Prophylactic Immunity in the Mealworm Beetle *Tenebrio molitor*

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Prophylactic Immunity in the Mealworm Beetle *Tenebrio molitor*

Andrew I. Barnes

Summary

Immune systems are thought to be costly to maintain and express in a variety of taxa. Evidence for this comes from observations that mechanisms which deal with pathogenic challenge are often extremely variable and are induced in the presence of an immune challenge, rather than being constitutively active. This thesis presents work aimed at testing predictions arising from these ideas, using the mealworm beetle *T. molitor* (Coleoptera: Tenebrionidae) as a model system. Assays were developed which reflected three specific aspects of immunity (haemocyte count, phenoloxidase levels and resistance to a generalist fungal pathogen), thereby allowing aspects of immune function to be quantified. It was shown that the level of fungal resistance differed in beetles reared gregariously (higher resistance) and solitarily (lower resistance). Conspecifics are a source of disease (Freeland 1983), so this is an example of immune defences being induced in situations with a higher risk of pathogenesis (density dependent prophylaxis). A strong predictor of fungal resistance was the degree of melanisation of the adults' cuticles. This trait was shown to be highly heritable (59%), as was the total haemocyte count of an individual: an important aspect of general invertebrate immunity. Selection for cuticular melanisation resulted in a rapid response, confirming the existence of large amounts of additive genetic variance for this trait. Fungal resistance showed a correlated

response to selection for cuticular melanisation, indicating that this too has additive genetic variance. Lines selected for darker cuticles showed higher levels of fungal resistance than those selected for lighter cuticles. Cuticular melanisation and fungal resistance are therefore genetically correlated, and the former can be used as an indicator of the latter in *T. molitor*. No specific costs of cuticular melanisation or fungal resistance were identified. A correlated response to selection for cuticular melanisation on larval competitive ability was investigated, but no such response was seen. Thus the mechanisms maintaining variability and inducibility in cuticular melanisation and fungal resistance are unknown. This thesis has therefore identified patterns of immune expression consistent with the hypothesis that immunity has associated costs, although these costs have not been shown. It has also identified a potentially novel role for cuticular melanisation, as an indicator of immunity to fungal pathogens.

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Chapter 1
Introduction

1.1. An historical view of parasitism

1.1.1. Pre-evolutionary views of parasites and disease

It has long been recognised that associations between small, disease causing biological entities (parasites or pathogens) and the larger organisms that support them (hosts) are common phenomena in nature. The earliest records explicitly linking human disease with parasitism are from c.1500 BC, in this case infection with tapeworms. The strong medical tradition of the Greeks established that parasites were not only common, but that they were ubiquitous, and this led to the erroneous belief that they were spontaneously and abiotically generated (abiogenesis) (Mayr 1982). A *Germ Theory of Disease* (the idea that biological agents too small to see are causative in disease) was proposed in the mid-sixteenth century by Girolamo Fracastoro, and strengthened by van Leeuwenhoek's exposure of the previously unexplored microscopic world (Pelczar et al. 1993). Various forms of the theory circulated for the next three hundred years, provoking controversy and much scepticism, as evidenced by the following popular satirical verse:

So, naturalists observe, a flea
Hath smaller fleas that on him prey;
And these have smaller still to bite 'em;
And so proceed ad infinitum.

(Jonathon Swift)

It was not until the nineteenth century that zoologists began to recognise that parasites were biological entities, and started fitting them into the Linnean classification system (Mayr 1982). Simultaneously, Louis Pasteur was putting the final nails in the coffin of abiogenesis and establishing what we know now as the 'modern' *Germ Theory of Disease*. The role of micro-organisms as causative agents in at least some diseases was established by Pasteur in definitive experiments using the protozoan parasite *Nosema bombysis* and its silkworm host, *Bombyx mori* (Brey & Hultmark, 1998)

1.1.2. The role of parasites in evolution

In *The Origin of Species* Darwin repeatedly refers to the observation that living organisms could act as selective forces in the evolution of others. This he called the effect of 'mutual relations' (Darwin 1859). For the next ninety years, however, mutual relations (now called coevolution) were neglected, with the prevalent view of evolution being one of a process in which an organism struggles to adapt to an environment that does not evolve in response (see e.g. Huxley 1942). Discussions of interactions between species were generally limited to those between predators and prey.

J.B.S. Haldane (1949) was the first author to explicitly state that interactions between parasites and their hosts represent an interesting and unique evolutionary paradigm (Haldane 1949). He noted that the evolutionary interactions were unusual because parasites have the ability to reproduce, and hence evolve, at a faster rate than their hosts. This, together with the ubiquity of potential parasites, means that disease is a major factor affecting host fitness. Parasites, therefore, not only cause the evolution of physiological immune defences, but also affect many other aspects of life history, such as social behaviour, reproduction and dispersal. Haldane also observed that the host

mechanisms directly responsible for interacting with parasites are often extremely variable. He hypothesised that host genotypes rarely encountered by parasites may be advantageous over those that are common in a population. Parasites can thus cause polymorphism and maintain genetic diversity (Haldane 1949; modelled by Gillespie 1975). A few years later this was supported by the finding that the alleles responsible for sickle cell anaemia (a fatal human blood condition) are maintained in human populations in central Africa because they confer resistance to malaria, an infectious disease caused by *Plasmodium spp.*, and a major killer in that part of the world (Allison 1954). This was the first empirical example of an infectious disease maintaining genetic diversity in a host.

