase. It has been suggested that there are thermoregulatory benefits to having a melanized cuticle. Low temperatures, as well as crowding, can trigger melanization (Johnson *et al.* 1985; Goulson 1994; Gunn 1998), and dark *Mamestra brassicae* larvae were found to have higher body temperatures when exposed to bright illumination than pale larvae (Goulson 1994). This could allow melanized individuals to develop faster due to an increased metabolic rate, and have greater activity levels. In fact, it is well documented that activity levels and development rate of crowded and melanized larvae are higher than their solitary-reared and pale counterparts (Hodjat 1970; Simmonds & Blaney 1986; Tojo 1991). However,

evidence that increased activity or development rate is directly related to melanization of the cuticle is lacking.

It has also been suggested that the black coloration of the cuticle may serve as an aposematic signal to predators (Iwao 1968; Wilson 2000), but observations of melanized larvae being consumed in the field by predators seem to discount this theory. However, the same argument was used to dismiss an aposematic function for the coloration of the gregaria phase of locusts. Two recent studies on two closely related locust species have now provided evidence that the coloration may indeed function as an aposematic signal (Sword 1999, 2000). However, the black and yellow striped coloration of the nymphs of gregaria phase desert locusts may be more memorable to predators than the uniform black coloration typical of the gregarious phase larvae of many Lepidopteran species (Wilson 2000).

The density-dependent prophylaxis (DDP) hypothesis provides an alternative explanation for density-dependent melanism (Wilson & Reeson 1998). Maintaining an effective immune system is expected to be costly. Therefore, larvae would benefit from increasing their allocation of resources to immune function when the risk of infection was high. The DDP hypothesis proposes that the increased levels of cuticular melanization observed in crowd-reared individuals is linked to immunity, such that gregarious individuals invest more in immune function than those reared solitarily, due to the increased risk of pathogenesis at high densities (Wilson & Reeson 1998).

There have been several studies on Lepidoptera, in which resistance to a specific pathogen has been compared between rearing densities. Kunimi and Yamada (1990) found that solitary-reared larvae of the armyworm, *Mythimna separata* were more susceptible to both NPV and GV than gregariously-reared larvae. Goulson

and Cory (1995) obtained similar results with larvae of the cabbage moth, *Mamestra brassicae* and its NPV although in this case larvae that were reared at exceptionally high densities experienced an increase in susceptibility. This trend was also found to hold across species, with species that feed gregariously developing greater resistance to NPV with age than those that feed solitarily (Hochberg 1991). The phenomenon has also been tested in the archetypal phase polyphenic species, the desert locust, *Schistocerca gregaria.* The solitary-phase locusts were significantly more susceptible to the entomopathogenic fungus, *Metarhizium anisopliae*, with the daily mortality risk being 1.47 times greater than for the gregarious-phase locusts (Wilson *et al.* 2002).

In some studies, the resistance to a specific pathogen has been compared between the colour phases whilst controlling for density. Melanic *Mythimna separata* larvae were more resistant to an entomopathogenic fungus (Mitsui & Kunimi 1988) and a nucleopolyhedrovirus (Kunimi & Yamada 1990); resistance to NPV was also higher in melanic *Spodoptera exempta* (Reeson *et al.* 1998). Cuticular colour was found to be a better predictor of resistance to *Metarhizium anisopliae* in the mealworm beetle, *Tenebrio molitor* as the palest beetles experienced significantly higher percentage mortality than the melanic beetles – though the melanic beetles were more common at high rearing densities (Barnes & Siva-Jothy 2000).

Despite the evidence that gregarious-phase individuals are more resistant to disease, few studies have examined the underlying investment in immune function that must underpin this relationship. Reeson *et al* (1998) found that gregarious phase African armyworm, *Spodoptera exempta,* were more resistant to NPV and had higher levels of haemolymph phenoloxidase (PO) than their solitary-phase counterparts (Reeson *et al.* 1998). It has previously been shown that PO in the cuticle of *S. exempta* was

higher in gregarious larvae than solitary larvae, and that this corresponded to increased resistance to both an entomopathogenic fungus and an ectoparasitoid (Chapter 4). However, the two studies on non-Lepidopteran species found no significant difference in PO activity between the rearing densities (Barnes & Siva-Jothy 2000; Wilson *et al.* 2002).

Phenoloxidase is a key enzyme in the synthesis of melanin, the pigment responsible for the darkening of the cuticle of gregarious phase insects, and it has been implicated in resistance to a range of pathogens (Rowley *et al.* 1990; Ourth & Renis 1993; Hagen *et al.* 1994; Hung & Boucias 1996; Washburn *et al.* 1996; Bidochka & Hajek 1998; Reeson *et al.* 1998). Haemolymph PO levels have been shown to be a good predictor of encapsulation ability in solitary-reared *Spodoptera littoralis* (Figure 3.2). It has been shown in *Helicoverpa zea* that cells infected with NPV are encapsulated and melanized in the trachea associated with the midgut (Washburn *et al.* 1996) and that fungal hyphae can be encapsulated in the cuticle of the silkworm, *Bombyx mori* (Tanada & Kaya 1993). Active phenoloxidase has been found in the midgut and the cuticle, as well as the haemolymph and hence it is an important part of the insect immune response (Ashida & Brey 1995).

However, other components of the immune system are also important in providing resistance to parasitic infection. Variation in haemocyte density has been shown to correlate with encapsulation ability both within and across *Drosophila* species (Eslin & Prévost 1996, 1998). There are also a range of antibacterial proteins produced by haemocytes and the fat body, such as lysozymes, attacins, defensins and cecropins, that can lyse bacterial cells or inhibit their growth (Gillespie *et al.* 1997; Chapman 1998). In addition to their role in fighting bacterial infection, there is evidence that these inducible proteins provide resistance against other

microorganisms. Defensins have been shown to be important in reducing the prevalence and intensity of microfilarial infection in the yellow fever mosquito, *Aedes aegypti* (Lowenberger *et al.* 1996). Lines of *Aedes aegypti* selected for high and low inducible anti-*Escherischia coli* activity showed differential susceptibility to microfilarial infection, with a reduction of up to 82% of larvae completing development in the high line (Ham *et al.* 1996). In addition, gregarious phase locusts were found to have significantly higher lysozyme activity in their haemolymph than solitary phase locusts (Wilson *et al.* 2002).

The Egyptian armyworm, *Spodoptera littoralis,* is a species that exhibits density-dependent phase polyphenism. At low densities, larvae tend to be pale brown-grey but when reared at high densities most larvae develop into the dark form which has a highly melanized cuticle (Hodjat 1970). Although development of the phenotype is mainly determined by rearing density, it does have a genetic component (Tojo 1991; Goulson 1994). This means that some larvae reared solitarily will develop into the melanized phenotype, and some larvae reared in groups will remain pale, the typical solitary phenotype. This allows the effects of colour and rearing density to be disentangled.

It has previously been shown that the melanic form of this species has higher resistance to an entomopathogenic fungus than the pale form (Chapter 4), but no studies have shown any variation in immune parameters between phases. So, in order to understand more fully the basis of improved resistance to pathogens found in the gregarious phase of this species, the following questions were addressed:

1. Are there quantifiable differences in immune investment between rearing densities, or between the pale and dark phases?
2. Is investment in all immune parameters elevated or reduced, or are some increased at the expense of others? i.e. is there evidence for phenotypic trade-offs within the immune system?

Methods

Insect rearing

First instar larvae of *Spodoptera littoralis* were placed in 25ml polypots, either singly or in groups of three, and reared on artificial diet (Appendix 1) at 25°C under a 12:12 light:dark regime until the final instar. Larvae were scored for colour (pale, medium or dark), and the medium larvae were discarded to give solitary pale, solitary dark, crowded pale and crowded dark larvae. As solitary-dark and crowded-pale larvae are rare, large numbers of larvae were reared so that sixty larvae of each type could be randomly selected, giving four treatment groups.

Haemolymph sampling

At the onset of the final instar, the larvae were weighed and a haemolymph sample taken from each individual by piercing the final proleg with a fine needle and allowing the haemolymph to pool onto parafilm. Each haemolymph sample was divided between 3 Eppendorfs. For the haemocyte counts, 10 μl of haemolymph

were added to 5 µl of EDTA anticoagulant in phosphate buffered saline (Appendix 2) and 5 µl of glycerol to protect the haemocytes during storage in the freezer. For the PO and protein assays, 8 µl of haemolymph were added to 400 µl of PBS (Appendix 2) and for the lysozyme assays, the remaining haemolymph was left undiluted. All the samples were then frozen at -20°C until they were to be measured. Twenty four hours after the first haemolymph sample, a second was taken, the haemolymph divided up as before and the larvae frozen.

Haemocyte counts

Fifteen microlitres of the EDTA/glycerol/haemolymph mixture were pipetted onto a haemocytometer with improved Neubauer ruling. Five non-adjacent squares were counted on each side of the haemocytometer and summed to give an estimate of the haemocyte density of each individual. Counts can be multiplied by 5000 to give the number of cells per ml of haemolymph.

Lysozyme assays

Lytic activity against the bacterium, *Micrococcus lysodeikticus* was determined using a lytic zone assay. Agar plates containing 10 ml of 1% agar with 5 mg per ml freeze-dried *M. lysodeikticus* were prepared as described in Kurtz et al (2000) (Chapter 2). Holes with a diameter of 2 mm were punched in the agar and filled with 70% ethanol saturated with phenylthiourea (PTU), PTU inhibits melanization of the haemolymph. After the ethanol had evaporated, 1 µl of haemolymph was placed in each well, two replicates per sample. The plates were incubated at 33°C

for 24 hours. After this time the plates were photographed using a *Polaroid DMC* digital camera and the diameter of the clear zones calculated using *Image Pro Plus* software (Media Cybernetics 1999). Standard curves were obtained using a serial dilution of hen egg white lysozyme. Concentration of "hen egg white lysozyme equivalents" was then calculated.

Haemolymph PO assays

Triplicate 100 µl samples of the haemolymph/PBS mixture were added to 100 µl of 20 mM L-Dopa. After 20 minutes incubation at 25°C, the absorbance was measured on a *Versamax tuneable microplate reader* (Molecular Devices Corporation, Sunnyvale, CA) at 492 nm. Previous results have shown the reaction to be in the linear phase during this time period (Figure 2.1a).

Protein assays

Protein was measured using the *BioRad* protein assay kit with BSA as the protein standard. Triplicate 10 µl samples of the haemolymph/PBS mixtures were used to measure the protein in each sample. Absorption was measured at 600 nm on a *Versamax tuneable microplate reader* (Molecular Devices Corporation, Sunnyvale, CA). The amount of protein in each sample was then calculated from the standard curve.

Repeatability of haemolymph parameters

It is important to establish that the measures of the different immune parameters are repeatable if they are to be used as an indication of an individual's investment in immunity. The repeatability, r, (Lessells & Boag 1987) was determined both within and across days.

Two haemolymph samples were taken from each individual, 24 hours apart. For the PO and protein assays, three measurements were taken for each sample and for the haemocyte counts and lytic assays, two measurements were taken for each sample. This allowed the within-sample repeatability to be calculated for each assay, giving an estimate of the accuracy of the measurement techniques. Taking measurements from the same individual on two different occasions allowed the calculation of the between-day repeatability, which estimates fluctuations in the parameters over time. Separate repeatabilities were also calculated for "challenged" larvae (larvae that had been implanted with a small piece of nylon) and "unchallenged" larvae.

Midgut and cuticular PO assays

The midgut and cuticle were dissected from each larva, fixed in 2% formaldehyde and 0.5% glutaraldehyde in phosphate buffer (Appendix 2). After fixation the cuticles and midguts were washed three times and stored in phosphate buffer (Wolfgang & Riddiford 1981). The cuticles and midguts were then cut in half and each piece weighed. One half of each midgut and cuticle was placed in 250 and 500 µl of 20 mM L-Dopa respectively. The other half was placed in 20 mM L-Dopa saturated with PTU as a control. Samples of 20 mM L-Dopa were incubated along with the experimental samples to control for any spontaneous darkening of the

substrate during the experiment. Absorbance was measured at 492 nm after 30 minutes. PO activity was expressed as PO units per gram of cuticle/midgut.

Encapsulation assays

Half of the larvae in each treatment group had a piece of nylon monofilament (*Orvis*, fishing line, diameter 0.5 mm) approximately 3mm long, inserted into the haemocoel after the first haemolymph sample had been taken. The nylon implants were dissected out after the larvae had been frozen, subsequent to the second haemolymph sample being taken. The implants were then mounted on slides and photographed using a *Polaroid DMC* digital camera. The level of melanization and the area of cell cover were separately quantified using *Image Pro-Plus* software (Media Cybernetics 1999). Melanization was scored as the mean level of blackness of the nylon (1/*Mean Density* in *Image Pro-Plus*) and this was independent of the length of the implant (*Major Axis*). The area of the attaching cell mass (*Area*) was also quantified but as this score correlated with nylon length, the residuals of the regression of nylon length on area were used as a corrected score. Both of these measures have previously been shown to correlate with a visual assessment of the level of encapsulation (chapter 3).

Results

Repeatability of haemolymph parameters

Haemocyte density showed high within-sample repeatability on both days ($r > 0.82$; Table 5.1). The between-day repeatability for this measure of immune function was

also highly significant, though considerably lower than the values obtained for the other parameters (Table 5.1). There was no significant difference between the estimates obtained for the "challenged" and the "unchallenged" groups (+ nylon: $r = 0.350 \pm 0.080$; - nylon: $r = 0.295 \pm 0.084$, $t = -0.47$, $df = 238$, $p = 0.635$; Table 5.1).

Lysozyme activity showed high repeatability within samples on both days ($r > 0.95$; Table 5.1). Again, there was no significant difference between the repeatabilities obtained for the "challenged" and the "unchallenged" groups (+ nylon: $r = 0.706 \pm 0.046$; - nylon: $r = 0.677 \pm 0.050$, $t = -1.46$, $df = 238$, $p = 0.145$; Table 5.1).

Haemolymph PO activity also had high within-sample repeatability on both days ($r > 0.89$; Table 5.1). Levels were repeatable between days and there was no significant difference between the repeatabilities obtained for the "challenged" and the "unchallenged" groups (+ nylon: $r = 0.582 \pm 0.060$; - nylon: $r = 0.507 \pm 0.068$, $t = 0.83$, $df = 238$, $p = 0.409$; Table 5.1).

The repeatability of haemolymph protein levels, a putative non-immune haemolymph parameter, was calculated for comparison. The within-sample repeatability of protein levels was also high ($r > 0.82$; Table 5.1). The between-day repeatability was significant and comparable to that of haemolymph PO. Again, there was no difference between the repeatabilities of the different treatment groups (+ nylon: $r = 0.576 \pm 0.061$; - nylon: $r = 0.658 \pm 0.052$, $t = -1.02$, $df = 238$, $p = 0.307$; Table 5.1).

Table 5.1 – Repeatability of immune parameters within and between days.

Repeatabilities, calculated according to the methods of Lessells and Boag (1987), for all measured haemolymph parameters ± SE. Values in the first two rows represent within-sample repeatabilities, a comparison of the multiple measures made on each haemolymph sample. The other three rows represent a comparison of the mean values per sample obtained on day 1, with those obtained on day 2, for larvae that had received a nylon implant between each haemolymph sample, "+ nylon", for larvae that had not "- nylon", and for both groups combined, "all data". Repeatabilities were calculated on data after the effect of "day" had been corrected for.

ns $P > 0.05$, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$

Repeatability	Haemocyte counts	Lysozyme activity	Haemolymph PO activity	Haemolymph protein
Within samples day 1	0.907 ± 0.011 ***	0.955 ± 0.006 ***	0.901 ± 0.009 ***	0.824 ± 0.011 ***
Within samples day 2	0.822 ± 0.021 ***	0.967 ± 0.004 ***	0.891 ± 0.006 ***	0.836 ± 0.019 ***
Between days + nylon	0.350 ± 0.080 ***	0.706 ± 0.046 ***	0.582 ± 0.060 ***	0.576 ± 0.061 ***
Between days - nylon	0.295 ± 0.084 ***	0.677 ± 0.050 ***	0.507 ± 0.068 ***	0.658 ± 0.052 ***
Between days all data	0.323 ± 0.058 ***	0.698 ± 0.033 ***	0.545 ± 0.045 ***	0.612 ± 0.040 ***

Table 5.2 – The effect of the nylon implant on immune parameters.

The results of two way ANOVAs, testing whether immune parameters differ between day 1 and day 2, and whether they are affected by the insertion of a nylon implant.

ns $P > 0.05$, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$

		Haemocyte counts	Lysozyme activity	Haemolymph PO	Haemolymph protein
Mean ± SE, - nylon	**Day 1**	148.47 ± 4.84	1.45 ± 0.04	-0.69 ± 0.03	-1.67 ± 0.04
	Day 2	104.72 ± 3.99	1.30 ± 0.04	-0.85 ± 0.04	-1.32 ± 0.03
Mean ± SE, + nylon	**Day 1**	142.42 ± 4.51	1.45 ± 0.03	-0.68 ± 0.03	-1.66 ± 0.05
	Day 2	85.91 ± 3.34	1.57 ± 0.04	-0.90 ± 0.04	-1.30 ± 0.05
F and p-values for each term in the model	**Nylon**	$F_{1,445} = 8.69$ **	$F_{1,454} = 11.75$ ***	$F_{1,465} = 0.32$ ns	$F_{1,467} = 0.01$ ns
	Day	$F_{1,445} = 141.38$ ***	$F_{1,454} = 0.27$ ns	$F_{1,465} = 30.52$ ***	$F_{1,467} = 64.32$ ***
	Nylon :Day	$F_{1,444} = 2.30$ ns	$F_{1,454} = 13.14$ ***	$F_{1,464} = 0.75$ ns	$F_{1,466} = 0.01$ ns

Effect of nylon implant on immune parameters

The baseline levels of immune parameters in "unchallenged" insects may not accurately reflect an individual's ability to respond to parasitic infection. It was for this reason that we compared the levels of all immune parameters in haemolymph sampled from "challenged" and "unchallenged" larvae, before assessing the effect of colour and rearing density on immune function.

Haemolymph PO activity and haemolymph protein levels declined significantly between day 1 and day 2 in both "challenged" and "unchallenged larvae". (Haemolymph PO: $p < 0.001$; Haemolymph protein: $p < 0.001$; Table 5.2).

There was also a significant reduction in haemocyte count from day 1 to day 2, but the reduction was larger in the treatment group that had received a nylon implant (day, $p < 0.001$; nylon, $p = 0.003$; Table 5.2). However, the decrease in haemocyte count (haemocyte count on day 1 minus the haemocyte count on day 2) in the group that had received a nylon implant relative to the group that had not, was not explained by the variation in capsule size (Linear regression: haemocyte decrease ~ capsule size: $r^2 = 0.015$, $df = 112$, $p = 0.18$).