Mathematical models suggested that a variety of evolutionary outcomes can arise from the interactions between parasites and hosts: host extinction, parasite extinction, evolution of mutualism and continued coexistence are all possibilities, depending on a number of factors, including host mortality, suitability of the host as a habitat, host immunity and the necessity of transmission (Anderson & May 1979). One possible outcome is the existence of stable cycles of host abundance and prevalence of infection (Anderson & May 1981). These cycles can occur because the prevalent parasite causes selection for rare resistant hosts. These hosts increase in frequency in the population, in turn causing selection for rare parasites which are able to infect them. These rare parasites will then increase in frequency until they become prevalent, and once again cause selection for rare resistant hosts. This cycle has the effect of increasing genetic diversity of hosts and parasites, and means that the alleles controlling mechanisms of both the development of disease (pathogenesis) and resistance have fluctuating fitness optima i.e.

selective advantage will alternate between the alleles available in the population (Hamilton & Zuk 1982).

1.2. Parasites as mediators of sexual selection

More recently, the ideas outlined in section 1.1 have generated interest among evolutionary biologists because of their potential for solving one of the enduring problems of the field, the paradox of the lek (Kirkpatrick & Ryan 1991). When exercising mate choice, one sex (usually females) often choose mates on the basis of exaggerated secondary sexual traits. Frequently, the female receives nothing from her mate other than sperm so the benefits to being choosy are assumed to be genetic (Andersson 1994). However, there is expected to be little or no genetic variation in fitness traits (Fisher 1930), and hence little benefit to choice (Kirkpatrick & Ryan 1991). If secondary sexual traits reflect an organism's condition, however, then choosing relatively elaborate (i.e. more costly) traits will be equivalent to choosing more parasite resistant males. Genetic variation will never be exhausted because of the fluctuating fitness values of alleles involved in parasite resistance (Hamilton & Zuk 1982). Certain hormones (notably testosterone) are responsible for the development of secondary sexual traits and, in some situations, have immunosuppressive effects, resulting in the possibility that such hormones may mediate a trade-off between sexual signals and susceptibility to pathogenesis. This provides a possible mechanistic basis for the Hamilton and Zuk hypothesis and is the basis of the 'Immunocompetence Handicap' theory (see figure 1.1) (Følstad & Karter 1992). Although the immunocompetence handicap theory is an explicitly vertebrate model, the principle that immune function may be traded-off with

other aspects of life history is a potentially important concept for all taxa (see section 1.4.2).

1.3. The invertebrate immune system

1.3.1. Immune systems mediate interactions between parasites and hosts

The above discussion illustrates that potential pathogens provide a ubiquitous fluctuating selection pressure for potential host organisms. The ubiquity and potentially large costs (up to and including death) of pathogenesis, have resulted in the evolution of mechanisms for the minimisation of these costs in the host. These include barriers for the entry of parasites and physicochemical responses against parasites once they are inside the body. These responses are collectively called the immune system, and they are responsible for mediating the costs of interactions between potential pathogens and potential hosts.

Vertebrate and invertebrate immune systems can be treated as effectively distinct systems, because the latter do not employ immunological 'memory', in the form of antibody/antigen interactions, unlike the former. This thesis is concerned only with the invertebrate system, which can be broken up into three conceptually different components: cellular, chemical (humoral) and mechanical (Ratcliffe et al. 1985). These components are not discrete, but interact to form complex and varied immune reactions in which parasites are dealt with broadly by type, with a single stereotypic 'innate' response occurring to each.

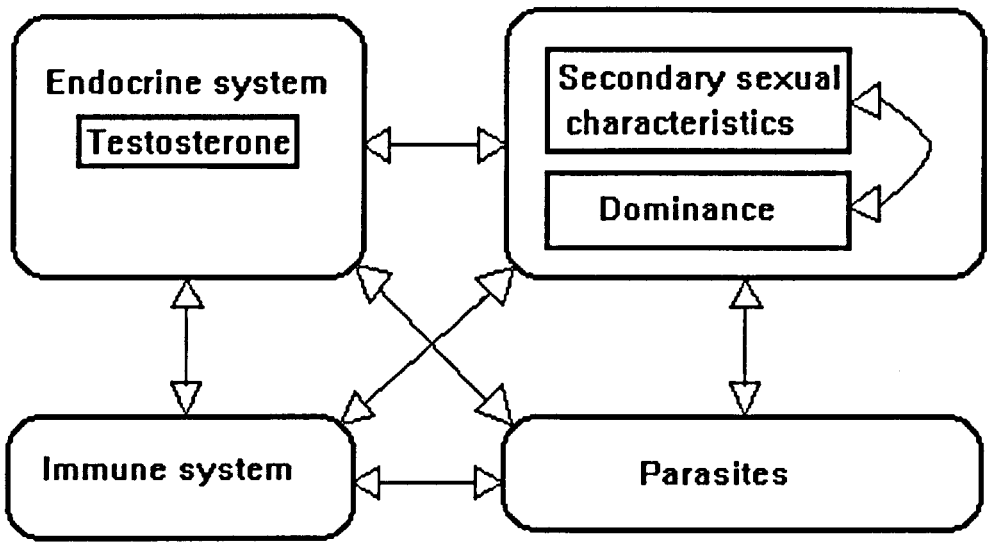

Figure 1.1: (from Folstad & Karter 1992): The model of the immunocompetence handicap theory, as formulated by Folstad and Karter (1992). In this model, sexual signals indicate the ability of their bearer to resist parasites because both secondary sexual characters and parasite resistance are mediated by the endocrine system. Vertebrate hormones (specifically testosterone) are known to promote the development of secondary sexual characters as well as suppressing the immune system. High quality individuals having higher levels of parasite resistance are able to express greater levels of testosterone and have larger secondary sexual traits. Variability in resistance ability is maintained by cyclical interactions between host and parasite genotypes (see section 1.2). Although explicitly a vertebrate model, the concept is applicable to all taxa if both sexual signals and immune systems are costly to express

1.3.2. Mechanical barriers to infection

All organisms employ mechanical barriers which prevent potential agents of pathogenesis entering the haemocoel. These include the exoskeletons, tests and shells found in many invertebrates (Ratcliffe et al. 1985). In adult insects, for instance, the external cuticle is made of cross-linked ('sclerotised') quinones (Chapman 1982), making it relatively tough and impenetrable. Most parasites, with the exception of a few fungi, cannot invade through intact cuticle (Hajek & St. Leger 1994). The external cuticle shows extensive melanisation (Chapman 1982). It is, however, unclear if melanisation is causal in, or correlated to, the thickening of tissues.

1.3.3. The recognition of non-self material in the haemocoel

Mechanical barriers to infection represent the first line of defence against parasites. Once they have entered the haemocoel they are dealt with by the cellular and humoral components of the immune system. 'Non-self' material is partly recognised by the physicochemical properties of surface immunogens expressed by the parasite, specifically the charge and wettability (hydrophobicity) (Lackie 1983). This has been shown by introducing otherwise chemically inert substances (e.g. nylon monofilaments or sepharose beads), which vary with respect to these properties, into the haemocoel and monitoring the host's immune responses. Responses to these substances are due to the same genetic pathway as responses to real parasites (Gorman et al. 1996). The substrate surface properties which elicit immune responses vary from host to host (reviewed in Lackie 1988), but in general the less neutral either property is, the larger the response (Lackie 1983; Weisner 1992). However, it appears that many invertebrates are able to

discriminate with higher precision than this system allows (Ratcliffe et al. 1985), suggesting a model in which more specific mechanisms of recognition are superimposed on the physicochemical method (Ratner & Vinson 1983).

The pathway by which non-self material is recognised and the immune system switched on is controversial, with many classes of substance potentially being involved (Carton & Nappi 1997). Much recent interest has centred on the prophenoloxidase cascade's role in this process (Söderhäll 1982). The cascade is a series of apparently proteolytic reactions culminating in the activation of phenoloxidase (PO) from its zymogen (pro-PO). The first reactions involve pattern recognition molecules capable of recognising pathogenic immunogens (Yoshida et al. 1986) and this, combined with the observation that melanin production is almost ubiquitous in invertebrate cellular immune responses (Nappi & Sugumaran 1993), have lead to speculation that the cascade is a recognition system in itself (Ashida & Brey 1998). The end product of the cascade (PO) is a key enzyme in the production of melanin (Mason 1955; Mason 1965), a pigment associated with both cuticular tanning and immune responses (see section 1.3.4) (Chapman 1982).

What is clear, is that once non-self material has been recognised, the pro-PO cascade is quickly activated. Activation can be due to the protease-mediated breakdown of immunogenic compounds, or by other chemical reagents (Nappi & Sugumaran 1993). Immunogens include β-1,3-glucans (fungal cell wall components) (Söderhäll 1981), lipopolysaccharides (LPS; from bacterial cell walls) and peptidoglycans (membrane proteins) (Söderhäll & Häll 1984). Other activators include chemicals such as lipids (Heyneman & Vercauteren 1968), nitro-cellulose (Brey et al. 1991), detergents and heavy

metals (Lario et al. 1993; Nappi & Sugumaran 1993), or processes such as cell adhesion (Hyun et al. 1999). Interestingly many of the activators of the pro-PO cascade also activate other components of the immune system (Söderhäll 1983; Leonard et al. 1985; Söderhäll et al. 1985a; Lackie 1988; Gillespie et al. 1997), further suggesting that activated PO may be central to the invertebrate immune response.

1.3.4. Cellular immune responses

Once activated, the recognition mechanism is responsible for the release of what have vaguely been termed 'wound factors' (Lackie 1988), which signal the presence of non-self material to the immune system as a whole. Components of the pro-PO cascade can putatively act as wound factors for cellular immune responses (Söderhäll 1982; Lackie 1988). Cellular responses are the main line of defence against pathogens in invertebrates, and involve the action of the cells within the haemocoel, or 'haemocytes' (Ratcliffe et al. 1985). Haemocytes are a group of up to eight different classes of cells (Lackie 1988) distinguishable by their morphology (Söderhäll & Smith 1983; Lackie 1988) and/or their reactivity to monoclonal antibodies (Chain et al. 1992; Mullett et al. 1993; Willott et al. 1994; Strand & Johnson 1996).

There are several stereotyped immune responses involving the haemocytes (following Ratcliffe et al. 1985):

1. *Haemolymph coagulation* – the haemocytes migrate to the site of an external wound and adhere to it to prevent loss of haemocoelic turgor. This is frequently followed by melanin deposition to seal the wound.

2. *Phagocytosis* – this is the primary defence against low level infection of smaller pathogens (viruses, protozoa, bacteria and some fungi). Haemocytes migrate to non-self material and ingest it before subjecting it to lysosomal discharge (Ratcliffe & Walters 1983).

3. *Nodule formation* – large micro-organisms are dealt with by surrounding it with phagocytic haemocytes, isolating the infection from the haemocoel. This is usually followed by melanisation of the nodule (Hoffman 1995). Nodulation is the most important defence against bacterial pathogens (Miller et al. 1999).

4. *Entrapment by sinus lining cells* – haemocytes fixed into the haemocoel wall remove non-self material from circulation and ingest it.