The only significant interaction between sampling day and whether or not a nylon implant had been received was obtained for lysozyme activity (Table 5.2). There was a significant reduction in lysozyme activity from day 1 to day 2 in the "unchallenged" treatment group ($t = 3.54$, $df = 238$, $p < 0.001$). However, in the group that received a nylon implant, lysozyme levels were significantly increased on day 2 relative to day 1 ($t = -2.59$, $df = 238$, $p = 0.01$; Table 5.2).

Table 5.3 – The effects of colour and rearing density on body condition and immune function – multivariate analysis.

Results of the MANOVAs on body condition and immune function. "Condition" refers to larval body weight and haemolymph protein levels. "Encapsulation" refers to capsule size and capsule melanization. "Other immune parameters" refers to haemocyte counts, lysozyme activity, haemolymph PO, midgut PO and cuticular PO. The two encapsulation measurements were analysed separately as their sample size was only half that of the other immune parameters. Values given in black are for the uncorrected data; values in blue are for the data corrected for the relationship with larval body weight.

ns $P > 0.05$, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$

	F values for each term in the model		
	Density	**Colour**	**Density x Colour**
Condition	$F_{2,231} = 68.97$ ***	$F_{2,231} = 14.59$ ***	$F_{2,230} = 1.03$ ns
Encapsulation	$F_{2,106} = 14.96$ ***	$F_{2,106} = 3.25$ *	$F_{2,105} = 0.79$ ns
	$F_{2,106} = 6.85$ **	$F_{2,106} = 4.42$ *	$F_{2,105} = 1.04$ ns
Other immune parameters	$F_{5,209} = 8.37$ ***	$F_{5,209} = 5.24$ ***	$F_{5,209} = 4.13$ ***
	$F_{5,207} = 7.43$ ***	$F_{5,207} = 6.95$ ***	$F_{5,207} = 4.00$ **

In all the analyses presented here, the change in immune parameters from day 1 to day 2 was independent of the colour and rearing density of the larvae, i.e. there were no significant interactions between colour or density and day or nylon. Therefore, the baseline levels (the measurements for day 1) of all the immune parameters were an adequate measure of an individual's investment in immune function and so were used in all subsequent analyses.

Effects of rearing density and colour phenotype on "condition"

The first thing to be assessed was the effect of the two treatments (colour and rearing density) on body condition, as measured by larval weight at the onset of the final instar, and haemolymph protein levels. As these two traits were highly correlated ($r = 0.398$, $p < 0.001$), the effect of rearing density and colour were analysed using multivariate analysis of variance (MANOVA) in *Minitab*, version 13.1 (Minitab, Inc.). This allows multiple responses to be analysed as a single multivariate response, rather than a collection of univariate responses, so allowing the covariation between multiple measurements to be explicitly modelled and the probability of Type I errors to be minimised. For statistically significant MANOVAs, univariate ANOVAs can then be performed for each response variable using sums of squares adjusted for the other dependent variables in the model *(S-Plus 2000*, Guide to Statistics, Volume 1).

The effects of colour and rearing density on "condition" were highly significant (density: $p < 0.001$; colour: $p < 0.001$; Table 5.3). Univariate ANOVAs showed that solitary-reared larvae were significantly heavier than gregarious larvae and had higher protein levels in the haemolymph, though this was not significant; pale

larvae were significantly heavier and had significantly higher haemolymph protein levels than dark larvae (Weight: density, $p < 0.001$, colour, $p = 0.002$; Protein: density, $p = 0.196$, colour, $p < 0.001$; Table 5.4, Figure 5.1). There was no interaction between colour and density.

This suggests that solitary-reared and pale larvae are in better "condition" than crowded or dark larvae. Previous studies have found similar differences between phases even when food is provided *ad libitum.* Pupae and adults of the crowded phase of *S. littoralis* tend to be smaller than solitary reared individuals (Hodjat 1970). As it is unclear what proportion of these differences are due to differing competition for resources between treatments and adaptation to crowding, subsequent analyses were performed on both the raw, uncorrected data and on data corrected for "condition". Although haemolymph PO levels were previously corrected for "condition" with haemolymph protein levels (chapter 3), larval weight was used in this case (residuals of the regression of weight on each immune parameter) as not all the parameters measured were haemolymph parameters. These values will subsequently be referred to as "corrected" and "uncorrected".

Effects of rearing density and colour phenotype on immune parameters

In the main analysis, the following immune parameters were examined: haemocyte density, lysozyme activity, haemolymph PO, midgut PO and cuticular PO. As there were significant correlations between many of these parameters, the effect of rearing density and colour on the different measurements of immune function were also analysed using MANOVA.

Table 5.4 – The effects of rearing density and colour on body condition and immune function – univariate analyses

The significance of colour and rearing density were determined using MANOVA. Below are the results of univariate ANOVAs performed for each response variable using sums of squares adjusted for the other dependent variables in the model. Values given in black are for the uncorrected data; values in blue are for each response variable corrected for the relationship with larval body weight.

ns $P > 0.05$, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$

	F values for each term in the model		
	Density	**Colour**	**Density x Colour**
Weight	$F_{1,234} = 126.45$ ***	$F_{1,234} = 9.82$ **	$F_{1,233} = 1.26$ ns
Protein	$F_{1,234} = 1.69$ ns	$F_{1,234} = 28.07$ ***	$F_{1,233} = 0.91$ ns
Haemocyte count	$F_{1,216} = 0.55$ ns	$F_{1,216} = 0.60$ ns	$F_{1,216} = 0.92$ ns
	$F_{1,214} = 1.28$ ns	$F_{1,214} = 0.55$ ns	$F_{1,214} = 1.17$ ns
Lysozyme activity	$F_{1,216} = 8.83$ **	$F_{1,216} = 7.26$ **	$F_{1,216} = 3.54$ ns
	$F_{1,214} = 11.74$ ***	$F_{1,214} = 9.37$ **	$F_{1,214} = 4.40$ *
Haemolymph PO	$F_{1,216} = 0.83$ ns	$F_{1,216} = 1.05$ ns	$F_{1,216} = 2.97$ ns
	$F_{1,214} = 11.01$ ***	$F_{1,214} = 8.57$ **	$F_{1,214} = 0.27$ ns
Midgut PO	$F_{1,216} = 12.09$ ***	$F_{1,216} = 11.67$ ***	$F_{1,216} = 12.60$ ***
	$F_{1,214} = 7.23$ **	$F_{1,214} = 11.76$ ***	$F_{1,214} = 13.79$ ***
Cuticular PO	$F_{1,216} = 20.26$ ***	$F_{1,216} = 4.45$ *	$F_{1,216} = 0.03$ ns
	$F_{1,214} = 0.07$ ns	$F_{1,214} = 0.77$ ns	$F_{1,214} = 0.42$ ns
Capsule melanization	$F_{1,109} = 30.20$ ***	$F_{1,109} = 4.67$ *	$F_{1,109} = 0.62$ ns
	$F_{1,109} = 13.83$ ***	$F_{1,109} = 6.92$ **	$F_{1,109} = 1.14$ ns
Capsule size	$F_{1,109} = 1.64$ ns	$F_{1,109} = 0.68$ ns	$F_{1,109} = 1.31$ ns
	$F_{1,109} = 0.81$ ns	$F_{1,109} = 0.50$ ns	$F_{1,109} = 1.46$ ns

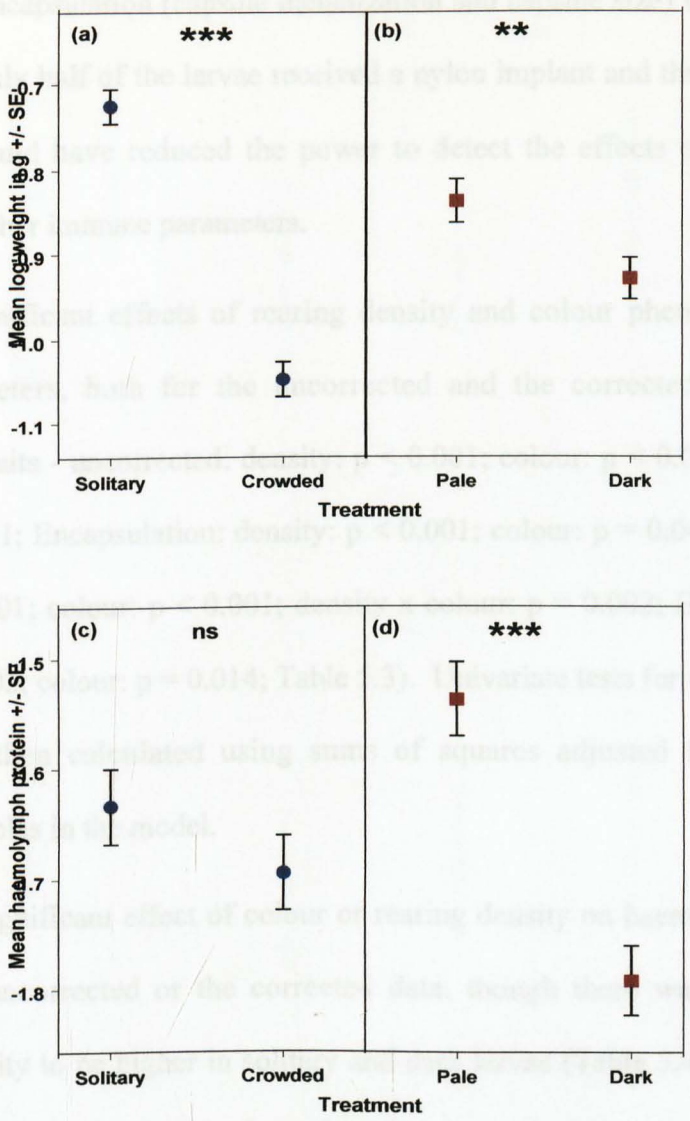


Figure 5.1 – The effect of rearing density and colour on larval weight and protein.

Variation in larval weight is plotted against (a) rearing densities and (b) colour phenotypes and variation in haemolymph protein levels is plotted against (c) rearing densities and (d) colour phenotypes. The bars represent the group mean $\pm$ 1 SE, corrected for the other terms in the model.

ns $P > 0.05$, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$

The effects of encapsulation (capsule melanization and capsule size) were analysed separately, as only half of the larvae received a nylon implant and the reduction in sample size would have reduced the power to detect the effects of colour and density on the other immune parameters.

There were significant effects of rearing density and colour phenotype on the immune parameters, both for the uncorrected and the corrected data (Non-encapsulation traits - uncorrected: density: $p < 0.001$; colour: $p < 0.001$; density x colour: $p < 0.001$; Encapsulation: density: $p < 0.001$; colour: $p = 0.043$; corrected: density: $p < 0.001$; colour: $p < 0.001$; density x colour: $p = 0.002$; Encapsulation: density: $p = 0.002$; colour: $p = 0.014$; Table 5.3). Univariate tests for each response variable were then calculated using sums of squares adjusted for the other dependent variables in the model.

There was no significant effect of colour or rearing density on haemocyte density for either the uncorrected or the corrected data, though there was a trend for haemocyte density to be higher in solitary and dark larvae (Table 5.4, Figure 5.2). There were significant effects of rearing density and colour on uncorrected lysozyme activity, with the highest values being obtained for solitary and pale larvae (Table 5.4, figure 5.3). The trend was the same for the corrected data but the interaction between colour and density was significant ($p = 0.037$). This is because the solitary-pale larvae had much higher lysozyme activity than the other three treatment groups (Table 5.4, Figure 5.3).

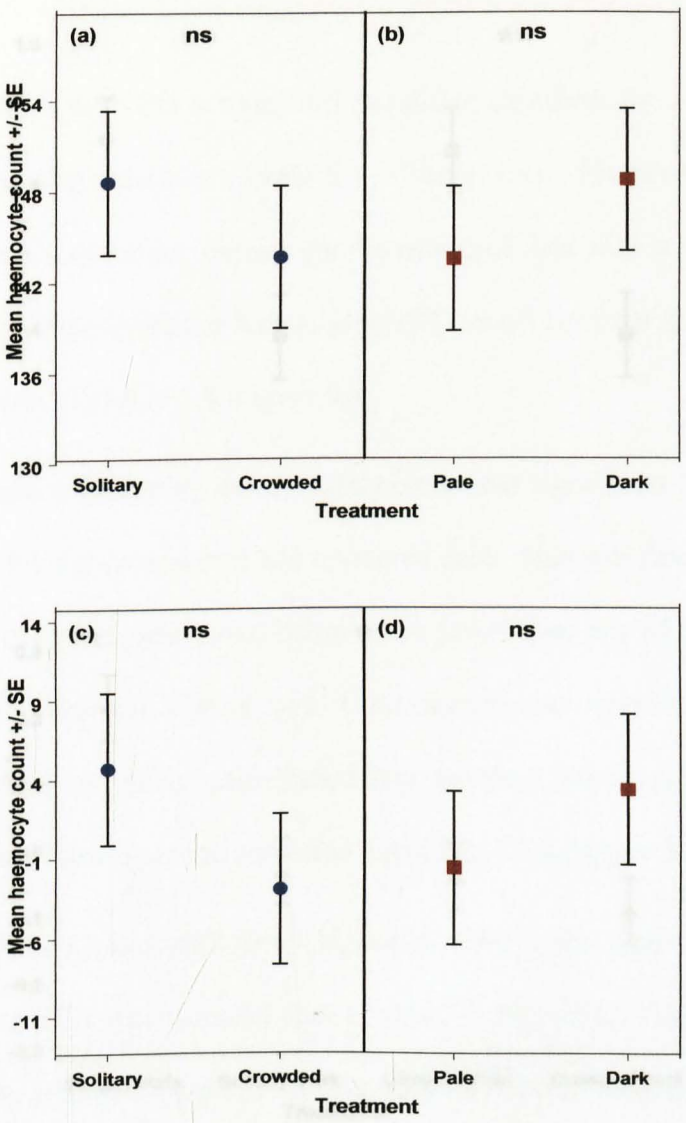


Figure 5.2 – The effect of rearing density and colour on haemocyte counts.

Variation in haemocyte counts between is plotted against rearing densities for uncorrected data (a) and corrected data (c) and against colour phenotypes for uncorrected data (b) and for corrected data (d). The bars represent the group mean $\pm$ 1 SE, corrected for the other terms in the model.

ns $P > 0.05$, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$

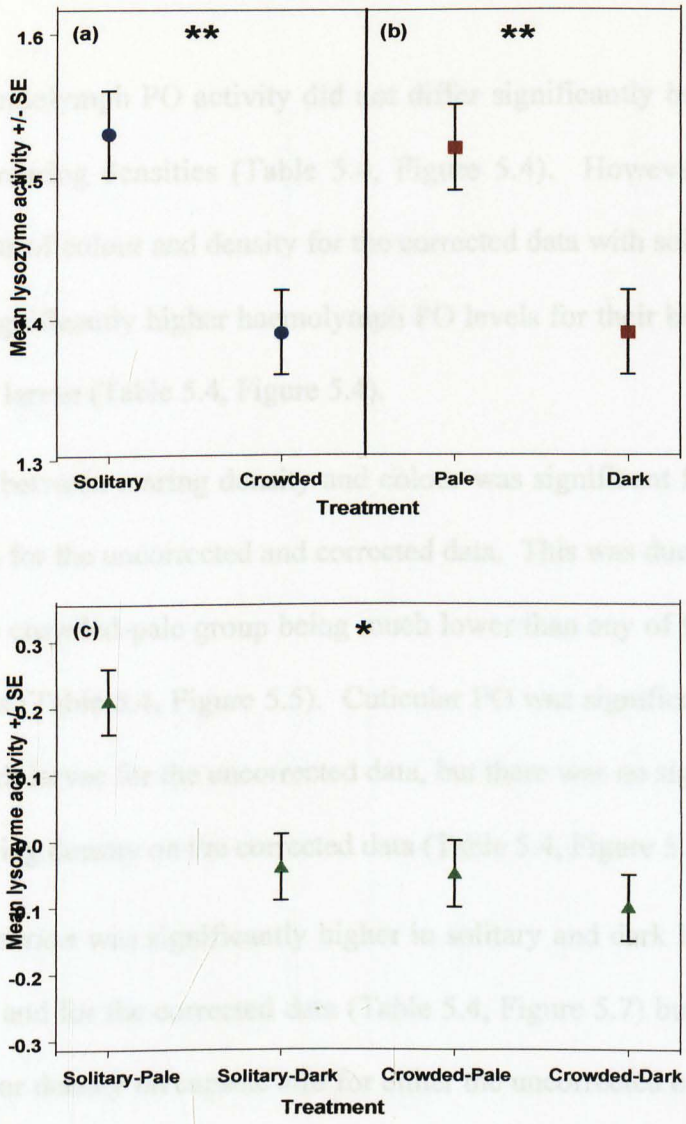


Figure 5.3 – The effect of rearing density and colour on lysozyme activity.

Variation in lysozyme activity is plotted against rearing densities (a) and against colour (b) for uncorrected data. The four treatment groups illustrate the colour*density interaction for the corrected data (c). The bars represent the group mean $\pm$ 1 SE, corrected for the other terms in the model.

ns $P > 0.05$, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$

Uncorrected haemolymph PO activity did not differ significantly between colour phenotypes or rearing densities (Table 5.4, Figure 5.4). However, there were significant effects of colour and density for the corrected data with solitary and dark larvae having significantly higher haemolymph PO levels for their body mass than crowded or pale larvae (Table 5.4, Figure 5.4).

The interaction between rearing density and colour was significant for the midgut PO activity both for the uncorrected and corrected data. This was due to the midgut PO levels in the crowded-pale group being much lower than any of the other three treatment groups (Table 5.4, Figure 5.5). Cuticular PO was significantly higher in crowded and dark larvae for the uncorrected data, but there was no significant effect of colour or rearing density on the corrected data (Table 5.4, Figure 5.6).

Capsule *melanization* was significantly higher in solitary and dark larvae both for the uncorrected and for the corrected data (Table 5.4, Figure 5.7) but there was no effect of colour or density on capsule *size* for either the uncorrected or the corrected data (Table 5.4, Figure 5.8).