5. *Encapsulation* – this is the chief defence against metazoan parasites, although it can be used against cells infected with viruses (Washburn et al. 1996). Non-self material is surrounded by a multicellular sheath of haemocytes. The cells become flattened, releasing their contents into the centre of the sheath, which is isolated from the haemolymph. The capsule usually becomes melanised (Hoffman 1995). Some Diptera form a capsule which consists entirely of a melanin sheath apparently formed independently of the haemocytes (Götz 1986).

6. *Cytotoxicity* – toxic chemicals are released by the haemocytes which lyse the target cell's membrane.

The mechanisms by which pathogens are killed within these cellular responses are not entirely clear. Physical isolation from the haemolymph may cut off the nutrient supply, with melanisation of the capsules increasing their impermeability, effectively 'starving' encapsulated pathogens. Thicker capsules appear to show greater success in

killing pathogens (Kuris et al. 1980; Ennesser & Nappi 1984). However, it seems likely that cellular responses are invariably accompanied by cytotoxic reactions (Lackie 1988) which involve the production of quinoids, reactive oxygen species (H_2O_2 and •OH; Nappi & Vass 1993) and superoxides (O_2-; Tôru 1994) by the haemocytes (Söderhäll et al. 1985b). These substances disrupt the cellular integrity of parasites, and are produced as intermediates during the conversion of tyrosine to melanin, the reaction mediated by products of the pro-PO cascade (Nappi et al. 1995). Additional anti-microbial activity is provided by the humoral immune system, which consists of a series of highly conserved peptides synthesized by the fat body and released into the haemolymph (Hoffman 1995), although many proteins become associated with haemocytes (Wang et al. 1995, Gillespie 1997). These peptides act synergistically with cellular responses and are probably activated by the same recognition mechanism (Ashida & Brey 1998).

1.3.5. Haemocytes and the pro-PO cascade are central to invertebrate immunity

The pro-PO cascade and the haemocytes are key components of the invertebrate immune response. Activated PO is involved in the formation of mechanical barriers to invasion such as the cuticle and midgut. The enzyme cascade that produces active PO may be responsible for the activation of the immune response as a whole, and the conversion of tyrosine to melanin produces many cytotoxic compounds capable of killing micro-organisms. The haemocytes, meanwhile, are the chief medium of the immune response, carrying out a range of actions aimed at isolating pathogens. In addition, haemocytes synthesize much of the active PO, both for building integumental defences

(Ashida & Brey 1995), and for melanisation reactions during immune responses (Söderhäll et al. 1985b; Ashida & Brey 1998).

1.4. Phenotypic plasticity and immunity

1.4.1. The inducibility of immune defences

The high costs associated with parasitism mean that immune defences are important mediators of fitness. Thus it might be expected that the immune system is maintained at a constitutively high level of expression to minimise the potential costs of pathogenesis. What is actually observed, however, is that immune responses are inducible: i.e. they are only expressed when the threat of pathogenesis is high. Many aspects of the immune response (including the pro-PO cascade and cellular reactions) are activated only when non-self immunogens from potential pathogens are encountered within the haemocoel (Johansson & Söderhäll 1989). Haemocyte populations increase exponentially in the presence of parasites (Sequeira et al. 1996), or even if the cuticle is breached, providing a potential entrance point for parasites (Nayar & Knight 1995). Specific responses such as phagocytosis, coagulation, nodulation and encapsulation all occur in response to the presence of signal molecules expressed in response to the encounter of non-self material (Ratner & Vinson 1983; Miller et al. 1999). Induction of the expression of genes controlling humoral components of the immune response is also seen after exposure to dead parasites or parasite components (Hultmark 1993; Hoffman 1995; Wang et al. 1995; Nicolas et al. 1996). The cuticle itself can become thickened at sites of attempted invasion (Hajek & St. Leger 1994). Furthermore, the levels of

resistance to parasites can change as the risk of pathogenesis changes, e.g. due to fluctuations in population density (Wilson & Reeson 1998).

Another way of saying this is that the expression of immune defences in invertebrates shows a high degree of phenotypic plasticity, i.e. a single genotype can be induced to produce a range of phenotypes under different environmental conditions (Stearns 1992). Phenotypic changes occur in a host in response to cues associated with biotic agents, which often reduce the subsequent effects these agents are able to have on that host. Immune responses are thus 'inducible defences' (Harvell & Tollrian 1999). Harvell and Tollrian (1999) list four prerequisites for the evolution of inducible defences:

1) The selection pressure of the inducing agent is variable and unpredictable, but sometimes strong
2) A cue which reliably signals the proximity of a threat is available.
3) The defence is effective
4) The inducibility is cost saving, i.e. the expression of the defence in the absence of a threat incurs a significant fitness cost. Without this cost then the trait will become fixed in the genome.

1.4.2. Immune systems are potentially costly

There has been much empirical and theoretical work which suggests that both invertebrate and vertebrate immune systems are costly to maintain and their responses costly to produce (Gemmill & Read 1998). The innate nature of invertebrate immunity, and the high conservation of the responses within this group, means that identifying costs of immunity may be more tractable in invertebrates. Potential costs have been identified

at a proximate biochemical level as well as in terms trade-offs with other life history traits. It can, however, be difficult to tease the cost of immunity apart from costs of pathogenesis.

One potential cost of immunity is the misdirection of a host response against self, rather than non-self, tissues ('autoimmunity'). The open circulatory system of invertebrates means that any parasite killing mechanism has the potential to harm the host expressing it. Activated invertebrate immune systems generate reactive oxygen species (ROS), both as cytotoxic agents against parasites and as a by-product of melanin production (Nappi & Vass 1993). Excess is mopped up by enzymes such as superoxide dismutase but overproduction allows ROS to linger in the haemocoel, where it can block further melanin production (Napolitano et al. 1996), produce cytotoxic quinoids (Nappi & Vass 1996) and disrupt cellular integrity ('oxidative stress'; von Schantz et al. 1999).