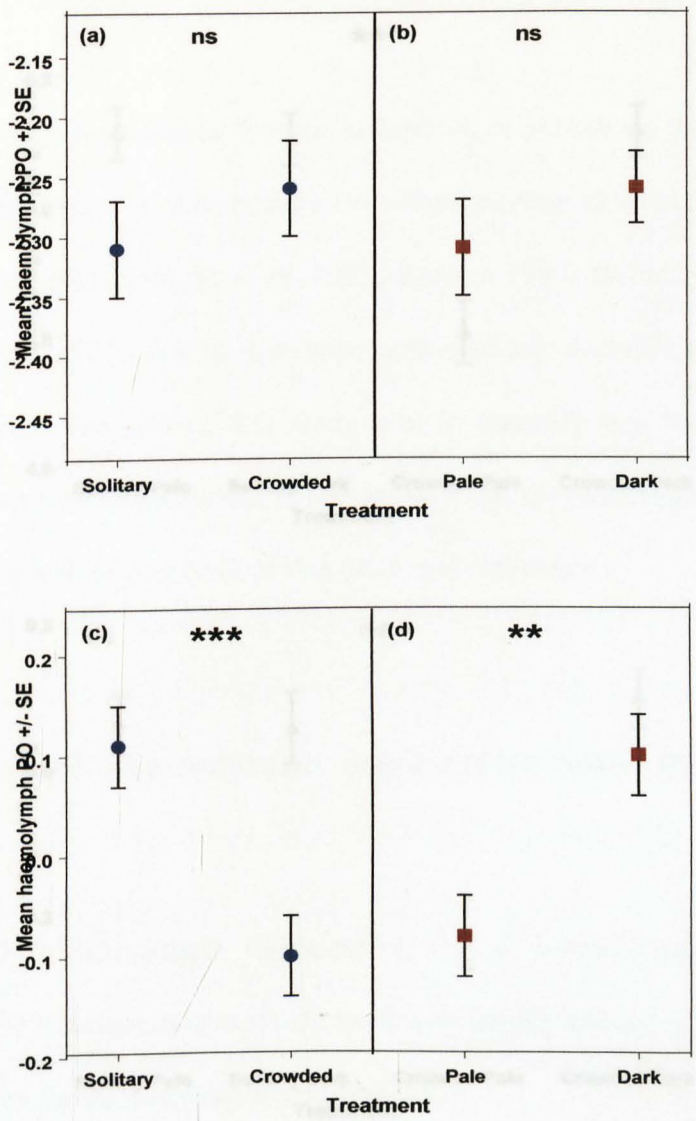


Figure 5.4 – The effect of rearing density and colour on haemolymph PO activity.

Variation in haemolymph PO activity is plotted against rearing densities for the uncorrected data (a) and the corrected data (c) and against colour phenotypes for uncorrected data (b) and the corrected data (d). The bars represent the group mean $\pm$ 1 SE, corrected for the other terms in the model.

ns $P > 0.05$, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$

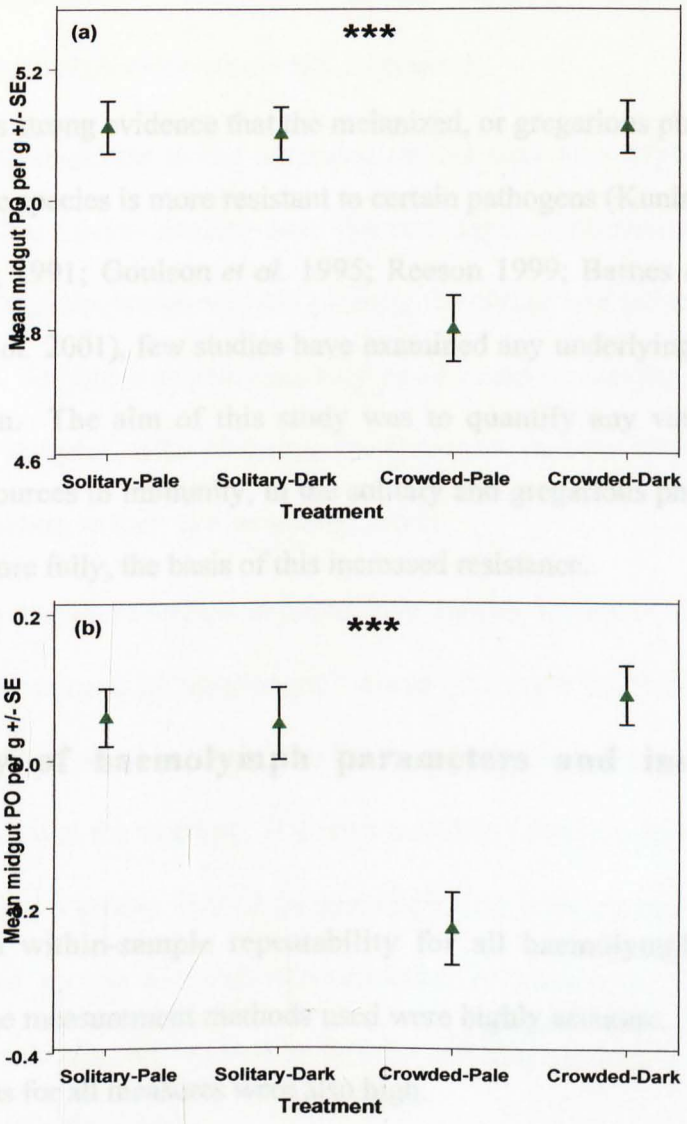


Figure 5.5 – The effect of rearing density and colour on midgut PO.

Variation in midgut PO activity is plotted against the four treatment groups to illustrate the colour*density interaction for the uncorrected data (a) and the corrected data (b). The bars represent the group mean $\pm$ 1 SE, corrected for the other terms in the model. ns $P > 0.05$, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$

Discussion

Although there is strong evidence that the melanized, or gregarious phase of certain phase-polyphenic species is more resistant to certain pathogens (Kunimi & Yamada 1990; Hochberg 1991; Goulson *et al.* 1995; Reeson 1999; Barnes & Siva-Jothy 2000; Wilson *et al.* 2001), few studies have examined any underlying alteration of immune function. The aim of this study was to quantify any variation in the allocation of resources to immunity, in the solitary and gregarious phases, in order to understand, more fully, the basis of this increased resistance.

Repeatability of haemolymph parameters and induction of immunity

There was high within-sample repeatability for all haemolymph parameters indicating that the measurement methods used were highly accurate. The between-day repeatabilities for all measures were also high.

We found no evidence for induction of haemolymph PO activity, or increase in haemocyte density, following immune challenge – at least over a 24 hour period. In fact, both measures of immune function were significantly lower on day 2 than on day 1. This is probably due to a combination of loss of haemolymph during sampling, and the recruitment of haemocytes to the site of the wound. It may be that the larvae seek to increase haemolymph volume following blood loss by retaining water from the diet and from metabolism of the fat body (Chapman 1998). This would result in dilution of the haemolymph that would reduce levels of all

haemolymph components. The fact that haemolymph protein content was also reduced by day 2 provides evidence for this hypothesis.

Recruitment of haemocytes to the wounded cuticle may also explain the lower repeatability of haemocyte density over the two days. Unfortunately, it is not possible to sample haemolymph without piercing the cuticle and inducing a wound-healing response. Higher repeatabilities may be obtained by leaving a longer time period between samples, thus allowing the larvae to recover from the loss of haemocytes and return to their pre-wounding levels.

In addition to an overall reduction in haemocyte density by day 2, the haemocyte count was further reduced in "challenged" larvae (i.e. those that had received the nylon implant). This could be explained by the recruitment of haemocytes to the capsule formed around the implant. It is also possible, however, that the insertion of the nylon caused a greater loss of haemolymph than piercing the cuticle alone. This is supported by the fact that the reduction in haemocyte density is not explained by capsule size, but it could be that this effect is swamped by variation in blood loss between individuals.

Although lysozyme activity levels on day 2 were significantly lower in larvae that did not receive a nylon implant, they were significantly higher in the challenged group, despite any dilution of the haemolymph. This provides further evidence that the artificial parasite is recognised as non-self and stimulates an enhanced immune response. However, the extent of the increase in lysozyme activity and decrease in haemocyte density and PO activity was consistent across colour and rearing density treatment groups. Therefore, any observed variation in immune function between groups should be due to different basal levels of the immune parameters rather than differential induction or up-regulation of immunity in response to infection.

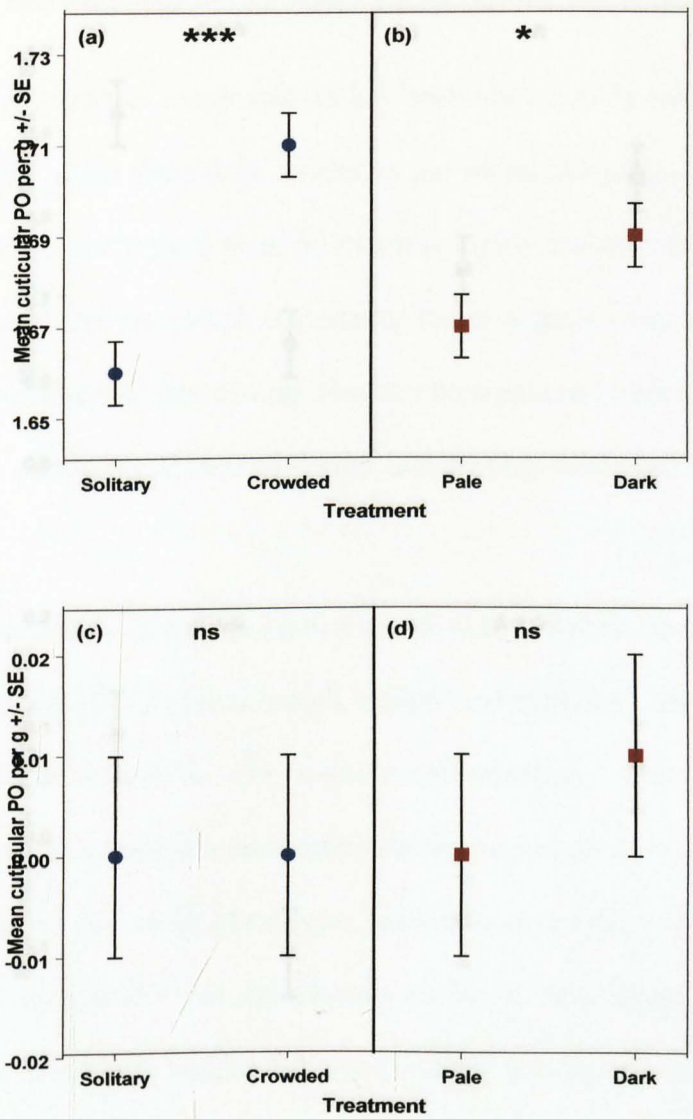


Figure 5.6 – The effect of rearing density and colour on cuticular PO activity.

Variation in cuticular PO activity per gram of tissue is plotted against rearing densities for the uncorrected data (a) and the corrected data (c) and against colour phenotypes for uncorrected data (b) and the corrected data (d). The bars represent the group mean $\pm$ 1 SE, corrected for the other terms in the model.

ns $P > 0.05$, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$

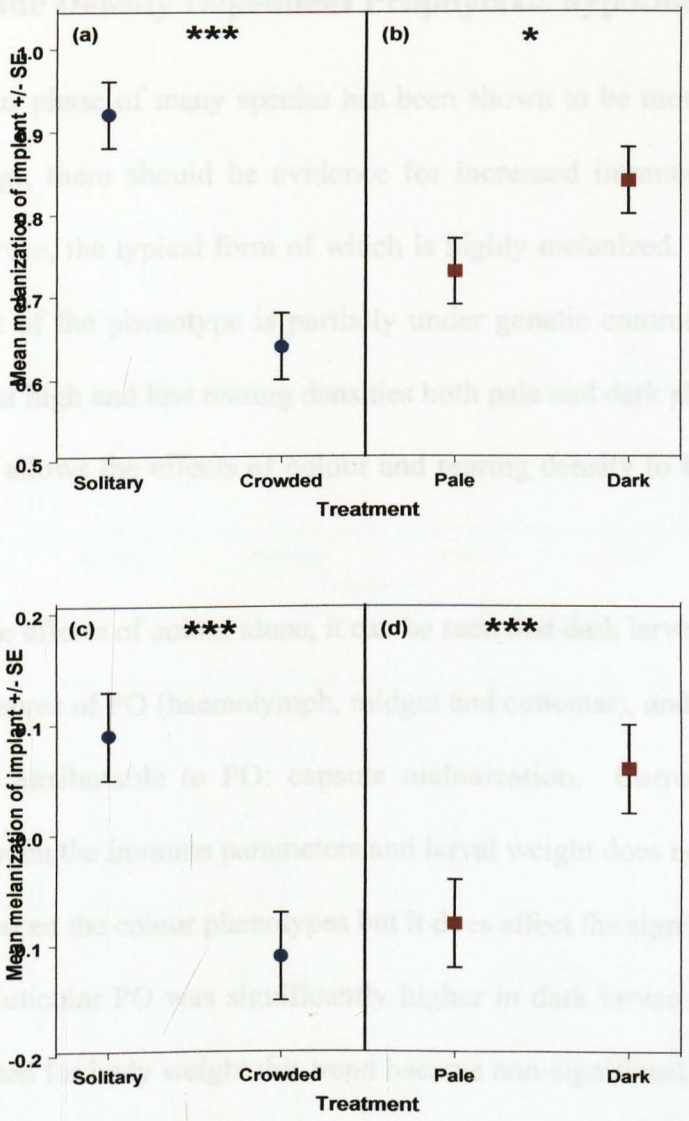


Figure 5.7 – The effect of rearing density and colour on melanization of the nylon implant.

Variation in the melanization of the nylon implant is plotted against rearing densities for the uncorrected data (a) and the corrected data (c) and against colour phenotypes for uncorrected data (b) and the corrected data (d). The bars represent the group mean $\pm$ 1 SE, corrected for the other terms in the model.

ns $P > 0.05$, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$

Evidence for the Density Dependent Prophylaxis hypothesis

As the gregarious phase of many species has been shown to be more resistant to viruses and fungi, there should be evidence for increased immune function in crowd-reared larvae, the typical form of which is highly melanized. However, as the development of the phenotype is partially under genetic control (Tojo 1991; Goulson 1994), at high and low rearing densities both pale and dark phenotypes are produced. This allows the effects of colour and rearing density to be considered separately.

If we consider the effects of colour alone, it can be seen that dark larvae have higher levels of all measures of PO (haemolymph, midgut and cuticular), and higher levels of the response attributable to PO: capsule melanization. Correcting for the relationship between the immune parameters and larval weight does not change any of the trends between the colour phenotypes but it does affect the significance of the relationships. Cuticular PO was significantly higher in dark larvae but when the data were corrected for body weight this trend became non-significant. Conversely, dark larvae had higher haemolymph PO levels, but the trend became significant only when the relationship with weight was taken into account.

Pale larvae had significantly higher lysozyme activity and there was a trend for them to produce a larger capsule around the nylon implant. This is suggestive of a trade-off in the immune system. If the dark form was investing in immune function (as indicated above), then the expectation would be that all immune parameters would increase. The fact that lysozyme activity was higher in the pale form suggests that the dark larvae may be unable to invest in both responses (Figure 5.10b).

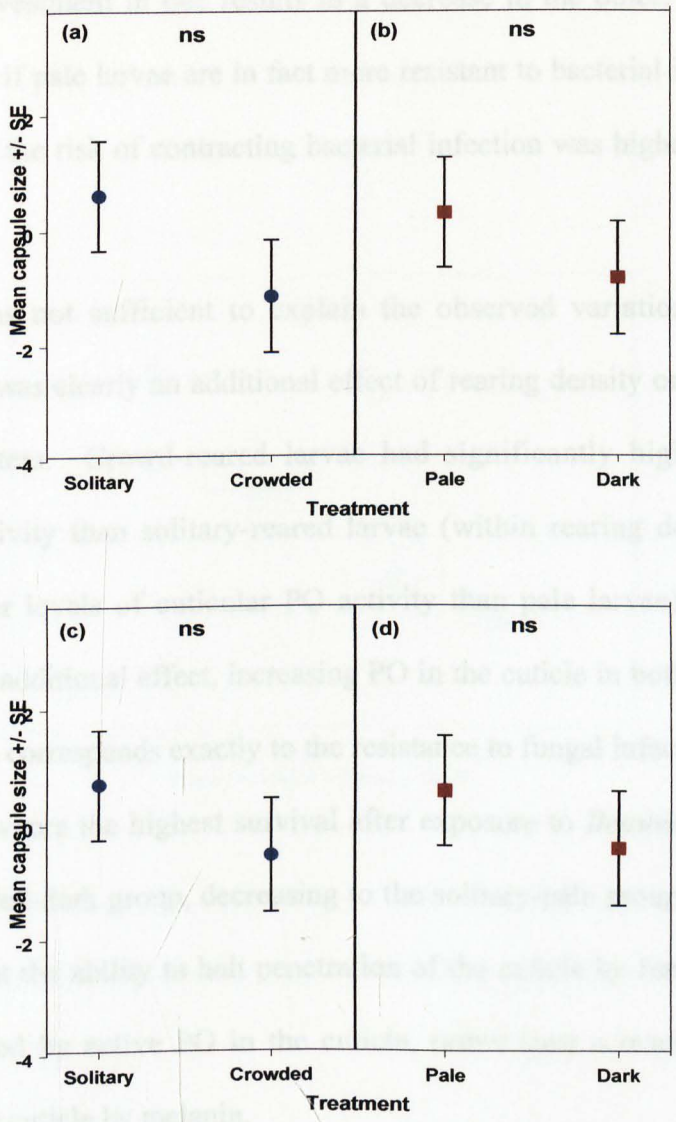


Figure 5.8 – The effect of rearing density and colour on capsule size.

Variation in capsule size is plotted against rearing densities for the uncorrected data (a) and the corrected data (c) and against colour phenotypes for uncorrected data (b) and the corrected data (d). The bars represent the group mean $\pm$ 1 SE, corrected for the other terms in the model.

ns $P > 0.05$, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$

It may be that lysozyme activity and PO are negatively genetically correlated and that increased investment in one results in a decrease in the other. It would be interesting to see if pale larvae are in fact more resistant to bacterial infection than dark larvae, or if the risk of contracting bacterial infection was higher for solitary individuals.

Colour alone was not sufficient to explain the observed variation in immune function. There was clearly an additional effect of rearing density on many of the immune parameters. Crowd-reared larvae had significantly higher levels of cuticular PO activity than solitary-reared larvae (within rearing densities, dark larvae had higher levels of cuticular PO activity than pale larvae). Therefore, crowding had an additional effect, increasing PO in the cuticle in both the pale and dark forms. This corresponds exactly to the resistance to fungal infection exhibited by this species, where the highest survival after exposure to *Beauvaria bassiana* was in the crowded-dark group, decreasing to the solitary-pale group (Figure 5.9). This suggests that the ability to halt penetration of the cuticle by fungal hyphae is probably mediated by active PO in the cuticle, rather than a result of physical toughening of the cuticle by melanin.

Although within rearing densities dark larvae melanized the nylon implant more strongly than pale larvae, the level of melanization was higher in solitary than crowded larvae. The levels of capsule melanization correspond well to levels of haemolymph PO corrected for larval weight (Figure 5.10a). This may be because crowd-reared larvae are smaller, and although they invest more in PO per unit of body weight than solitary larvae, their absolute levels are lower. It may be that the total amount of PO in the haemolymph is responsible for capsule melanization rather than its concentration.

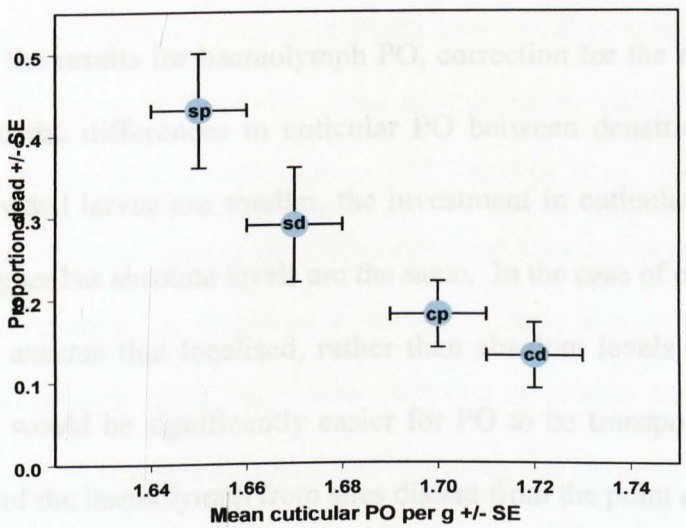


Figure 5.9 – The relationship between cuticular PO activity and resistance to fungal infection.

The proportion of larvae dying from fungal infection after exposure to *Beauvaria bassiana* was averaged over two fungal doses. The graph shows the relationship between resistance to fungal infection and levels of cuticular PO activity across treatment groups. The bars represent the group mean $\pm$ 1 SE. Fungus data is taken from chapter 4.

Again, it would be interesting to see if solitary-reared larvae were more resistant to parasitoids, for example, than crowded larvae, or if the risk of parasitoid attack was higher for solitaries. It could be that bacterial infection and parasitoid attack are linked as the piercing of the cuticle by the ovipositor causes a wound through which bacteria can directly enter the haemocoel.

In contrast to the results for haemolymph PO, correction for the relationship with weight caused the differences in cuticular PO between densities to disappear. Again, as crowded larvae are smaller, the investment in cuticular PO per unit of cuticle was higher but absolute levels are the same. In the case of cuticular PO, it is reasonable to assume that localised, rather than absolute levels of PO are more important. It would be significantly easier for PO to be transported through the fluid medium of the haemolymph from sites distant from the point of infection, than through the extra-cellular matrix of the cuticle. Evidence for this comes from the silkworm where abrasion of the cuticle induced the production of lysozyme proteins in the epicuticle directly beneath the abrasion but not in surrounding areas (Brey *et al.* 1993).

Although levels of PO in the midgut were higher in dark than pale larvae, the levels were not increased in the crowded group. This is in contrast to results obtained with the closely related species, *Spodoptera exempta*, where levels in the midgut corresponded well to resistance to viral infection (Figure 4.5). The results obtained here do not explain the results of many studies showing increased resistance to viral infection in response to crowding. There are many possible explanations for this. Crowding induces a variety of responses in phase polyphenic species. As well as the typical melanization of the cuticle, larvae tend to be smaller, be more active and consume greater quantities of food. They are also more resistant to starving and

unpalatable food than solitary larvae (Iwao 1968). The larval development tends to be quicker but the resultant pupae and adults are generally smaller (Hodjat 1970). As the passage of infection for viruses is through the midgut, other aspects of midgut physiology may be important in resistance. For example, the mechanism that allows larvae to consume unpalatable foods may contribute to reduced viral infection, or greater turnover of gut contents may result in virus particles being flushed from the body more rapidly. One mechanism to reduce infection is the sloughing of infected midgut cells (Briese 1981; Keddie *et al.* 1989). The faster development of crowded larvae may increase cell turnover in the midgut and hence reduce the opportunity for infection to spread. These are all mechanisms not covered by the present study. It is also possible, of course, that the results for midgut PO obtained here are spurious and repeating the experiment may produce different results. It is notable, perhaps, that midgut PO was the only trait that generated significant interaction terms for both the corrected and uncorrected data.

This study provides evidence that *S. littoralis* exhibits prophylactic investment in disease resistance mechanisms. The melanized form of this species, typical of crowded environments, invests in PO, a general immune response implicated in resistance to a range of pathogens. However, the investment in PO seems to preclude investment in lysozyme activity, which was significantly higher in the typical pale, solitary form. Most of the studies investigating this phenomenon have concentrated on viruses and fungi. Further investigation is required to examine the susceptibility of the crowded phase to bacteria and parasitoids predicted by this study.

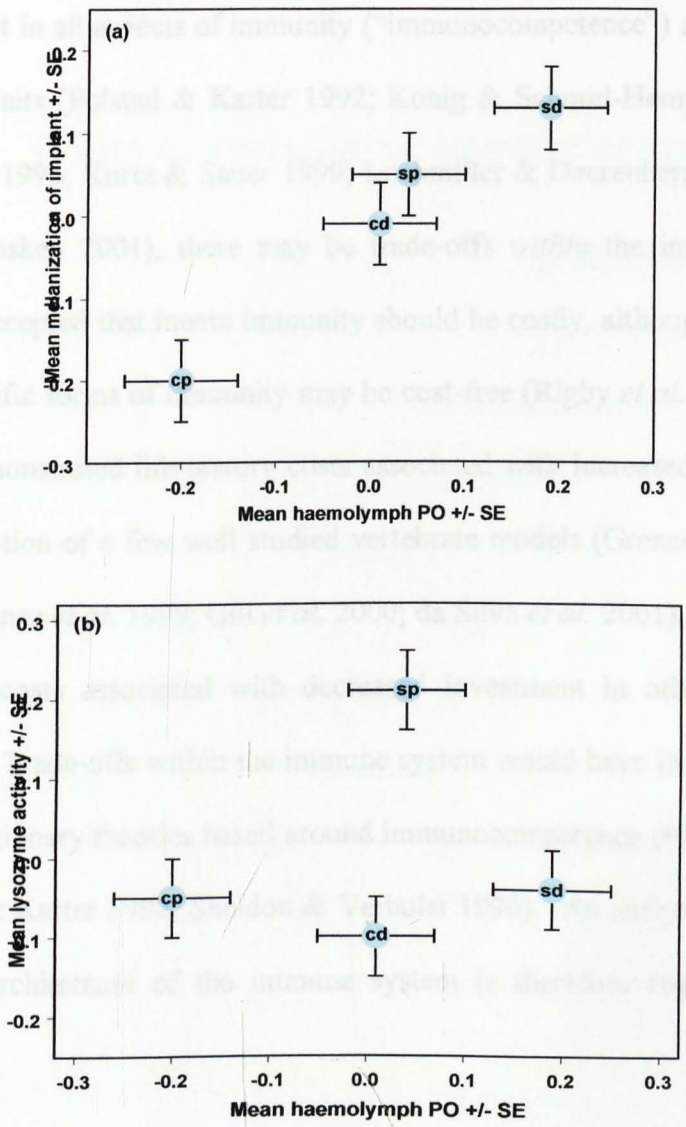


Figure 5.10 – The relationship between haemolymph PO and capsule melanization across treatment groups.

This shows the results from the MANOVA of the corrected data. The treatment means of haemolymph PO are plotted (a) capsule melanization and (b) lysozyme activity to show the relationship across groups. The bars represent the treatment means +/- 1 SE, corrected for the other terms in the model.

In conclusion, it is possible that, contrary to the current opinion that an individual can invest in all aspects of immunity ("immunocompetence") at the expense of life-history traits (Folstad & Karter 1992; Konig & Schmid-Hempel 1995; Sheldon & Verhulst 1996; Kurtz & Sauer 1999; Lochmiller & Deerenberg 2000; Adamo *et al.* 2001; Hosken 2001), there may be trade-offs *within* the immune system. It is widely accepted that innate immunity should be costly, although it has been argued that specific forms of immunity may be cost-free (Rigby *et al.* 2002). Many studies have demonstrated life-history costs associated with increased resistance but, with the exception of a few well studied vertebrate models (Grencis 1997; Gehad *et al.* 1999; Ibanez *et al.* 1999; Gill *et al.* 2000; da Silva *et al.* 2001), have overlooked any possible costs associated with decreased investment in other types of immune function. Trade-offs within the immune system would have important ramifications for evolutionary theories based around immunocompetence (Hamilton & Zuk 1982; Folstad & Karter 1992; Sheldon & Verhulst 1996). An analysis of the quantitative genetic architecture of the immune system is therefore required to clarify this situation.

Figure caption: pale and dark final instar *S. littoralis* larvae.

Chapter 6

Costs of resistance: genetic correlations and potential trade-offs

S.C. Cotter, L.E.B. Kruuk & K. Wilson

Abstract

As parasitism can affect both survival and fecundity, investment in immunity should be closely linked to fitness. Using sib-analysis methods, the heritabilities of different measures of immune function were estimated, and the genetic correlations between these immune traits and other life-history traits were calculated. Analysis of the covariance matrix indicated a potential trade-off within the immune system. Investment in haemocyte density, haemolymph PO activity and cuticular melanization (indicative of resistance against parasitoids and fungi) was negatively genetically correlated with investment in antibacterial (lysozyme-like) activity (resistance to bacteria). There were also negative genetic correlations between three measures of immunity (haemocyte density, haemolymph PO activity and cuticular melanization) and body condition (larval weight and haemolymph protein levels) suggesting that immunity may be costly under conditions of intra-specific competition. A lack of fitness costs to immune function under low-competition conditions implies that cuticular melanization itself may be costly in the form of reduced crypsis. Potential mechanisms underlying these trade-offs are discussed.

Introduction

Immunity to parasites and pathogens has been studied in many organisms. By definition, parasitic infection is costly to the host. Parasites can reduce host fecundity directly by means of castration (Baudoin 1975), or indirectly, by using resources that would otherwise be put into maintenance or reproduction thereby rendering the infected individual less attractive to potential mates (Hamilton & Zuk 1982). Parasites may also affect survival; particularly virulent parasites can kill hosts rapidly. If this occurs in pre-reproductive individuals, the direct fitness of the individual is effectively reduced to zero. It is clear, therefore, that the ability to mount an efficient immune response should be closely related to fitness.

Traits closely related to fitness should be under strong selection in a population. However, for a trait to respond to selection, there must be significant levels of additive genetic variation for that trait present within the population (Fisher 1930; Stearns 1992). The assumption that a proportion of the observable phenotypic variation is accounted for by additive genetic variance, i.e. that a trait is heritable, is implicit in the theories of natural and sexual selection (Andersson 1982); (Kirkpatrick & Ryan 1991); parasite-mediated sexual selection - (Hamilton & Zuk 1982); and the immunocompetence handicap hypothesis - (Folstad & Karter 1992); (Sheldon & Verhulst 1996). However, theory suggests that traits closely related to fitness should have low heritabilities. Strong directional selection on these traits should reduce the amount of additive genetic variation present, as alleles conferring the highest fitness benefits should be driven to fixation by natural selection (Fisher 1930; Stearns 1992).

Some studies provide support for this idea. For example, Gustaffson (1986), showed a negative correlation between the magnitude of a trait's heritability and it's correlation to fitness. Mousseau and Roff (1987) compared estimates for different types of traits in the genus *Drosophila* and found that the heritabilities of morphometric traits tended to be the highest, behavioural and physiological traits were intermediate, and life-history traits had the lowest heritabilities. Two more recent studies provide comparable results. Kruuk et al (2000) measured the heritabilities of a number of traits in a wild population of red deer, including total fitness (defined as the number of offspring produced by an individual in its lifetime). Five of the six morphometric traits measured had significant heritabilities. In contrast, the only life-history traits with heritabilities significantly different from zero were fecundity and age at first breeding in females; none of the estimates for males were significant. Merila and Sheldon (2000) found similar results with collared flycatchers. The heritability estimates for the number of offspring and recruits produced by males, and the number of offspring produced by females were not significant, though the number of recruits produced by females was heritable. It is important to note, however, that whilst these studies show lower heritabilities for traits closely related to fitness, many studies show that these traits still have significant levels of additive genetic variation. The results from studies examining genetic variation in immune function paint a similar picture. Contrary to the predictions of Fisher's fundamental theory of natural selection (Fisher 1930), many studies show high levels of additive genetic variation being maintained in disease resistance (Fuxa & Richter 1989; Boots & Begon 1993; Kraaijeveld & Godfray 1997; Yan *et al.* 1997; Fuxa & Richter 1998; Fellowes *et al.* 1999).

The heritable component of immunity has been determined in two different ways. First, it has been quantified as the ability of an individual to resist infection by a specific parasite or pathogen. Invertebrate studies that have used this approach include selection for resistance to granulosis virus in the Indian mealmoth, *Plodia interpunctella* (Boots & Begon 1993); selection for resistance to the parasitoid, *Asobara tabida,* in the fruit fly, *Drosophila melanogaster* (Kraaijeveld & Godfray 1997); selection for resistance to *Plasmodium gallinaceum* in the yellow fever mosquito, *Aedes aegypti* (Yan *et al.* 1997); selection for resistance to NPV in the fall armyworm, *Spodoptera frugiperda* (Fuxa & Richter 1989, 1998) and selection for resistance to the schistosome, *Schistosoma mansoni* in the freshwater snail, *Biomphalaria glabrata* (Webster & Woolhouse 1999). The response to selection shows that there is additive genetic variation in the selected trait.

Other studies have calculated heritability estimates for resistance to specific parasites. Selection for resistance to the parasitoid, *Leptopilina boulardi,* in the fruit fly *Drosophila melanogaster* yielded a heritability estimate of 0.24 (no standard error was reported) (Fellowes *et al.* 1998a). Estimates for the heritability of resistance to *Bacillus thuringiensis* in the European corn borer, *Ostrinia nubilalis* ranged from 0.17-0.31 (no standard errors were reported) (Huang *et al.* 1999). The heritability of resistance to *Trichostronglyus colubriformis* nematodes in Romney sheep was estimated to be 0.27-0.34 (SE = 0.07; (Shaw *et al.* 1999). Resistance to *Mycobacterium bovis* in red deer, *Cervus elaphus* was estimated to have a heritability of 0.48 $\pm$ 0.10 (Mackintosh 2001). Resistance to gastro-intestinal nematodes in Soay sheep, *Ovis aries*, was found to be heritable, with estimates ranging from 0.11-0.14 (SE = 0.01-0.03; (Coltman *et al.* 2001).

The second way that the heritability of disease resistance has been measured has been to quantify an individual's investment in immune function by assaying components of the immune system. This generally gives an indication of the non-specific immune response rather than immunity to a specific parasite or pathogen. Fewer invertebrate studies have used this approach, but results are similar in that many of the general immune responses have significant levels of additive genetic variation. Ham *et al* (1996) showed that inducible antibacterial activity in the mosquito *Aedes aegypti* was heritable and they successfully selected for both high and low responder lines. Kurtz and Sauer (1999) found significant heritabilities of antibacterial (lysozyme-like) activity (mothers/daughters, $h^2 = 0.75 \pm 0.28$) and haemocyte phagocytic activity (fathers/daughters, $h^2 = 0.77 \pm 0.27$; fathers/sons, $h^2 = 0.83 \pm 0.27$) in the scorpionfly, *Panorpa vulgaris*. Ryder and Siva-Jothy (2001) calculated the heritabilities of haemocyte density and capsule volume around a nylon implant in the haemocoel of the house cricket, *Acheta domesticus,* as 0.30 ± 0.17, and 0.20 ± 0.12, respectively. Hosken (2001) found haemolymph phenoloxidase (PO) activity to be heritable (0.50 ± 0.15) for males of the yellow dung fly, *Sarcophaga stercoraria.* This is in reasonable agreement with the heritability of PO activity estimated for *S. littoralis* ($h^2 = 0.69 \pm 0.07$; Table 3.2).

So, how are the high levels of genetic variation maintained in these traits? There are a number of mechanisms that could allow the maintenance of additive genetic variation in fitness-related traits. These include mutation-selection balance, genotype by environment interactions, flat fitness profiles and antagonistic pleiotropy (Stearns 1992). Antagonistic pleiotropy occurs when a gene has a positive effect on one trait but a negative effect on another. This is the genetic basis

of trade-offs and is the mechanism most often invoked as maintaining additive genetic variation in these systems.

Two studies examined correlated responses to selection for resistance to NPV in larvae of the noctuids, *Spodoptera frugiperda* and *Anticarsia gemmitalis.* Resistant *Spodoptera frugiperda* lines showed a shorter adult life span, lower fecundity and lower egg viability than controls (Fuxa & Richter 1989), and resistant lines of *Anticarsia gemmitalis* had lower fecundity, survival and pupal weights with an increased life span (Fuxa & Richter 1998). The Indian meal moth, *Plodia interpunctella* selected for increased resistance to granulosis virus showed a lengthening of their development time, a reduction in egg viability and an increase in pupal weight (Boots & Begon 1993). Diamondback moth, *Plutella xylostella,* resistant to the bacterium, *Bacillus thuring*iensis, had lower survival, fecundity and percentage egg hatch than the susceptible strain (Groeters *et al.* 1994) and lines of the cabbage looper *Trichoplusia ni,* resistant to TnSNPV laid fewer eggs (Milks 1997). Webster and Woolhouse (1999) artificially selected lines of the snail, *Biomphalaria glabrata,* resistant and susceptible to infection with the schistosome, *Schistosoma mansoni,* and found resistant lines to have lower fecundity than susceptible lines in the absence of parasites. Twelve generations of forced polyandry in the dung fly, *Sarcophaga stercoraria,* resulted in increased testis size and a corresponding decrease in phenoloxidase activity. This suggests that there is a negative genetic correlation between sperm production and investment in at least one aspect of immunity and in this species (Hosken 2001). Thus, a number of studies have demonstrated that there may be genetic trade-offs between resistance to parasites and life-history traits. Therefore, additive genetic variation in immune

parameters could be maintained because high levels can be bought only at the expense of other important functions or life-history traits.

However, costs are not always apparent; they can be hidden by individual variation in resources levels. This is because there can be genetic variation in the ability to acquire resources from the environment, as shown by variation in individual "condition". Genetic variation in the way these resources are then allocated between different traits leads to trade-offs. However, if the variation in resource *acquisition* is greater than the variation in resource *allocation*, trade-offs between traits may be hidden (van Noordwijk & de Jong 1986; Houle 1991). Some traits are particularly affected by an individual's condition. Immune function is likely to show such condition-dependence, with individuals in good condition being better able to mount an immune response than individuals in poor condition (Møller *et al.* 1998; Westneat & Birkhead 1998).