Stress proteins, such as the heat shock family of proteins (HSPs) have long been known to be costly in vertebrate immune responses because of their ability to trigger autoimmune diseases (Feige & Mollenhauer 1992). These proteins act to protect the molecular integrity of cellular proteins when the cell as a whole is under high stress (e.g. at physiologically intolerable temperatures). However, pathogen HSPs often act as antigens in vertebrate hosts and can trigger an immune response. The high degree of conservation among HSPs means that there is potential for cross-reactivity and autoimmunity (Råberg et al. 1998). There are large costs associated with HSP expression in invertebrates (Krebs & Bettencourt 1999), and expression can result from the loss of cellular integrity, such as that which occurs in haemocytes during an encapsulation response (Feige & Mollenhauer 1992). If invertebrate immune responses cause a rise in

the haemocoelic levels of host HSPs then it is possible that they may also be causal in invertebrate autoimmunity (Coleman et al. 1995).

As well as the potential to do damage at the biochemical level, the expression of immune function has also been observed to be traded-off with other life-history traits in invertebrates. Decreased encapsulation of artificial substrates has been found in two insect taxa in response to energetic stresses. Bumble bees allowed to forage show lower encapsulation responses than bees remaining in the nest (König & Schmidt-Hempel 1995) and damselflies show a similarly reduced response after copulation and oviposition (Siva-Jothy et al. 1998). A trade-off between predator avoidance and immune defence has also been demonstrated in freshwater snails (Rigby & Jokela 2000). The simultaneous expression of many genes is energetically expensive (Harshman & James 1998) and may deplete the host's energy reserves: such reserves can be important in determining life history strategies in insects (Plaistow & Siva-Jothy 1996). Mosquitoes take significantly longer to oviposit and show slower ovarian development when infected with parasites, possibly because both resistance mechanisms and egg production require large amounts of tyrosine (Ferdig et al. 1993). Tyrosine is a precursor to melanin, and is thus used in cuticular tanning during all life stages, for immune responses and egg chorion formation. Although tyrosine has not been demonstrated to be a limiting resource, it can only be synthesized from phenylalanine, an essential amino acid (Chapman 1982). Therefore, because tyrosine can only be derived from a limited number of external sources and has important metabolic roles, its usage is a potential cost of immunity.

The above examples are all phenotypic trade-offs. Trade-offs between immune function and other life history traits have been demonstrated on the genotypic level by

laboratory selection experiments. In the best studied insect system, *Drosophila*, selection for increased encapsulation of an endoparasite causes a decrease in the competitive ability of larvae (Kraaijeveld & Godfray 1997), probably due to a decrease in feeding rate (Fellowes et al. 1998). Selection for viral resistance in Indian meal moths caused an increase in larval period, a reduction in egg viability and an increase in pupal weight (Boots & Begon 1993). In the meal moth study, a two-fold increase in resistance resulted in an estimated drop in fitness of about 15% in the absence of parasites.

The downregulation of immune function may be adaptive for a number of reasons. The above examples assume that immune suppression allows resources to be reallocated to other metabolic demands. This resource allocation hypothesis (suggested by Wedekind and Følstad; 1994), assumes that there are considerable amounts of resources directed into immune responses. Care must be taken, however, because apparent suppression of immunity, as suggested by assays of single aspects of an immune response, may be misleading if resources have been redirected into other areas that are involved in parasite resistance that have not been assayed (the 'immunoredistribution hypothesis'; Braude et al. 1999). Thus immune responsiveness as a whole may be maintained while its individual components fluctuate in expression. Finally, it has been suggested that under conditions of stress, the immune system is more likely to be overexpressed and the costs mentioned above (autoimmunity, etc.) be incurred, and that suppression may be adaptive in counteracting this (Råberg et al. 1998).

1.5. Synopsis of the introduction

Pathogenesis is one of the most significant selection pressures encountered by metazoan organisms during their lives. The threat of pathogenesis is ever present and infection can reduce fitness to the point of killing the host. This threat has resulted in the evolution of complex mechanisms for the avoidance of paying these fitness costs. However, the expression of these mechanisms can itself incur a cost, possibly due to using up valuable resources or due to the production of harmful substances. Because of this, immune systems are rarely constitutively active, but are usually induced into activity by the threat of pathogenesis. Thus in order to optimise the costs and benefits of immunity, opposing selection pressures must be balanced. Underexpression of immunity can lead to loss of fitness due to the costs of pathogenesis, whereas overexpression can lead to loss of fitness due to the costs of immunity

1.6. The study organism: *Tenebrio molitor*

This thesis investigates aspects of the patterns of investment in immunity in the mealworm beetle *T. molitor* (Coleoptera: Tenebrionidae) (figure 1.2). Tenebrionids are economically important pests, living in and feeding on stored grain. They are hosts for specific metazoan parasites (Hurd 1996), as well as being susceptible to a range of generalist entomopathogens (e.g. Steinkraus et al. 1991; Beeman et al. 1992; Geden et al. 1998). Their short generation time (c. 2 months), ease of culturing and relatively large

size makes them amenable to laboratory studies. Tenebrionids also show plasticity in their life histories, undergoing variable number of larval moults, depending on the environment (Weaver & Macfarlane 1990). They are usually maintained at high population densities and are facultatively cannibalistic, suggesting that disease may be an important factor in their evolution (Steinhaus 1958; Pfennig et al. 1998).