Some studies on birds have provided evidence for the condition-dependence of immune function. In magpies, immune function (measured as the size of the spleen) was found to be positively correlated with condition (measured as the size of the breast muscle over the sternum; (Blanco *et al.* 2001). The phenotypic correlation between levels of testosterone, an immunodepressant hormone, and infection status in wild male house finches was positive; in hormonally-manipulated birds the relationship was negative (Duckworth *et al.* 2001). This suggests that the trade-off was masked by variation in resource levels between wild birds, indicating that the relationship was condition-dependent. Thus variation in body condition can hide potential trade-offs between traits.

Immune function, or immunocompetence, is often thought of as a "black box"; a single trait that is improved with the input of increased resources (Owens & Wilson

1999; Rigby *et al.* 2002). If this is the case, immune traits should positively covary, with each other and with body condition. It could be that any costs associated with increased immune function become apparent only after controlling for the covariation with body condition. However, a study on peacocks found that whilst two measures of immune function (the phytohaemagglutin response and heterophil-lymphocyte ratio) positively covaried with condition, a third (the response to sheep red blood cells) was independent of body condition (Møller & Petrie 2002). Even the simple innate immune system of invertebrates is made up of many parts, humoral and cellular, that work in concert to fight off infection. It is possible that increased production of one component of this system, results in a decrease in another component. By examining the quantitative genetics of immune function, body condition and other life-history parameters, the following questions were addressed:

1. Is additive genetic variation maintained in immune traits such as haemocyte density and lysozyme activity?
2. Are levels of additive genetic variation in immune traits higher than those found in typical life-history traits such as development rate and pupal weight?
3. Are there costs to resistance in the form of negative genetic correlations between the different measures of immune function, or between measures of immunity and other life-history traits?

Methods

Spodoptera littoralis culture

A stock culture of *Spodoptera littoralis* was maintained in the laboratory under a 12:12 light/dark regime. Larvae were reared at 25°C and fed on a semi-artificial wheatgerm-based diet (Appendix 1). The culture was set up from larvae collected in Egypt in 1998. Each generation, eggs were collected from at least 100 adults to reduce inbreeding and larvae from Egypt were added each year to maintain genetic variation.

Experimental design

A full-sib/half-sib design was used to determine heritabilities of immune parameters and life-history traits (Falconer & Mackay 1996). Fourteen virgin males were mated to four virgin females each. Each mated female was then placed in a pot (10 cm diameter x 5 cm) with *ad libitum* access to 5% sucrose solution, filter paper and nappy liner on which to lay eggs. Fifty larvae from each female were removed two days after hatching and placed in individual 25 ml polypots containing semi-artificial wheatgerm-based diet.

Measuring condition and immune parameters

Approximately thirty final instar larvae from each family were weighed and the colour of each was scored as pale, medium or dark. This qualitative measure of cuticular melanization was subsequently converted to a quantitative score by determining the mean darkness of cuticles dissected from each phenotype with

image analysis software (Chapter 2). Cuticular melanization was considered to be an immune trait due to its association both with cuticular PO levels (Figure 5.6), and resistance to fungal infection (Figure 5.9).

To measure the haemolymph-based immune parameters, a proleg was pierced with a fine, sterile needle and the haemolymph pooled onto parafilm. For the PO assays, 8 µl of haemolymph were added to 400 µl of ice-cold phosphate buffered saline (PBS; Appendix 2) and vortexed. For the haemocyte counts, 10 µl of haemolymph were added to 5 µl of EDTA anticoagulant in PBS (Appendix 2) and 5 µl of glycerol to protect the haemocytes during storage in the freezer and for the antibacterial assays, the remaining haemolymph was left undiluted. Samples were frozen at -20°C at this point and thawed when all the samples were ready to be measured. All of the measurement techniques are highly repeatable ($r > 0.82$; Table 5.1) and the immune parameters have also been shown to be repeatable within individuals ($r = 0.32$-0.71; Table 5.1)

PO and protein assays

Triplicate 100 µl samples of the haemolymph/PBS mixtures were added to 100 µl of 20 mM L-Dopa. After twenty minutes incubation at 25°C, the absorbance was measured on a *Versamax tuneable microplate reader* (Molecular Devices Corporation, Sunnyvale, CA) at 492 nm. Previous results have shown the reaction to be in the linear phase during this time period (Figure 2.1a).

Protein was measured using the *BioRad* protein assay kit with BSA as the protein standard. Triplicate 10 µl samples of the haemolymph/PBS mixtures were used to measure the protein in each sample. Absorption was measured at 600 nm. Larval

weight before haemolymph sampling and haemolymph protein levels were used as measures of condition.

Haemocyte counts

Fifteen microlitres of the EDTA/glycerol/haemolymph mixture were pipetted onto a haemocytometer with improved Neubauer ruling. Five non-adjacent squares were counted on each side of the haemocytometer and summed to give an estimate of the haemocyte density of each individual. Counts can be multiplied by 5000 to give the number of cells per ml of haemolymph.

Lysozyme assays

Lytic activity against the bacterium, *Micrococcus lysodeikticus* was determined using a lytic zone assay. Agar plates containing 10 ml of 1% agar with 5 mg/ml freeze dried *M. lysodeikticus* were prepared as described in Kurtz et al (2000). Holes with a diameter of 2 mm were punched in the agar and filled with 70% ethanol saturated with phenylthiourea (PTU), PTU inhibits melanization of the haemolymph. After the ethanol had evaporated, 1 µl of haemolymph was placed in each well, two replicates per sample. The plates were incubated at 33°C for 24 hours. After this time the plates were photographed using a *Polaroid DMC* digital camera, and the diameter of the clear zones calculated using *Image Pro Plus* software (Media Cybernetics 1999). Standard curves were obtained using a serial dilution of hen egg white lysozyme. Concentration of "hen egg white lysozyme equivalents" was then calculated.

Measuring life-history traits

After the haemolymph had been sampled, larvae were placed in fresh polypots with artificial diet and left to pupate. For each individual, the following data were collected: number of days as a larva, pupal weight, number of days as a pupa, sex and adult longevity (Table 6.1). Development rate was measured as the number of days spent in either the larval or pupal stage. Consequently, high values of these traits correspond to slow development rates. Transformation of these traits to rate measures resulted in a skewed distribution, and although the correlations were all very similar to those obtained with the raw data, the standard errors were inflated. Therefore, negative correlations with either days spent as a larva or pupa are discussed as positive correlations with larval or pupal development rate.

Variance components estimation (VCE)

Estimates for the heritability of each trait and genetic correlations between traits were obtained using restricted-estimate, maximum likelihood (REML) models in the program *VCE* (Groeneveld & Kovac 1990). This involves fitting an individual *Animal model* where the phenotype of each individual is separated into additive genetic components of variance plus other random and fixed effects, such that:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Za} + \mathbf{Mc} + \mathbf{e}$$

Where **y** is a vector of phenotypic values; **b** is a vector of fixed effects; **a** and **c** are vectors of additive genetic and maternal random effects; **e** is a vector of residual values; and X, Z and M are the corresponding design matrices relating records to the appropriate fixed or random effects (Lynch & Walsh 1998).

The phenotypic variance of each trait, V_P is thus described as:

$$V_P = V_A + V_M + V_R$$

Where V_A is the additive genetic variance, V_M is the variance attributable to maternal effects and V_R is the residual variance which includes non-additive sources of genetic variance such as dominance variance or epistatic effects, environmental effects and error variance. The heritability of each trait is then calculated as the ratio of additive genetic variance to phenotypic variance.

$$h^2 = V_A / V_P$$

Life-history traits are likely to be influenced by a greater number of genes than other traits and hence, are likely to exhibit more environmental variance, which would swamp any additive genetic variance present in the trait (Houle 1992). Therefore, coefficients of additive genetic variance (CV_A) and residual variance (CV_R) were also calculated for each trait. These are measures of variance, scaled by the trait mean (X) rather than the total variance where:

$$CV_A = (V_A)^{0.5} / X \quad \text{and} \quad CV_R = (V_R)^{0.5} / X$$

Genetic correlations between each pair of traits, r_A, were estimated from the genetic covariance estimate between the two traits **Cov [x,y],** and the estimate of additive genetic variance for each trait V_{Ax} and V_{Ay} where:

$$r_A = \text{Cov } [x,y] / [(V_{Ax})(V_{Ay})]^{0.5}$$

The *VCE* program returns standard errors for all estimates, the significance of which could then be determined with t-tests. An assumption of REML models is normality of the data, therefore some of the traits had to be log-transformed prior to analysis to conform to this assumption (Table 6.1). After transformation all of the

traits were normally distributed except cuticular melanization, therefore the results for this trait should be viewed with caution.

The effect of *Sex* on each trait was determined with linear mixed models in *Genstat 5.1*. *Sex* was included as a fixed effect, and *Sire* and *Dam* (nested within *Sire*), were included as random effects. Where *Sex* was found to be significant in the mixed models, it was included as a fixed effect in the animal model. Larval weight and haemolymph protein levels were considered to be measures of individual "condition". The effects of both measures on each life-history and immune trait were also determined in *GenStat* as described above.

Heritability estimates were obtained by running each trait individually in *VCE*. The mothers' identity (*Dam*) was included as a random effect to provide an estimate for maternal effects on each trait. As all the estimates for maternal effects were non-significant, they were removed from the models. Genetic correlations were estimated by running traits pairwise in *VCE*, correcting for *Sex* effects where necessary. As the sex was known only for two thirds of the individuals, the analyses were run twice, with and without *Sex* included as a fixed effect. The inclusion of *Sex* did not significantly alter the heritability estimates or genetic correlations but the reduction in sample size meant that the standard errors were increased, consequently *Sex* was dropped from the models. The analyses were also repeated with each trait corrected for *Weight* or *Protein* to control for variation in body condition. Again, the resulting heritability estimates and genetic correlations were not markedly different from the basic analysis. Therefore condition was not considered to be an important factor in the resulting correlations between traits and was dropped from the analysis. The simplified model then allowed the genetic correlations between traits to be estimated from a single multivariate analysis.

Table 6.1 – Life-history and immune parameters measured in the genetic analysis

Below is a description of each trait, along with the type, the mean, the standard error (SE) and number of observations available. Development rate was measured as the number of days spent in either the larval or pupal stage, consequently, high values of these traits correspond to low development rates.

Trait	Description	Trait type	Mean	SE	n
Larval weight	Larval weight in g at sampling – $\log_e$ transformed.	*Condition*	-0.65	0.011	606
Haemolymph Protein	Protein content of haemolymph at sampling in mg – $\log_e$ transformed	*Condition*	-1.68	0.012	574
Development rate – larva	Number of days from hatching to pupation.	*Life-history*	15.56	0.046	562
Pupal weight	Weight of the pupa in g.	*Life-history*	0.31	0.002	545
Development rate - pupa	Number of days from pupation to eclosion.	*Life-history*	7.88	0.042	480
Adult longevity	Number of days from eclosion to death.	*Life-history*	11.21	0.229	458
Cuticular melanization	Mean cuticular melanization for each colour.	*Immune function*	3.08	0.043	608
Haemolymph PO activity	PO content of haemolymph – $\log_e$ transformed.	*Immune function*	-2.18	0.009	602
Lysozyme activity	"lysozyme" content of haemolymph in ng/µl – $\log_e$ transformed.	*Immune function*	1.55	0.035	588
Haemocyte density	Number of haemocytes counted in 0.2 µl of haemolymph.	*Immune function*	229.91	3.750	594

Results

Heritability estimates

All traits exhibited significant additive genetic variation, with heritability estimates ranging from 0.22-0.85 (Table 6.2). The highest estimates were for the two life-history traits *larval development rate* and *pupal weight* (traits that are strongly positively correlated with adult fecundity; Figure 6.4). The lowest estimates were also for life-history traits: *pupal development rate* and. *adult longevity* (Table 6.2). Estimates for the condition traits, and immune traits were intermediate. There was no significant difference between the heritability estimates obtained for the different types of traits ($h^2_{\text{Life-history}} = 0.531 \pm 0.175$, $h^2_{\text{Immunity}} = 0.480 \pm 0.070$, $h^2_{\text{Condition}} = 0.387 \pm 0.027$, Kruskal-wallis $\chi^2 = 0.185$, df = 2,, p = 0.912; Figure 6.1a).

Coefficients of variation

Coefficients of additive genetic ($\mathbf{CV_A}$) and residual ($\mathbf{CV_R}$) variation were calculated for each trait. As coefficients of variation are meaningless for transformed data (Houle 1992), each trait was reanalysed using the untransformed data and the heritability estimates compared for both analyses. There was no significant difference between the estimates obtained for the transformed data compared to the untransformed data (paired t-test: t = 1.38, df = 3, p = 0.262). Therefore the $\mathbf{CV_A}$ and $\mathbf{CV_R}$ were calculated using the $\mathbf{V_A}$ and $\mathbf{V_R}$ values obtained using the untransformed data. There was no significant difference between the $\mathbf{CV_A}$ or the $\mathbf{CV_R}$ estimates obtained for the different types of traits ($\mathbf{CV_A}$, Kruskal-wallis $\chi^2 = 0.87$, df = 2, p = 0.646; $\mathbf{CV_R}$, Kruskal-wallis $\chi^2 = 2.67$, df = 2, p = 0.263; Figure 6.1b).

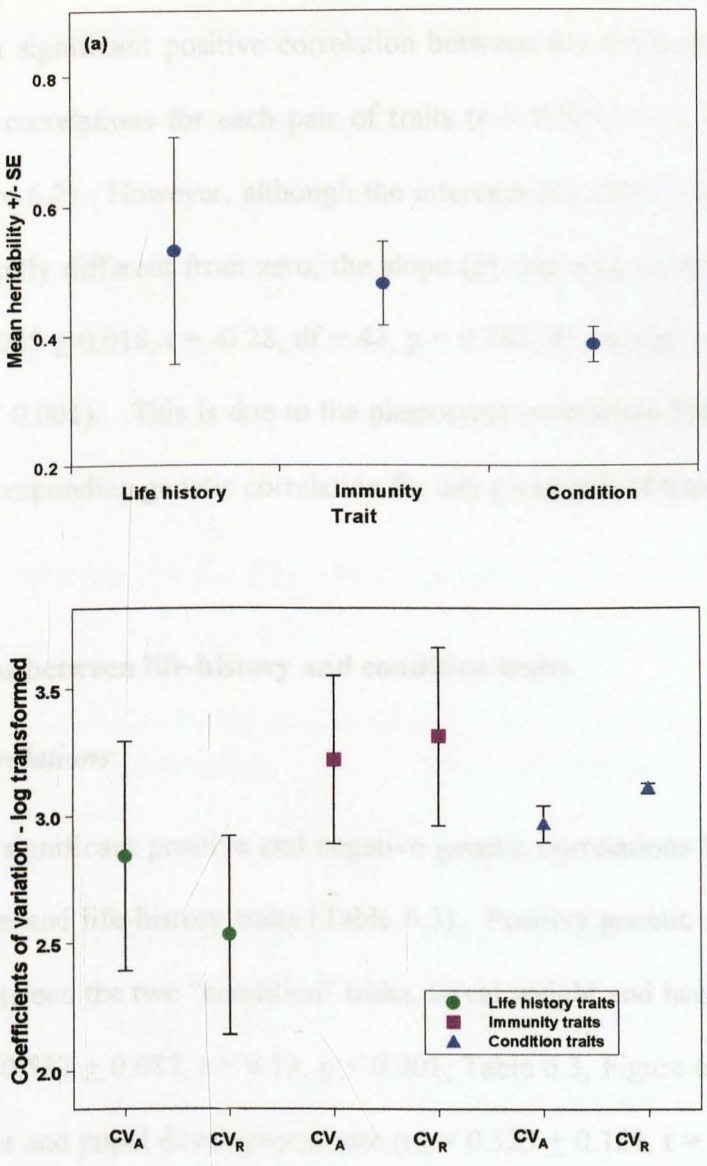


Figure 6.1 – A comparison of the genetic variation present in life-history and immune traits.

Figure (a) shows the comparison of the mean heritabilities for life-history, immune traits and other traits, figure (b) shows the difference in mean CV_A and CV_R obtained for all types of traits.

Phenotypic and genetic correlations

There was a significant positive correlation between the estimates for phenotypic and genetic correlations for each pair of traits ($r = 0.589$, $t = 4.78$, $df = 43$, $p < 0.001$; Figure 6.2). However, although the intercept (α) of the regression line was not significantly different from zero, the slope (β) was significantly different from one ($\alpha = -0.005 \pm 0.018$, $t = -0.28$, $df = 43$, $p = 0.783$; $\beta = 0.304 \pm 0.063$, $t = 4.825$, $df = 43$, $p < 0.001$). This is due to the phenotypic correlation being much weaker than the corresponding genetic correlation for any given pair of traits.

Correlations between life-history and condition traits

Genetic correlations

There were significant positive and negative genetic correlations between many of the condition and life-history traits (Table 6.3). Positive genetic correlations were observed between the two "condition" traits, larval weight and haemolymph protein levels ($r_A = 0.533 \pm 0.087$, $t = 6.13$, $p < 0.001$; Table 6.3, Figure 6.3a) and between larval weight and pupal development rate ($r_A = 0.323 \pm 0.121$, $t = 2.67$, $p = 0.007$). Larval development rate was positively genetically correlated with pupal weight ($r_A = 0.240 \pm 0.050$, $t = 4.80$, $p < 0.001$) and pupal development rate ($r_A = 0.201 \pm 0.081$, $t = 2.48$, $p = 0.013$). Pupal weight was positively genetically correlated with adult longevity ($r_A = 0.412 \pm 0.100$, $t = 4.12$, $p < 0.001$).

Table 6.2 – Heritability estimates for each trait

This includes the estimates for the proportion of phenotypic variance accounted for by additive genetic variance (the heritability), and the proportion of phenotypic variance accounted for by residual variance. Non-significant maternal effects were removed from the model and so are not reported. Coefficients of additive genetic (CV_A) and residual (CV_R) variance calculated for untransformed data are also reported.

ns $P > 0.05$, * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$

Trait	Heritability	Residual Variance	CV_A	CV_R
Larval weight	0.365 ± 0.081 ***	0.635 ± 0.081 ***	17.58	21.93
Haemolymph Protein	0.505 ± 0.092 ***	0.495 ± 0.092 ***	22.35	23.50
Development rate – larva	0.822 ± 0.030 ***	0.178 ± 0.030 ***	47.29	9.35
Pupal weight	0.845 ± 0.032 ***	0.155 ± 0.032 ***	16.61	6.51
Development rate - pupa	0.201 ± 0.064 **	0.799 ± 0.064 ***	5.29	10.55
Adult longevity	0.219 ± 0.070 **	0.781 ± 0.070 ***	20.81	39.34
Cuticular melanization	0.360 ± 0.082 ***	0.640 ± 0.082 ***	20.99	27.97
Haemolymph PO activity	0.650 ± 0.111 ***	0.350 ± 0.111 **	13.13	11.17
Lysozyme activity	0.631 ± 0.108 ***	0.369 ± 0.108 ***	63.02	61.05
Haemocyte density	0.358 ± 0.081 ***	0.642 ± 0.081 ***	22.80	30.56

Negative genetic correlations were found between larval weight and adult longevity (r_A = -0.434 ± 0.134, t = -3.24, p = 0.001; Table 6.3, Figure 6.3a). Haemolymph protein levels were negatively genetically correlated with larval development rate (r_A = -0.379 ± 0.080, t = -4.74, p < 0.001). Pupal development rate was negatively genetically correlated with pupal weight (r_A = -0.339 ± 0.088, t = -3.85, p < 0.001) and adult longevity (r_A = -0.485 ± 0.112, t = -4.33, p < 0.001).