1.7. Outline of the thesis

Chapter 1 outlines the growing importance of studies of immune function in evolutionary biology, and introduces the unique paradigm that is the interaction between parasites and hosts. It also demonstrates that immune expression is not at a constitutive maximum, but is induced when it is required, and that this may be due to associated costs of expression.

Chapter 2 reports the development of four assays of components of immunity in *T. molitor*, and discusses their biological significance, their relevance to studies of the ideas of Hamilton and Zuk (1982), and Følstad and Karter (1992) (the field known as 'ecological immunology'), and in particular their relationship to ideas about 'immunocompetence'.

Chapter 3 tests the idea that investment in immunity is increased as the risk of pathogenesis increases. Specifically, investment in immunity is looked at in beetles reared at two different rearing densities, with the prediction that those reared at higher densities should show greater resistance to a generalist entomopathogen. Correlations

between resistance and an immunological trait (phenoloxidase levels) and a morphological trait (cuticular melanisation) are also studied.

Chapter 4 investigates the quantitative genetics of cuticular melanisation. Its heritability is assessed in order to quantify the amount of additive genetic variation in the population for this putative immune marker, and by implication, the amount of additive genetic variation in fungal resistance. The existence of genetic correlations between cuticular melanisation and another trait involved in immunity, total haemocyte count, are investigated, and findings discussed in the light of ideas about definitions of immunocompetence.

The relationship between cuticular melanisation and fungal immunity investigated in chapter 3 is a phenotypic correlation. **Chapter 5** attempts to show that there is a genetic correlation between cuticular melanisation and fungal resistance, thus indicating that the former is a reliable indicator of the latter. A genetic correlation between cuticular melanisation and another life history trait (larval competitive ability) is investigated in order to elucidate the causes of maintenance of the additive genetic variation indicated in **chapter 4**.

Chapter 6 synthesises the ideas investigated in the thesis, and discusses them in terms of current thinking on ecological immunity and immunocompetence, as well as suggests areas for future research suggested by the findings of the thesis.

Figure 1.2: The adult mealworm beetle *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae).

Chapter 2
Assays of Immune Function

2.1. Introduction

2.1.1. Problems with the definition and measurement of immunocompetence

Testing ideas such as Følstad and Karter's (1992) and Hamilton and Zuk's (1982), requires that differences between individuals in their levels of immunity are quantifiable. Likewise, to quantify costs of immunity it is necessary that immunity is quantifiable. However, the concept that 'immunity' is a simple measurable trait, is controversial. The recent interest in immunity amongst evolutionary biologists caused by the 'Immunocompetence Handicap Theory' (Følstad & Karter 1992) has provoked much debate about what, if anything, it means to be 'immunocompetent', and how this can be assayed.

Attempts to define immunocompetence have been heavily confounded by the need to make it testable. The term has been used in an immunological sense to mean the ability of the immune system to respond to pathogenesis (Roitt 1988; Gillespie et al. 1997). This is the definition that Folstad and Karter (1992) used in their original paper, although this is not explicitly stated. However, studies of ecological immunity require that variability between individuals in immune investment is assayable, and this has led to a definition of immunocompetence as being the relative ability of different individuals

in a population to respond to pathogens (Sheldon & Verhulst 1996). Tests of this definition have tended to measure single immunological characters, with the assumption that high values for single characters reflect high immunocompetence. The validity of this is questionable, because few studies ever relate immunology to the ability to resist pathogens, and a rationale linking the assay and immunocompetence is often not provided (Owens & Wilson 1999). Also, the complexity of immune systems means that resources may not be invested in immunity *en masse*, but may be distributed to different aspects of the immune system (Braude et al. 1999). The need to link immunology to resistance before immunocompetence can be quantified has been pointed out by several authors (Siva-Jothy 1995; Skarstein 1996; Svensson & Skarstein 1997). Attempts to do this, e.g. by using a battery of immune assays to assess immunocompetence (Lochmiller 1995), are not practical for use by empiricists.

Most organism are typically challenged by a variety of pathogen types. Insects can act as hosts for viruses, fungi, bacteria, nematodes, eggs of other insects (parasitoids) and a variety of other challenges (Ratcliffe et al. 1985). These are often dealt with by different stereotypical responses of the immune system (see section 1.3). Hence observations of high levels of resistance to one pathogen type (e.g. a nematode) may say little about resistance to another (e.g. a virus). Variation in immunity may exist both between individuals and within the immune system of a single individual. Thus immunocompetence as defined above (*the ability to respond to pathogens*) may not exist as a single, simple, measurable life history trait.

2.1.2. Aims of the chapter

In this chapter a number a number of assays of aspects of immune function are developed for *T. molitor*. These were chosen because of their potential to reflect the ability to resist actual pathogens (discussed separately in the sections listed below) and their apparent simplicity. The assays developed are:

1) The total number of haemocytes contained within the haemocoel (section 2.4).

2) The total activity of phenoloxidase (PO) within the haemocoel (section 2.5).

3) Resistance to a generalist entomopathogenic fungus, *Metarhizium anisopliae* (section 2.6).

These assays are not designed to measure immunocompetence *per se*. Instead they measure specific aspects of the immune system of *T. molitor*, and are discussed with specific reference to their biological relevance, and their role in the stereotypical immune responses seen in insects.

2.2. Beetle culturing

Beetles were reared at the University of Sheffield. They were maintained at 26°C and were kept in plastic tanks in a substrate of rat chow (Special Diet Services), which also served as their primary dietary source. This consisted of 77% cereal (wheat, maize, barley, wheatfeed), 15% vegetable proteins (soya bean meal), 5% animal protein (fish meal) and 3% vitamins, minerals (major and trace) and amino acids. *T. molitor* absorb liquid from the atmosphere, but this was supplemented with additions of lettuce or apple chunks, approximately biweekly. Original sources of cultures included the Sheffield

Mealworm Company, Blades Biological Supplies, Leeds University, Arizona University and Sheffield University stock cultures. Both stock populations (a large outbred population maintained for several years) and inbred isofemale lines (maintained for at least four years) are used in this thesis.

2.3. Total haemolymph extraction

2.3.1. Total haemolymph extraction - Introduction

In order to assay components of haemocoelic immune function it is necessary to standardise the amount of haemolymph being extracted from each beetle. However, collection of a small amount of haemolymph exuded from a wound was not possible, partly due to the small size of the insects, and partly due to the high viscosity of the fluid within the haemocoel (Wigglesworth 1972). Since non-destructive standardised sampling was difficult, the haemolymph was collected by washing the abdomen through with a buffer and collecting the perfused liquid. Using this method, it is not possible to quantify the volume of haemolymph collected. Thus in order to standardise the bleeds I elected to collect all of the haemolymph within the haemocoel. To quantify the amount of buffer needed to wash the entirety of the haemolymph from the haemocoel, a series of sequential bleeds were performed and the number of haemocytes collected in each were counted. These were then used as indicators of the relative amount of total haemolymph collected in each bleed.

2.3.2. Total haemolymph extraction - Materials and Methods

Adult beetles were collected at random from the stock population. They were chilled on ice for approximately ten minutes to immobilise them. They were then injected with chilled anti-coagulant (146mM NaOH, 216 mM NaCl, 25mM EDTA, 61 mM citric acid, pH = 4.5, mOs/Kg = 550) in the posterior opening of the terminal sclerites using a 1ml disposable syringe (Becton-Dickinson) until their abdomen became swollen and their genitals were extruded. This took approximately 10µl of solution. The extruded genitals were then severed with a pair of bowspring scissors to create a hole out of which haemolymph was collected. The beetles were injected with anticoagulant anteriorly through the first abdominal sclerite using a 5ml disposable syringe (Becton-Dickinson) and the perfused liquid was collected through the posterior hole into disposable tubes. Each beetle had a total of 10ml injected and collected, with each 1ml of extract being collected into separately labelled 1.5ml centrifuge tubes. Beetles, tubes and solutions were kept on ice throughout. The haemocytes were pelleted out by centrifugation of the collected liquid (800 G, 6 minutes, 4°C). The supernatants were discarded and the pelleted cells were resuspended by adding 200 µl of anticoagulant and vortexing. This was done in order to give a higher concentration of cells for counting.

30 µl of the suspension was used for cell counts. Counts were done on multiwell slides (Hendley-Essex). This enabled samples from all ten extracts from a single beetle to be plated out simultaneously, to minimise cell coagulation, which can occasionally be observed even in anticoagulant and on ice. A count of the total number of cells obtained in each 30µl sample was made using phase contrast microscopy at x40 magnification (Leitz Diaplan).

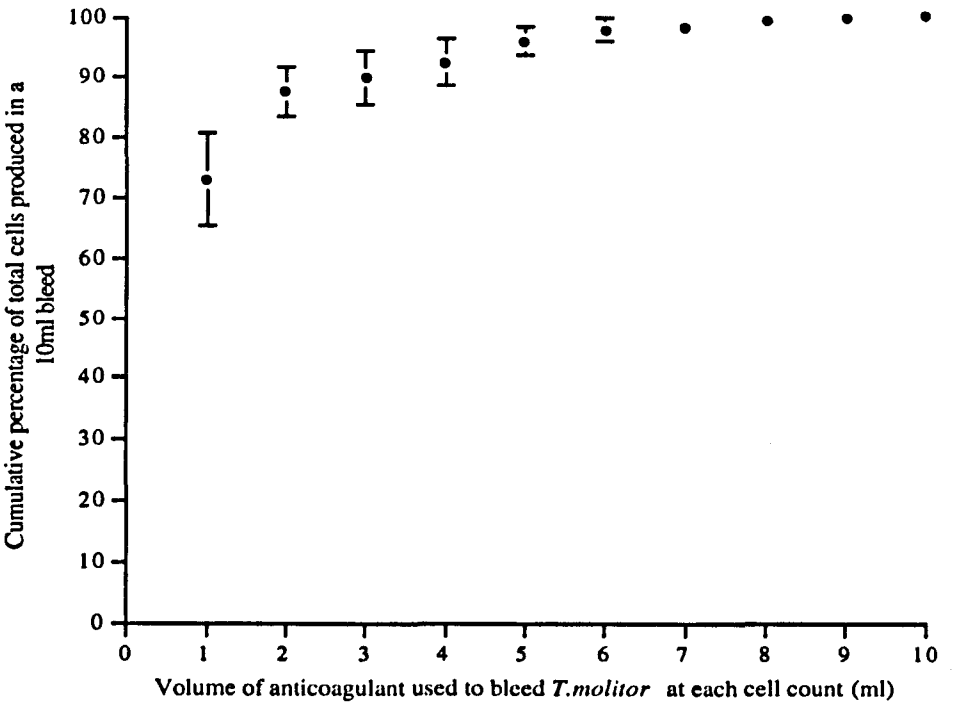

Figure 2.1: The percentage of the total number of haemocytes (i.e. the number of cells produced by washing the abdomen of *T.molitor* with 10ml of anticoagulant) produced by sequential 1ml washes. Error bars are 1 standard deviation and n=10 at each point (see section 2.3.)