Phenotypic correlations

Although the phenotypic correlations were generally in the same direction as the genetic correlations, they tended to be much weaker. However, due to the large sample size, weak correlations were significant. To reduce the probability of including spurious correlations, the critical significance level for phenotypic correlations was reduced to p = 0.01. Larval weight was positively phenotypically correlated with haemolymph protein (r_P = 0.523, t = 14.60, p < 0.001; Table 6.3, Figure 6.3b). Pupal weight was positively correlated with larval development rate (r_P = 0.210, t = 5.02, p < 0.001). Haemolymph protein levels were negatively phenotypically correlated with larval development rate (r_P = -0.181, t = -4.22, p < 0.001).

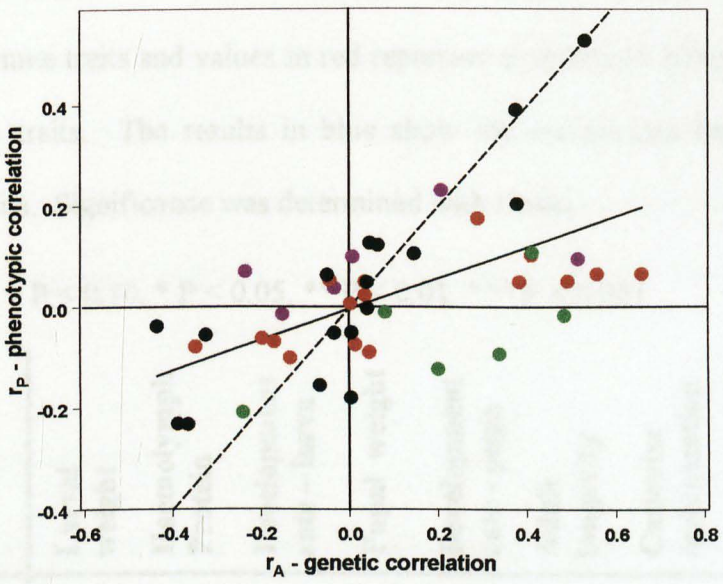


Figure 6.2 – The correlation between the phenotypic and genetic correlations for each pair of traits.

The dotted line represents a 1:1 relationship. There is a significant positive correlation between the two estimates that passes through the origin. The green circles represent correlations between life-history traits, the red circles represent correlations between life-history and immune traits and the purple circles represent correlations between the immune traits. The black circles represent any correlations with the condition traits.

Table 6.3 – Genetic and phenotypic correlations between traits.

Values above the diagonal show phenotypic correlations (Pearsons) between traits, values below show genetic correlations as estimated by *VCE*. Values in green represent correlations between life-history traits, values in purple are correlations between immune traits and values in red represent correlations between life-history and immune traits. The results in blue show the correlations between the two condition traits. Significance was determined with t-tests.

ns P > 0.10, + P < 0.10, * P < 0.05. ** P < 0.01, *** P < 0.001

	Larval weight	Haemolymph Protein	Development rate – larva	Pupal weight	Development rate - pupa	Adult longevity	Cuticular melanization	Haemolymph PO activity	Lysozyme activity	Haemocyte density
Larval weight		0.523 ***	-0.017 ns	0.057 ns	-0.053 ns	-0.018 ns	-0.163 ***	-0.238 ***	0.033 ns	-0.167 ***
Haemolymph Protein	0.533 ***		0.181 ***	-0.021 ns	-0.027 ns	0.114 *	-0.205 ***	0.105 *	0.360 ***	0.090 *
Development rate – larva	-0.033 ns	0.379 ***		0.210 ***	0.093 *	-0.107 *	0.035 ns	-0.102 *	0.075 +	0.069 ns
Pupal weight	-0.049 ns	0.042 ns	0.240 ***		-0.093 *	0.107 *	-0.018 ns	0.102 *	-0.075 +	-0.069 ns
Development rate - pupa	-0.323 **	0.006 ns	0.201 *	0.339 ***		0.018 ns	-0.051 ns	-0.049 ns	0.079 +	-0.064 ns
Adult longevity	-0.434 **	0.147 ns	-0.082 ns	0.412 ***	-0.485 ***		-0.104 *	0.064 ns	0.052 ns	0.022 ns
Cuticular melanization	0.030 ns	-0.360 ***	0.179 ***	0.003 ns	-0.430 ***	-0.220 ns		0.035 ns	-0.008 ns	0.090 *
Haemolymph PO activity	-0.387 ***	0.065 ns	-0.046 ns	0.410 ***	-0.384 ***	0.559 ***	-0.075 ns		0.098 *	0.230 ***
Lysozyme activity	0.041 ns	0.377 ***	-0.290 ***	0.014 ns	0.347 ***	-0.044 ns	-0.055 ns	0.008 ns		0.069 ns
Haemocyte density	-0.066 ns	0.047 ns	-0.036 ns	-0.169 **	-0.660 ***	0.036 ns	0.546 ***	0.208 *	-0.234 *	

Correlations between immune traits

Genetic correlations

There were significant positive and negative genetic correlations between the immune traits. There were positive genetic correlations between haemocyte density and both the degree of cuticular melanization ($r_A = 0.546 \pm 0.082$, $t = 6.66$, $p < 0.001$; Table 6.3, Figure 6.3a) and haemolymph PO activity ($r_A = 0.208 \pm 0.099$, $t = 2.10$, $p = 0.036$). However, the genetic correlation between haemocyte density and lysozyme activity was negative ($r_A = -0.234 \pm 0.102$, $t = 2.29$, $p = 0.022$).

Phenotypic correlations

The phenotypic correlations were mostly positive but significant only for haemolymph PO activity and haemocyte density ($r_P = 0.230$, $t = 5.75$, $df = 594$, $p < 0.001$; Table 6.3, Figure 6.3b).

Correlations between life-history and immune traits

Genetic correlations

Each of the immune traits exhibited significant genetic correlations with many of the life-history traits. Cuticular melanization was positively genetically correlated with larval development rate ($r_A = 0.179 \pm 0.055$, $t = 3.27$, $p < 0.001$; Table 6.3, Figure 6.3a). Haemolymph PO activity was positively genetically correlated with pupal weight ($r_A = 0.410 \pm 0.060$, $t = 6.83$, $p < 0.001$) and adult longevity ($r_A = 0.559 \pm 0.103$, $t = 5.43$, $p < 0.001$). Lysozyme activity was positively genetically

correlated with haemolymph protein levels ($r_A = 0.377 \pm 0.096$, $t = 3.93$, $p < 0.001$) and pupal development rate ($r_A = 0.347 \pm 0.094$, $t = 3.69$, $p < 0.001$).

Cuticular melanization was negatively genetically correlated with haemolymph protein levels ($r_A = -0.360 \pm 0.093$, $t = -3.87$, $p < 0.001$; Table 6.3, Figure 6.3a) and pupal development rate ($r_A = -0.493 \pm 0.100$, $t = -4.93$, $p = 0.001$). Haemolymph PO activity was negatively genetically correlated with larval weight ($r_A = -0.387 \pm 0.087$, $t = -4.45$, $p < 0.001$) and pupal development rate ($r_A = -0.384 \pm 0.105$, $t = -3.66$, $p < 0.001$). Lysozyme activity was negatively genetically correlated with larval development rate ($r_A = -0.290 \pm 0.076$, $t = -3.82$, $p < 0.001$), and haemocyte density was negatively genetically correlated with pupal weight ($r_A = -0.169 \pm 0.065$, $t = 2.60$, $p = 0.010$) and pupal development rate ($r_A = -0.660 \pm 0.089$, $t = -7.42$, $p < 0.001$).

Phenotypic correlations

There was a significant positive phenotypic correlation between lysozyme activity and haemolymph protein levels ($r_P = 0.360$, $t = 9.06$, $p < 0.001$). Negative phenotypic correlations were found between the level of cuticular melanization and larval weight ($r_P = -0.163$, $t = -4.05$, $p < 0.001$; Table 6.3, Figure 6.3b), and haemolymph protein ($r_P = -0.204$, $t = -4.98$, $p < 0.001$). Haemolymph PO activity and haemocyte density were also negatively phenotypically correlated with larval weight (PO: $r_P = -0.238$, $t = -5.99$, $p < 0.001$; haemocyte density: $r_P = -0.167$, $t = -4.11$, $p < 0.001$).

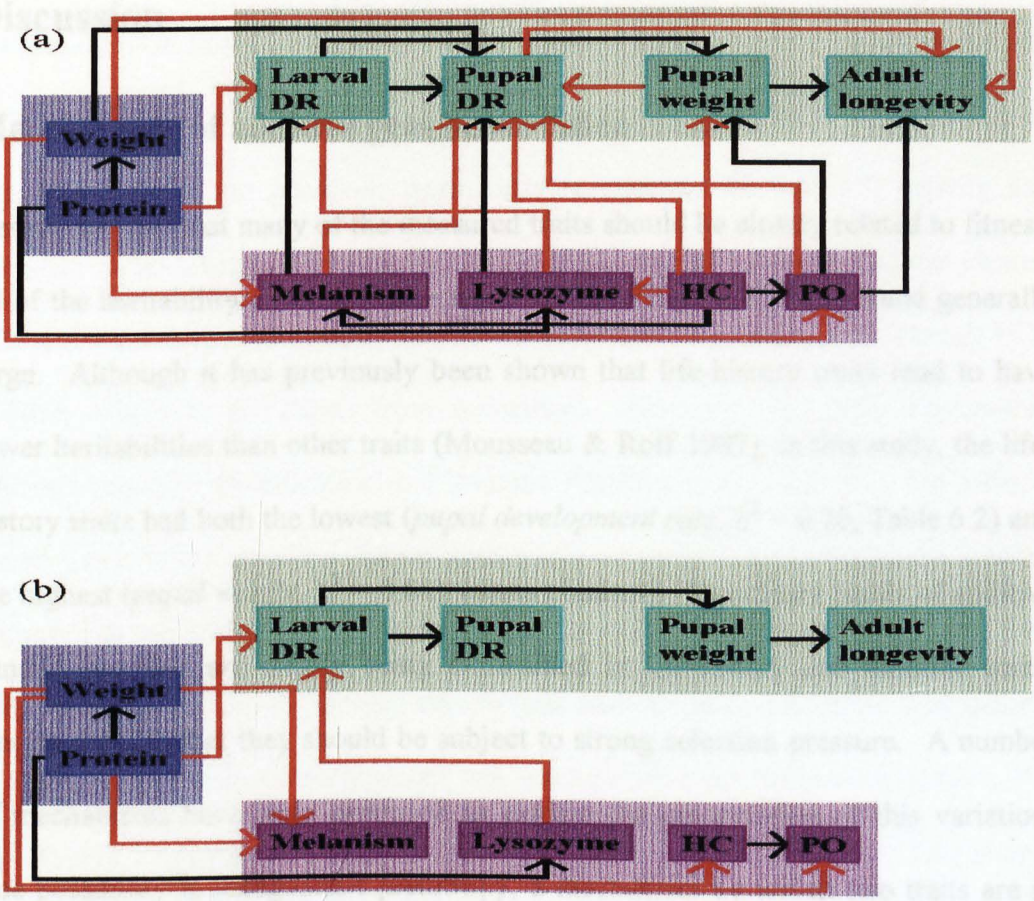


Figure 6.3 – Schematic of the genetic and phenotypic relationships between all traits.

Below is a representation of the (a) genetic and (b) phenotypic correlations between traits. The black lines represent positive correlations and the red lines represent negative correlations. Blue boxes represent condition traits, green boxes represent life-history traits and purple boxes represent immune traits. "Melanism" refers to cuticular melanization. Phenotypic correlations where $p > 0.01$ are not included.

Discussion

Maintenance of additive genetic variation

Despite the fact that many of the measured traits should be closely related to fitness, all of the heritability estimates were significantly different from zero and generally large. Although it has previously been shown that life-history traits tend to have lower heritabilities than other traits (Mousseau & Roff 1987), in this study, the life-history traits had both the lowest (*pupal development rate*, $h^2 = 0.20$, Table 6.2) and the highest (*pupal weight*, $h^2 = 0.85$) values obtained. Significant levels of additive genetic variation are clearly being maintained in life-history and immune traits, despite the fact that they should be subject to strong selection pressure. A number of mechanisms have been proposed to explain the preservation of this variation. One possibility is antagonistic pleiotropy, a mechanism by which two traits are in part influenced by the same genes, but the influence on one trait is positive and the other negative. A negative genetic correlation between two traits therefore implies a possible trade-off.

This study provided evidence for three types of costs that could be maintaining variation in immunity: trade-offs *within* the immune system, trade-offs *between* immune function and life-history traits and trade-offs *between* immune function and predator avoidance (crypsis).

Trade-offs within the immune system

An examination of the genetic correlations revealed a potential trade-off within the immune system. Haemocyte density was negatively genetically correlated with lysozyme activity but positively genetically correlated with both PO activity and cuticular melanization. The results for cuticular melanization should be viewed with a certain degree of caution, due to the non-normality of the data (though *VCE* is fairly robust to deviations from normality). However, the same relationship between cuticular melanization and immune function was apparent in the solitary reared-larvae in the previous chapter where pale larvae had higher lysozyme activity (Figure 5.3) but lower haemolymph PO activity than dark larvae (Figure 5.4). The immune traits also have opposing correlations with development rates. The genetic correlations with pupal development rate were positive for haemocyte density, PO activity and cuticular melanization, but negative for lysozyme activity. This results in larvae that, genetically, either invest in haemocyte production, haemolymph PO activity and cuticular melanization (i.e. dark larvae), or larvae that invest in lysozyme activity (i.e. pale larvae). Moret and Schmid-Hempel (2001) also suggested a possible trade-off between phenoloxidase activity and the antibacterial response in the bumblebee, *Bombus terrestris* based on a negative *phenotypic* correlation between the two measures of immune function. However, this study is the first to establish a genetic basis for the correlation.

The physiological basis of this potential trade-off could be considered from the point of view of resource allocation, or the pleiotropic action of hormones. Lysozyme is typically produced by the fat body (Boman & Hultmark 1987), whereas haemolymph PO and cuticular PO (responsible for cuticular melanization) are both produced in the haemocytes (Söderhäll & Smith 1986; Ashida & Brey

1997). Haemocytes are constantly being produced and so are likely to be energetically costly (Chapman 1998). It is possible that the production of haemocytes leads to reduced fat storage which could, in turn, lead to a reduced capacity for lysozyme production. At a more fundamental level, both the haemocytes and the fat body originate from the mesoderm (Vilmos & Kurucz 1998). Therefore, the levels of investment in the two may rely on the initial tissue allocation during larval development.

Hormones are excellent candidates for the mediation of life-history trade-offs as not only can a single hormone have different effects on different targets, but they can also act throughout different life stages of the organism (Finch & Rose 1995). There have been very few studies investigating the hormonal regulation of immune function in insects. However, a recent study found that juvenile hormone (JH) appears to inhibit PO activity in *Tenebrio molitor* (Rolff and Siva-Jothy 2002). It is also well documented that JH application can cause *gregaria* locusts to change into the green solitary form, and that the pale, solitary forms of many phase-polyphenic Lepidoptera have higher JH titres than their crowded, dark counterparts (Yagi & Kuramochi 1976; Ikemoto 1983; Fescemyer & Hammond 1988; Pener 1991). It is therefore possible that the trade-off in the immune system is hormonally regulated with JH inhibiting haemocyte production, PO and cuticular melanization whilst promoting lysozyme activity. Further studies are clearly required to clarify the role of hormones in insect immunity.

There is also evidence of possible trade-offs within the vertebrate immune system. In mice and sheep, infection with gastrointestinal nematodes causes up-regulation of the Th-2 mediated immune response, components of which down-regulate the Th-1 response, which is effective against microparasites such as bacteria and viruses

(Grencis 1997; Gill *et al.* 2000). Also, mice selected for increased antibody production had a higher susceptibility to chemically-induced skin tumours than the low antibody line (Ibanez *et al.* 1999). Similar results have been found in birds. In chickens, selection for high antibody production against sheep red blood cells resulted in lower lymphocyte mitogenic activity compared to lines selected for low antibody production. This suggests that selection for a greater humoral response may be at the expense of cellular responses (Gehad *et al.* 1999). In addition, the assumption that specific resistance to one parasite may not confer resistance to another, is the basis of parasite-mediated sexual selection (Hamilton & Zuk 1982). This relies on co-evolution between parasites and hosts and provides an explanation for the maintenance of additive genetic variation in immunity. But both of these trade-offs are based around specific immune responses, the types of immune response that provide resistance to a particular parasite or pathogen, either through exposure and memory, or co-evolution. That there should be conflicts between such specific responses is perhaps less surprising than the possibility of a trade-off between general components of the innate immune response.

Trade-offs between immunity and other life-history traits

The trade-off within the immune system observed in the present study seems to be linked to the pale and dark phases associated with density-dependent polyphenism (Chapter 5). The density dependent prophylaxis (DDP) hypothesis predicts that larvae reared in crowds, i.e. dark larvae, will invest more resources in immune function due to the increased risk of pathogenesis at high densities (Wilson & Reeson 1998). Implicit in this hypothesis is the assumption that immune function is

costly. We would therefore expect that in conditions where the risk of pathogenesis was low, pale larvae would outcompete dark larvae. Therefore, in the absence of pathogens, there must be significant costs associated with being dark. An examination of the genetic covariance between all of the traits revealed a complex array of positive and negative correlations (Figure 6.3). In order to clarify these relationships, theoretical life-history trajectories were predicted from the point of view of phase, i.e. pale or dark larvae (Table 6.4).

In addition to having higher PO activity and haemocyte density, dark larvae pupated sooner and at a heavier weight than pale larvae, and their adult lifespan was longer (Table 6.4). Haemocyte density and PO have been shown to be good predictors of encapsulation ability, both within and across species (chapter 3; Eslin & Prévost 1996, 1998; Wilson *et al.* Submitted). Also, darker larvae have higher cuticular PO activity, which corresponds to resistance to both fungal infection and ectoparasitoids (Chapter 4). Consequently, dark larvae should be better equipped to survive attack by parasites or pathogens. Larval development rate and pupal weight are good predictors of fecundity in this species (Figure 6.4). This is to be expected as adult moths ingest no protein, and as a result the resources with which the adult emerges limits its production of eggs (Nijhout 1994). The longer adult lifespan of dark larvae suggests that their lifetime reproductive success should also be higher as not only are they more fecund, but they also have a longer time period over which to lay eggs. However, there may be differences in the length of the pre-reproductive phase between adults from pale or dark larvae (Gunn & Gatehouse 1993).

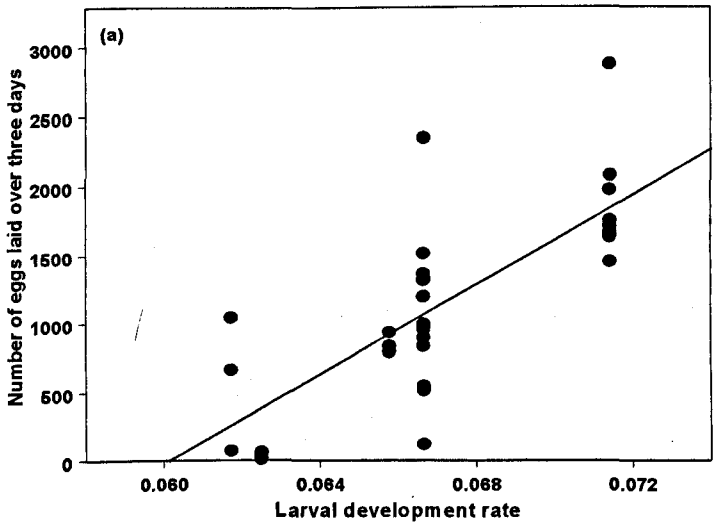
Table 6.4 – Costs of immune function

Traits in italics are not directly genetically correlated with cuticular melanization and so the correlations were inferred from relationships with other traits. Traits that are presumed to be beneficial are in black and those presumed to be detrimental to individual fitness are in red.

Trait	Pale	Dark
Haemolymph protein	High protein	Low protein
Larval weight	*High weight*	*Low weight*
Lysozyme activity	*High lysozyme*	*Low lysozyme*
Haemolymph PO activity	*Low PO*	*High PO*
Haemocyte density	Low HC	High HC
Pupal weight	*Low pupal weight*	*High pupal weight*
Larval development	Slow larval development	Fast larval development
Pupal development	Fast pupal development	Slow pupal development
Adult longevity	*Short adult lifespan*	*Long adult lifespan*

The costs associated with the dark phase are low larval weight, low protein, low lysozyme activity and a slow pupal development rate (Table 6.4). As there is greater variation in larval than pupal development rate (Table 6.2), the period of time spent as a juvenile (hatching to eclosion) is primarily determined by the larval development rate. It seems unlikely, therefore, that a decreased pupal development rate inflicts a large enough cost to counter the benefits of a fast larval development rate. In addition, low larval weight at the final instar may not be costly as, contrary to expectation, it was not a good of predictor of pupal weight, at least for larvae with access to food *ad libitum*. Surprisingly, the dark larvae managed to pupate at a heavier weight despite their poorer "condition" at the start of the final instar.

Conversely, low haemolymph protein levels may be costly in terms of the ability to provision eggs. Proteins are taken up directly from the haemolymph during oogenesis (Chapman 1998), consequently, low protein levels may result in smaller or less well-provisioned eggs. Low lysozyme levels may also represent a cost to being dark. If the risk of bacterial infection was higher than the risk of parasitoid attack, fungal or viral infection, then low lysozyme levels may reduce the survival of dark larvae. Furthermore, in the tobacco hornworm, *Manduca sexta*, lysozyme is secreted into the midgut lumen prior to the larval/pupal ecdysis. It seems that during metamorphosis, when the midgut is broken down, the gut is "sterilised" via the action of lysozyme and other antibacterial proteins, to reduce the risk of septicaemia (Russell & Dunn 1996). As a result, dark larvae may run a greater risk of death during pupation than pale larvae. A high risk of death before adulthood should provide a strong enough selection pressure to counter the benefits of increased fecundity.



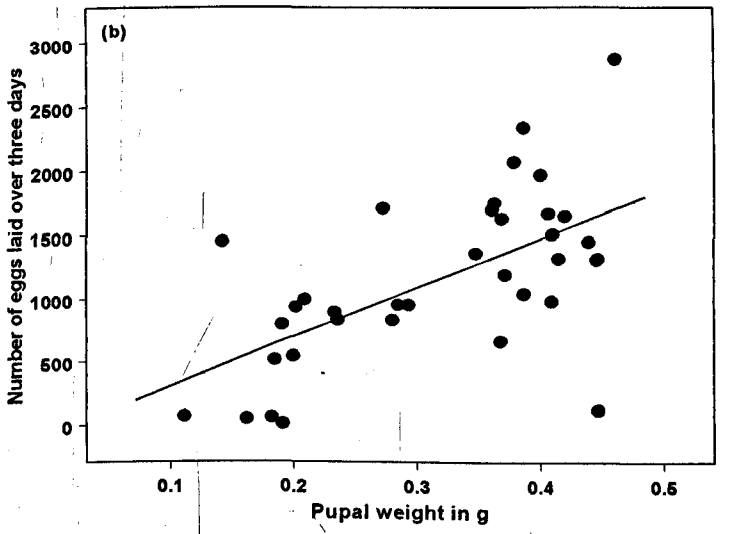


Figure 6.4 – The relationship between larval development rate, pupal weight and fecundity.

Fecundity, measured as the number of eggs laid over three days, is plotted against (a) larval development rate ($F_{1,33} = 23.17$, $p < 0.001$) and (b) pupal weight ($F_{1,33} = 12.82$, $p = 0.001$). (This data is taken from a separate experiment, see Appendix 3 for details)

It is possible that further costs associated with the dark phase are not apparent due to the conditions under which the larvae were reared. Dark larvae seemed to be in poorer "condition" as measured by weight and haemolymph protein levels. However, there was no evidence that any of the traits were condition-dependent, as controlling for body condition in the analysis did not alter the relationships between the traits (though direct manipulation of resources may have produced different results). Kraaijeveld and Godfray (1997) selected lines of *Drosophila melanogaster* for resistance to the parasitoid, *Asobara tabida*. Resistant lines had significantly higher encapsulation rates than controls, but appeared to suffer no fitness costs. Re-examination of the fitness traits under high competition-low food conditions revealed that the resistant lines had significantly lower larval competitive ability. Parallels can be drawn between this study and Spitze's work on the waterflea, *Daphnia pulex* (Spitze 1991). Artificial selection under conditions of high predation pressure resulted in a larger, more fecund waterflea, at no apparent cost. However, the life-history traits were measured under conditions of high resource availability; under low resource conditions, costs may have been apparent. High levels of variation in resource acquisition can result in seemingly counterintuitive positive phenotypic and genetic correlations between traits (van Noordwijk & de Jong 1986; Houle 1991).

The positive correlation between larval development rate and pupal weight may fall into this category. Classic life-history theory predicts a trade-off between age and size at maturity (Roff 1992). The positive correlation observed in this species between larval development rate and pupal weight therefore seems counterintuitive and may be apparent only under high-resource conditions. In natural conditions, resources are often limiting and under crowded conditions, the dark, fast developing

larvae tend to have lower pupal weights than pale larvae (Hodjat 1970). Therefore, any cost associated with the ability to *acquire* resources could result in the fast-developing larvae suffering more, when competition for food was high, due to their higher resource requirements. Additional support for this idea comes from another study on *Daphnia,* which found that adult clones with the highest body mass showed greater sensitivity to declining resources than smaller clones (Reznick *et al.* 2000; Tessier *et al.* 2000).

Trade-off between immunity and predation avoidance

Another possibility is that the dark cuticle is itself costly. If dark larvae are more conspicuous than pale larvae, then the benefits of the dark phase may be outweighed by the increased risk of predation. At low larval densities, the risk of pathogenesis should be low, as disease transmission is usually density-dependent. However, predation risks are often reduced by living in groups, therefore at low densities selection should favour crypsis over disease resistance, whereas at high densities disease resistance should be more important (Sword 2000; Wilson 2000). This potential trade-off between defence against parasites and predators has been observed in the freshwater snail *Lymnaea stagnalis* (Rigby & Jokela 2000). Snails respond to the risk of predation by retreating deep into their shell, but to accomplish this they must expel haemolymph, the loss of which resulted in reduced phagocytic capability. However, further experiments are required to test whether the trade-off between defence against parasites and predators occurs in this species.

Conclusions

In conclusion, there was significant additive genetic variation in all four immune traits, as well as the measures of body condition and the life-history traits. However, there was no evidence that the levels of additive genetic variation were higher in immune traits compared to life-history traits, as predicted by Fisher's fundamental theorem (Fisher 1958). There were negative genetic correlations both between the different immune traits, and between the immune traits and some of the life-history traits, suggesting possible trade-offs with immunity. Investment in haemocytes, phenoloxidase activity and cuticular melanization seemed to reduce investment in lysozyme activity, although the physiological basis for the negative genetic correlation is unclear.

The typical solitary form of *S. littoralis* is pale, and this is correlated with slow larval development, low pupal weight, (and low fecundity), high lysozyme activity, low haemocyte density and low phenoloxidase activity. Therefore, it could be the darkening of the cuticle itself that is the most important constraint on immune function in this environment. A lack of crypsis could increase the risk of predation sufficiently to outweigh the benefits of increased fecundity. Also, although there is no evidence that the correlations between any of the traits are condition-dependent, direct manipulation of resources would be required to test this hypothesis. It is possible that the correlation between larval development time and pupal weight is positive only in a resource-rich environment, and that further costs to investment in immunity would be apparent under poorer conditions.

This study has provided evidence both of potential trade-offs between general mechanisms of immune function and between defence against pathogens

(investment in immunity) and predators (crypsis). Future research should examine the importance of environmental effects on these trade-offs, particularly how the relationships change under conditions of high competition, as experienced in high-density, outbreak populations.

Figure caption: the pre-pupal stage of *S. littoralis* larvae.

Chapter 7

General discussion

The aim of this study was to use an insect model to examine the genetic basis of innate immunity; to consider possible costs (trade-offs) that could maintain genetic variation in immune function; and to assess the role of phenotypic plasticity in moderating those costs.

Additive genetic variation in immune function

A key finding of this study was that high levels of additive genetic variation were maintained in non-specific components of the insect immune system. Heritability estimates ranged from 0.36-0.65 (Table 6.2) and were comparable to estimates for life-history and condition traits (h^2 = 0.20-0.85; Table 6.2). This means that, contrary to expectation, selection is not removing alleles for low levels of basic immune parameters (e.g. circulating blood cells or antibacterial proteins) from the population. There are many, non-exclusive reasons why this should be so. If these measures of immune function are not related to fitness, they will not be under strong directional selection and hence these traits will retain genetic variability. However, if the measures of immune function *are* related to fitness, there are still a number of mechanisms by which variation can be maintained. These include mutation-selection balance, stabilising selection, antagonistic pleiotropy and genotype by environment interactions (Stearns 1992). Each of these explanations will be considered in turn.

Are the measures of immune function related to fitness?

The simplest explanation for the maintenance of additive genetic variation in immune system components is that these measures of immune function are not related to fitness. It is unlikely that resistance to parasites or pathogens is not related to fitness due to the, sometimes dire, consequences that infection can have on an individual's fecundity or survival. However, quantitative measurements of immune system components may not be directly related to fitness - if these components do not accurately reflect resistance. This difficulty with assessing "immunocompetence" has been raised previously, but many studies still measure immune traits without showing that they are, in some way, related to an individual's ability to resist infection (Siva-Jothy 1995; Owens & Wilson 1999).

In this study, several components of the immune system were used as indicators of an individual's investment in resistance. Haemocyte density, an estimate of the numbers of circulating blood cells per millilitre of haemolymph, has been shown to be correlated with resistance to parasitoids, both within and across *Drosophila* species (Eslin & Prévost 1996, 1998). *Drosophila* tend to have few circulating haemocytes, therefore the initial number available for encapsulation may be crucial in determining resistance. However, a recent study suggests that the importance of haemocyte density for immune function may be a more widespread phenomenon. Haemocyte density also correlates with encapsulation ability, both within and across Lepidopteran species (Wilson *et al.* Submitted), which have significantly higher numbers of haemocytes than most Dipterans (Chapman 1998). In addition, *Drosophila melanogaster*, selected for increased resistance to the parasitoid *Asobara tabida*, had twice as many circulating haemocytes as control flies. Thus, there is evidence for both a genetic and a phenotypic correlation between haemocyte

density and encapsulation ability. It seems, therefore, that haemocyte density *is* related to parasite resistance and hence to fitness.

Phenoloxidase (PO) is a key enzyme in the synthesis of melanin, which is found around encapsulated foreign bodies in the haemolymph, midgut and cuticle (Götz 1986; Paskewitz *et al.* 1989; Tanada & Kaya 1993; Washburn *et al.* 1996). Melanin is also responsible for the physical toughening of the cuticle and it may have chemical properties that aid in parasite resistance (Söderhäll & Ajaxon 1982; St. Leger *et al.* 1988). PO may also be involved in non-self recognition in the haemocoel as it is activated by components of microbial cell walls (Pye 1974). Products of the PO cascade are thought to be involved in agglutination of microbes in the haemocoel (Nigam *et al.* 1997), and the killing of encapsulated parasites via intoxication (Götz 1986). It is clear, therefore, that PO is fundamental in resistance to a variety of parasites. However, the idea that resistance could be related to the levels of PO present in the different compartments of the body had rarely been demonstrated (Reeson *et al.* 1998).

In this study, haemolymph PO activity was correlated with the ability to encapsulate an artificial parasite in the haemocoel (Chapter 3), levels of midgut PO activity were correlated with resistance to a virus (Chapter 4) and levels of cuticular PO activity were correlated with resistance to fungal infection (Chapters 4 & 5). Moreover, the levels of PO activity in all three compartments of the body were correlated in both *Spodoptera* species (Chapter 3 & 4), suggesting that resistance to a range of parasites can be improved by increasing investment in PO. It must be noted that these are correlational results and, as such, cannot alone reveal causation. However, a causal link between PO activity and melanization has been revealed in two Dipteran species. A mutant strain of *Drosophila melanogaster,* lacking in PO, was

unable to melanize capsules around parasites in the haemocoel (Rizki & Rizki 1990). Furthermore, Shiao *et al* (2001) demonstrated that by using a recombinant virus to knockout PO expression in the mosquito *Armigeres subalbatus*, melanization of microfilariae *in vivo* could be inhibited. Thus, the direct link between PO activity and encapsulation/melanization found in Diptera, and the correlation between PO activity and encapsulation in Lepidoptera, identified in this study suggest that PO activity *is* related to fitness.

Lysozyme is an important part of the immune response, working with inducible antibacterial peptides to destroy bacteria in the haemolymph (Boman & Hultmark 1987). Assuming that the up-regulation of lysozyme activity in response to infection is adaptive, absolute levels must be important (Boman & Hultmark 1987). Also the high repeatability of lysozyme activity over 24 hours (Table 5.1) suggests that initial levels are a good indication of inducible levels, at least over a short time scale. However, lysozyme activity can be continually increased for up to 7 days after infection (Wittwer *et al.* 1997). Therefore the relationship between standing levels of lysozyme activity and resistance to bacterial infection needs to be tested directly.

There is strong evidence that haemocyte density and PO activity are related to fitness. However, the relationship between standing levels of lysozyme in the haemolymph and immune function is less clear. It is probable, however, that lysozyme present in the haemolymph will allow a quick response to parasitism and will be vital in the early stages of infection. If these immune system components represent an individual's investment in resistance and, as such, are related to fitness, why isn't all the additive genetic variation eroded by directional selection?

Maintenance of additive genetic variation in fitness-related traits

Assuming that the components of immune function measured in this study *are* related to fitness, the mechanism maintaining variation in these traits must be addressed. A number of potential mechanisms have been suggested in the literature. These include mutation-selection balance, stabilising selection, antagonistic pleiotropy and genotype by environment interactions (Stearns 1992).

Mutation-selection balance is a mechanism by which the variation lost, due to alleles being removed from the population by selection, is replenished by constant mutation. Houle (1991) demonstrated that under certain circumstances, this alone could maintain variation in traits under weak directional selection. However, it is not clear whether the rates of mutation required to maintain variation in traits under strong directional selection occur in natural populations (Roff 2002).

Stabilising selection occurs if an intermediate value of a trait is the optimum. It is possible that certain immune components could be under stabilising selection, i.e. very high levels could reduce fitness. Many immune reactions result in the production of oxidative metabolites and free radicals, high levels of which are linked to the risk of cancer and reduced longevity. This is due to the extensive damage they can cause to DNA, proteins and lipids – referred to as oxidative stress (von Schantz *et al.* 1999). The PO cascade generates reactive oxygen species (ROS), which can damage host tissue. However, melanin, the end-product of the cascade, scavenges ROS produced during the reaction (Nappi & Vass 1993). Therefore, as PO activity produces both dangerous oxygen compounds and an efficient antioxidant, melanin, it is difficult to predict if high levels of PO could result in reduced fitness and hence be subject to stabilising selection. However,

although models of stabilising selection predict stable polymorphisms under certain conditions, there is little empirical evidence that this mechanism can maintain additive genetic variation in fitness-related traits (Roff 2002).

In contrast, antagonistic pleiotropy and genotype by environment interactions have received considerable attention and are often invoked as mechanisms to maintain additive genetic variation. As these two mechanisms are explicitally addressed in the present study they will be considered in greater detail.

Antagonistic pleiotropy

Antagonistic pleiotropy, where a gene has a positive effect on one trait but a negative effect on another, is a commonly cited mechanism to maintain additive genetic variation. This is the genetic basis of trade-offs, which are central to the study of life-history evolution. This study identified several potential trade-offs associated with immune function: trade-offs within the immune system; trade-offs between immune function and condition; and trade-offs between immune function and predator avoidance (Chapter 6).

Haemocyte density, haemolymph PO, cuticular melanization – and by association cuticular PO, were negatively genetically correlated with lysozyme activity (Chapter 6). This suggests that investing in resistance to fungi and parasitoids that enter the host via the cuticle could leave the host susceptible to bacterial infection. Immune function is often considered to be a single trait that can be improved with increased resources (Owens & Wilson 1999; Rigby *et al.* 2002). However, this potential trade-off within the immune system brings into question the idea of "immunocompetence", an all-encompassing measure of resistance.

Theories of sexual selection based around parasite resistance assume that an individual is able to signal its immunocompetence to potential mates, thereby allowing mate choice on the basis of good genes (Hamilton & Zuk 1982; Folstad & Karter 1992). However, a trade-off within the immune system would mean that an individual could invest in certain immune components, only at the expense of others. The importance of this potential trade-off relies on the mechanism generating it. If it is generated by resource allocation, it could be hidden by the variation in resource acquisition, likely to occur in natural conditions (van Noordwijk & de Jong 1986; Houle 1991). Individuals in good condition, i.e. those with the most resources could then invest more in immune function than those in poor condition, as predicted by the immunocompetence handicap hypothesis, despite a trade-off between immune components (Folstad & Karter 1992). However, if the trade-off is mediated by a hormone, whereby the hormone elicits transcription of genes coding for one component but inhibits transcription of another, it should be independent of body condition. Therefore, even individuals with plenty of resources available to invest in immune function would face the choice of which components to up-regulate. In this case, the sexual signals displayed by reproductive males could indicate the individual's ability to regulate the immune system such that it matched the risk of pathogenesis in that environment. In the light of this result, estimates of immune function that are based on a single measured variable should be viewed with caution.

The potential trade-off between components of the immune system, identified in this study, was found in the simple innate immune system of insects. However, this system shares many basic characteristics with the innate immune system of vertebrates, with many factors showing remarkable homology across species

(Vilmos & Kurucz 1998). Therefore, these findings may be applicable across a wide-range of taxa. Clearly, the mechanism generating the trade-off needs to be investigated if conclusions, regarding its impact on immunocompetence in natural populations, are to drawn.

This study also identified potential immune function trade-offs with body condition, measured as larval weight and protein content of the haemolymph at the start of the final instar, and predator avoidance (Chapter 6). A negative genetic correlation between the immune parameters associated with dark phase larvae (haemocyte density, haemolymph PO and cuticular PO) and body condition implies a strict re-allocation of resources from maintenance or storage to resistance. The environment in which the larvae were raised, *ad libitum* food and solitary conditions, meant that there was no competition for food and no energy was expended foraging. As such, there may have been very little variation in resource acquisition, which can mask variation in resource allocation (van Noordwijk & de Jong 1986). However, the dark phase larvae appeared to suffer few fitness costs, as they pupated sooner and at a heavier weight than pale larvae (larval development rate and pupal weight are highly correlated with fecundity; Figure 6.4). Therefore, in this environment, the potential trade-off between certain components of the immune system and body condition is not costly, as dark larvae can compensate for their poor condition during the final instar.

As there is an apparent lack of costs associated with investment in immune function in the low-density environment, dark larvae should be fitter than pale larvae. However, the typical low-density larva is pale, suggesting that there are costs associated with being dark that weren't measured in this study. One possibility is the lack of crypsis associated with a highly melanized cuticle (Owens & Wilson

1999; Wilson 2000)]. In low-density conditions, visibility to predators could be a more significant fitness cost than reduced immune function. This hypothesis could be tested by comparing predator-related mortality rates of pale and dark larvae in natural conditions. It is also possible that there may be trade-offs with traits not measured in this study. For example, immunity could trade-off with pheromone production or flight capability, which may impact upon mate-finding ability and hence fitness. This highlights the importance of measuring many variables when considering costs in life-history analysis, and considering trade-offs that may not be related to resource levels (Roff 2002).

Genotype by environment interactions

The trade-off analysis measured traits in a single environment. However, many traits are phenotypically plastic, i.e. a single genotype can display a range of phenotypes depending on the environment. Many of the traits measured in this study are phenotypically plastic. Cuticular melanization is one such trait, as it varies with population density. At low densities, larvae tend to be pale and at high densities they tend to be dark. However, the threshold density at which a larva produces a melanized cuticle is dependent on its genotype; some larvae will remain pale at very high densities whereas some will develop highly melanized cuticles in solitary conditions. As the optimal phenotype for low-density environments seems to be the pale phase, and for high-density environments the dark phase, phenotypic plasticity in cuticular melanization and its associated traits, allows a single genotype to maximise its fitness by developing the phenotype best suited to its environment. The way in which the phenotype of a single genotype varies with the environment is

called a "reaction norm". If the reaction norms of all genotypes were identical, a single genotype could outcompete others in all environments and would quickly become fixed in the population (Stearns 1989). If this were the case for cuticular melanization, there would be no genetic variation in the threshold density at which an individual started to melanize its cuticle. However, there *is* variation in this threshold; colour (measured under solitary conditions) is heritable and so is not entirely determined by population density (Table 6.2). This suggests that the reaction norms for different genotypes are not the same. This is referred to as a genotype by environment interaction and is another mechanism by which additive genetic variation can be maintained in traits closely related to fitness. This mechanism is likely to be very important for species such as *Spodoptera*, which can experience huge fluctuations in population density, both within and across generations.

Phenotypic plasticity in immune function

Many studies have shown that pathogen resistance varies with colour and/or rearing density (Mitsui & Kunimi 1988; Kunimi & Yamada 1990; Goulson & Cory 1995), but few have investigated any underlying variation in the allocation of resources to immunity (Reeson *et al.* 1998; Barnes & Siva-Jothy 2000; Wilson *et al.* 2002). This study was the first to demonstrate that basal levels of a range of immune system components were phenotypically plastic. Haemolymph PO activity, cuticular PO activity and capsule melanization were all higher in dark larvae, whereas lysozyme activity was higher in pale larvae (Chapter 5). As dark larvae are common only in high-density conditions, this suggests that *S. littoralis* up-regulate immune function

only when the risk of pathogenesis is high, as predicted by the density dependent prophylaxis hypothesis (Wilson & Reeson 1998). This phenotypic plasticity in immune function reduces the costs associated with maintaining the immune system by tailoring it to the environment. However, lysozyme activity, an important antibacterial response in the haemocoel, was not up-regulated in dark larvae; in fact, it was higher in pale larvae typical of low-density environments. Further experiments are required to ascertain whether the increased levels of lysozyme activity in pale larvae translate into increased resistance to bacterial infection. It is also important to assess whether the high levels of lysozyme activity present in pale larvae are adaptive, by determining the risk of bacterial infection in low-density versus crowded environments. This would provide information as to whether the trade-off was costly, or simply an adaptation to the way the risk of pathogenesis varies with the environment.

The results from both the genetic and the phenotypic analysis of immune function suggest that there is a trade-off in the immune system and that pale and dark phase larvae display differential investment in immune function. Although differing allocation of resources could explain some of the potential trade-offs identified in this study, the hormonal regulation of immunity should also be considered as it represents the intermediate level between genotype and phenotype (Stearns 1989).

A hormonal model of phenotypic plasticity and trade-offs in immune function

The role of juvenile hormone in phenotypic plasticity

It has been proposed that hormones may play a pivotal role in the mediation of life-history trade-offs. A single hormone can have different effects on different targets, both within a life-stage, and across life-stages of the organism (Finch & Rose 1995). Wing polymorphism in insects represents a classic life-history trade-off between reproduction and maintenance (Zera & Harshman 2001). The winged morphs tend to have functional flight muscles and lipid reserves, whereas the flightless morphs tend to have small, non-functional wings and reduced lipid storage (Roff 1986). In the wing polymorphic crickets *Gryllus firmus* and *Gryllus rubens,* it has been demonstrated that the flightless morph is more fecund and that there is a trade-off between allocation of reserves to flight muscles and ovaries (Zera & Mole 1994; Zera & Denno 1997). The flightless morph also has a higher titre of juvenile hormone (JH) than the winged morph (Zera & Harshman 2001). Exogenous application of the JH analogue, methoprene, to the non-dimorphic, flight-capable *Gryllus assimilis* resulted in increased ovary development and reduced flight muscles demonstrating that this trade-off is mediated by JH (Zera *et al.* 1998).

However, phase differences in JH titre are not restricted to wing-dimorphic species. Solitary phases of many phase polyphenic Lepidoptera and locusts also have high JH titres compared to the gregarious (Yagi & Kuramochi 1976; Nijhout & Wheeler 1982). As JH is involved in regulation of the moulting cycle, high titres usually result in longer juvenile development times (Yagi & Kuramochi 1976; Fescemyer & Hammond 1988). High JH titres are also associated with a lack of melanization of

the cuticle, with solitary phases tending towards pale or cryptic coloration (Ikemoto 1983; Fescemyer & Hammond 1988). Indeed, exogenous application of JH to gregarious-phase locusts resulted in a reversion to the green coloration typical of solitary-phase locusts (Pener 1991). In adults of many insect species, JH is involved in the development of reproductive tissue and the regulation of egg production (Nijhout 1994; Soller *et al.* 1999) and high titres have been shown to reduce adult longevity (Herman & Tatar 2001). Once titres fall below a certain level, development of the reproductive tissue is postponed and migration ensues (Nijhout 1994). Moreover, gregarious adults of many species tend to have larger lipid reserves than solitaries and have a greater propensity to migrate (Iwao 1968; Gunn & Gatehouse 1993). Therefore, JH is thought to play a key role in the phenotypic plasticity of many juvenile and adult traits in many insect species.

Hormones and immune function

So, could JH mediate the potential trade-offs with immune function and the phenotypic plasticity of certain immune traits observed in this study? There have been very few studies investigating the hormonal regulation of immune function in insects. However, a recent study found that JH could inhibit PO activity in *Tenebrio molitor* (Rolff & Siva-Jothy 2002). JH produced by mated adults caused a down-regulation of PO activity, though whether this was direct or indirect is unclear.

Other studies examining the activity of hormones on immune function have implicated an alternative insect hormone, ecdysone. Ecdysone is also important in moulting and is involved in cuticular melanization (Curtis *et al.* 1984; Nijhout

1994). In *Drosophila*, the application of ecdysone increased the phagocytic activity of haemocytes *in vitro*, and up-regulated the expression of inducible antimicrobial peptides such as diptericin and drosomysin (Dimarcq *et al.* 1997). Ecdysone also caused the induction of genes expressing membrane receptors, which are involved in the recognition of microorganisms. In the yellow fever mosquito, *Anopheles gambiae*, an ecdysone receptor site has been identified in the pro-PO 1 gene (Ahmed *et al.* 1999). Ecdysone up-regulated pro-PO two hours after application and levels increased up to 24 hours later before returning to normal. So, the evidence seems to point to either ecdysone-mediated *up-regulation* of immune function, or JH-mediated *inhibition* of immune function. Are these two mechanisms mutually exclusive, or could they work together?

It is commonly accepted that JH can inhibit the ecdysteroid-induced expression of certain genes. Throughout larval development, the presence of JH prior to the moult ensures that the larva will proceed to the next larval instar. However, prior to the pupal moult, JH titres fall, the ecdysone-mediated expression of pupal genes is no longer inhibited and the larva moults to a pupa (Nijhout 1994). There is also evidence that JH can inhibit ecdysone-induced apoptosis of developing oocytes in *Drosophila melanogaster* (Soller *et al.* 1999). Could JH also inhibit the ecdysteroid-mediated expression of certain genes relating to immune function? If it could, this would provide an explanation for JH-induced colour change, as it is ecdysone that is responsible for cuticular melanization. Furthermore, in JH-deficient *Manduca sexta* larvae, levels of Dopa decarboxylase (DDC), an enzyme that plays a role in cuticular melanization, were found to be twice those found in control larvae (Hiruma & Riddiford 1993). It could be JH-mediated inhibition of PO expression by ecdysone that is responsible for the reduced PO activity in mated *Tenebrio*

molitor. Mating was also found to reduce antibacterial activity in *Drosophila* males, measured as the clearance of *E. coli* from the haemocoel (McKean & Nunney 2001). Again, if mated *Drosophila* males have increased JH titres and this inhibits the ecdysone-mediated regulation of phagocytosis and the expression of inducible antimicrobial peptides such as diptericin and drosomysin, we would expect these males to have reduced antibacterial activity.

In this study, the phenotypic and genetic analysis of immune function suggested that certain immune traits, particularly PO and responses involving PO such as cuticular melanization and encapsulation, were up-regulated in the dark-phase larvae, typical of crowded conditions; exactly those individuals that should have the lowest JH titres – though this was not tested directly. In addition, there was a strong positive genetic correlation between PO and adult longevity; high JH titres can inhibit PO and reduce adult longevity. It is therefore possible that the trade-offs in the immune system and phenotypic plasticity in immune function are hormonally regulated.

If JH mediates the trade-off in the immune system, it should up-regulate lysozyme activity and down-regulate haemocyte density, PO etc. This could easily be tested by the removal of the corpora allata and by exogenous application of JH. *In vivo* studies on the role of ecdysone are more problematic due to the difficulty of removing the prothoracic glands. Consequently, most studies have concentrated on JH as the corpora allata can be removed intact and implanted into other insects. Chemical inhibition of ecdysone production, in conjunction with removal/transplantation of the corpora allata could clarify the relative importance of the two hormones. *In vitro* studies could also provide valuable information about the hormonal regulation of immune function. Work on *Drosophila* cell cultures demonstrated ecdysone-induced up-regulation of phagocytosis and antibacterial

activity (Dimarcq *et al.* 1997). Similar experiments using Lepidopteran cell cultures could clarify the generality of this result. In addition, by testing the effects on immune parameters of ecdysone and JH alone, and together, the regulatory effects of each could be elucidated.

Concluding remarks

This study provides evidence for additive genetic variation in basal levels of immune system components in larvae of the noctuid, *Spodoptera littoralis*. Two probable mechanisms for maintaining this variation were identified; antagonistic pleiotropy – the genetic basis of trade-offs, and genotype by environment interactions. Potential trade-offs within the immune system could provide an evolutionary constraint on selection for increased immunity in this species. Moreover, homology between the insect immune system and the innate immune system of vertebrates suggests that this finding could be applicable to a wide range of taxa (though the potential mechanism of hormonal regulation would necessarily differ). Phenotypic plasticity in immune system components and resistance to parasites identified in both *Spodoptera* species, suggests that the costs of maintaining an effective immune system are minimised by tailoring the immune system to the environment. This study highlights the importance of considering immune function in life-history analysis, and of considering both its genetic and phenotypic aspects.

Figure caption: two *S. littoralis* pupae.

Appendices

Appendix 1 – Artificial diet for the *Spodoptera littoralis* culture

Modified from Reeson (1999)

The following ingredients are mixed together and autoclaved:

Wheatgerm	72g
Casein	33g
Sugar	29.25g
Agar	18.75g
Yeast	14.25g
Wesson's salts	9.37g
Sorbic acid	1.5g
Cholesterol	0.93g
P-hydroxybenzoic acid	0.93g
Linseed oil	1.87g
Water	862.5ml

After autoclaving the mixture is cooled to 60°C and the following added:

Vitamin mix	0.093g
Antibiotic mix	5.53g
Choline	0.93g

The mixture is then thoroughly blended and transferred, either to a large tub or into individual polypots, and stored in a refrigerator.

Vitamin mix

Nicotinic acid	5g
Pantothenic acid	5g
Riboflavin	2.5g
Thiamine	1.25g
Pyridoxine	1.25g
Folic acid	1.25g
D-Biotin	0.1g
Cyanocobalamine	0.01g

Antibiotic mix

Streptomycin	25g
L-Ascorbic acid	100g

Appendix 2 – Buffers

Phosphate buffered saline (PBS) – pH 7.4

To make 1 litre:

1.	NaCl	8g
2.	KCl	0.2g
3.	Na_2HPO_4	1.44g
4.	KH_2PO_4	0.24g
5.	HCl	1 M

Add *1-4* to 800 ml of distilled water. Check pH and, if necessary, add *5* a drop at a time until the pH is 7.4, make up to 1 litre with distilled water, autoclave and store at room temperature.

Sodium cacodylate buffer (Na-cac) – pH 6.5

To make 1 litre:

1.	Na-cac	0.01 M
2.	$CaCl_2$	0.005 M

Add *1* & *2* to 1 litre of distilled water, autoclave and store at room temperature.

EDTA anticoagulant in PBS – pH 7.4

To make 100 ml:

1. EDTA 10mM

2. Citric acid 10mM

3. HCl 1 M

Dissolve *1* & *2* in 80 ml of PBS. Check pH and, if necessary, add *3* a drop at a time until the pH is at 7.4, make up to 100ml with PBS and store in a refrigerator.

Potassium phosphate buffer - 0.2M, pH 6.4

To make 100 ml:

1. KH_2PO_4 2.72 g

2. K_2HPO_4 1.74 g

Solution 1 - Dissolve *1* in 100 ml of distilled water. Solution 2 - Dissolve *2* in 50 ml of distilled water. To make the buffer mix 70 ml of solution 1 with 25 ml of solution 2. Check the pH and adjust with solution 2 to pH 6.4. Sterilise by filtering through a 0.2 μm filter and store in a refrigerator.

Appendix 3 – Starvation experiment

This experiment was designed to examine the costs of mounting an immune response under conditions of food limitation. Experiments performed in the laboratory usually supply food *ad libitum*. Under these conditions, costs to immune function may not always be apparent as the experimental animals can increase their food consumption to limit costs – a strategy that may not be possible under natural conditions. However a recent study found that there were survival cost to mounting an immune defence under conditions of food limitation (Moret & Schmid-Hempel 2000). The effects of starvation, haemolymph sampling and immune challenge (insertion of a nylon implant) were assessed independently as described below.

Methods

Larvae were randomly assigned to six treatment groups as shown below:

Group	Fed	Haemolymph sampled	Nylon inserted
1.	Y	N	N
2.	N	N	N
3.	Y	Y	N
4.	N	Y	N
5.	Y	Y	Y
6.	N	Y	Y

All larvae were weighed and those assigned to the two control groups were put into fresh polypots either with (treatment *1*) or without food (treatment *2*). For the remaining larvae, haemolymph was sampled from between the last pair of prolegs, as described previously. Larvae assigned to treatment *3* were then put into polypots with food and larvae assigned to treatment *4* were put into polypots without food. The remaining larvae had a small piece of nylon inserted into the haemocoel as an artificial parasite, and were then placed in polypots with (treatment *5*) or without food (treatment *6*). Each haemolymph sample was divided between three Eppendorfs, as described previously (Chapter 5) and the haemolymph PO, protein, haemocyte density and lysozyme activity quantified.

Survival of all larvae was then monitored daily for the remainder of the experiment and the stage of death recorded. The following life-history traits were recorded for each individual: days spent as larvae, days spent as pupae, sex, pupal weight, adult longevity and female fecundity. Female fecundity was measured as the number of eggs laid over a period of three days.

Figure caption: newly emerged *S. littoralis* moths. The male (top) has pale markings on the top of his wings and small blue patches towards the wing tips. The female (bottom) has a more cryptic coloration.

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