2.3.3. Total haemolymph extraction - Results

The absolute number of cells obtained in 10ml of extract varied considerably between samples, from 188 to 10,020 (mean = 1848±320 s.d.). However, the pattern with which cells were obtained was remarkably constant over the ten replicates. Figure 2.1 shows the number of cells obtained in each sequential bleed as a cumulative proportion of the total 10ml bleed. The first two 1ml bleeds cumulatively produced 87±5% of the total haemocytes produced in 10ml. By the 10^{th} bleed less than 0.4% of the total cell number was being produced, and in 5 of the 10 replicates no cells at all were counted at this stage.

2.3.4. Total haemolymph extraction - Conclusions

The results suggests that the first 2 washes with anticoagulant (i.e. 2ml) produced a consistently high proportion (about 90%) of the total number of haemocytes produced by 10 washes. The 10^{th} wash produced less than 1% of the total cells counted, and probably represents the point by which almost all of the cells have been collected. If the number of haemocytes collected directly reflects the volume of haemolymph collected then it would appear that most of the haemolymph can be collected by washing the abdomen through with 2ml of buffer. The low standard deviation of this value over the ten individuals sampled reflects a high repeatability of the proportion of cells collected in this volume. Moreover, the number of haemocytes collected probably represents a conservative estimate of the total volume of haemolymph, because many haemocytes adhere to the lining of the haemocoel and are not free within the haemolymph (Lackie 1988). Thus by washing the haemocoel through with 2ml of buffer, a consistent

approximation of the totality of the free haemocytes in the haemolymph of *T. molitor* can be made.

2.4. Total haemocyte counts

2.4.1. Total haemocyte counts - Introduction

Haemocytes play a central role in the invertebrate immune response, and are involved in resistance to a wide variety of pathogens (see section 1.3.4). They facilitate phagocytosis, nodulation and encapsulation, and these are the responses which deal with viruses (Washburn et al. 1996), bacteria, fungi and all metazoan parasites in most insect taxa (Ratcliffe et al. 1985). The number of circulating haemocytes has been shown to positively correlate with resistance to a parasitoid in *Drosophila melanogaster* (Eslin & Prévost 1996; Eslin & Prévost 1998), and in one invertebrate, the shore crab *Hemigraspus oregonensis*, the thickness of the capsule formed against metazoan pathogens was shown to determine resistance ability. Capsule thickness is a function of the number of haemocytes recruited (Kuris et al. 1980). Hence the number of haemocytes may be an important determinant of the efficacy of a wide range of immune responses.

2.4.2. Total haemocyte counts - Materials and Methods

Collection of haemolymph was performed as described in section 2.3, with 2ml of anticoagulant being washed through the abdomen. To perform haemocyte counts, however, the extracts were not subject to centrifugation. The crude extract was transferred to a haemocytometer (Weber Scientific) and counts taken from three non-

adjacent areas (1x1x0.1 mm each). The total haemocyte count was calculated as being the mean of the three readings scaled up from the size of a single counted volume ($0.1mm^3$) to the total 2ml bleed. This was used as an estimate of the total number of haemocytes present within an individual beetle.

Section 2.4.3. Total haemocyte counts – Repeatability

It is not possible to take repeated haemolymph samples from beetles because the sampling method used is destructive. It is possible, however, to take repeated readings from collected samples and test the repeatability of counting cells. This was done by taking haemolymph samples (as section 2.4.2) from 100 individuals taken at random from the stock cultures. Three subsamples were pipetted out onto separate haemocytometers (Weber Scientific), and counts were performed as above. Rather than taking the mean of the three counts they were not combined. Repeatabilities were calculated as described by Lessels and Boag (1987) using individual number as a factor and haemocyte count as a variable. There is a significant effect of within individual effect on haemocyte count ($df = 99$, $F = 14.411$, $p < 0.0001$) and the repeatability of counts within individual samples was 82%.

2.4.4. Total haemocyte counts - Conclusions

By collecting the totality of the haemolymph from a beetle's abdomen in anticoagulant, it is possible to assay the number of haemocytes within this sample in a significantly repeatable way. Only 18% of the total variation in cells counted was attributable to sampling and measurement error, whilst the remaining 82% was

partitioned into differences between individuals. This indicates that cells are not coagulating in small areas of the extracted sample, but that their distribution is fairly uniform throughout it.

Because sampling is destructive, it is not possible to test the repeatability of the sampling procedure within an individual. However, spatial repeatability should not be a problem because haemocytes are counted from a total bleed, not from samples taken from specific areas of the haemocoel. The temporal repeatability of sampling is also not possible to assess.

2.5. Assaying phenoloxidase

2.5.1. Assaying phenoloxidase - Introduction

PO is thought to be fundamental in a wide range of invertebrate immune reactions (see section 1.3). The pro-PO activating system has been implicated as a potential mechanism for the recognition of non-self material, with PO being activated as a result of recognition (Ashida & Brey 1998). Wounds (such as those created by a parasitoid's ovipositor) are repaired by the deposition of melanin (Nayar & Knight 1995), the product of PO catalysis, and melanisation is intimately associated with capsule formation and with the killing mechanism of cellular immune responses (Nappi et al. 1995). PO is hence an important immunological parameter. Higher levels of PO correlate with baculovirus resistance in the moth *Spodoptera exempta* (Reeson et al. 1998). In general, however, the relationship between absolute levels of PO and resistance has not been determined.

Phenoloxidase is assayed by monitoring the conversion of a melanin precursor (L-DOPA) to melanin, which can be observed as a colour change from clear to brown and quantified as a change in optical density using spectrophotometry. In order that this colour change reflects the amount of active enzyme present, it is necessary that the reaction being carried out is limited by nothing other than the amount of enzyme present, and the relationship between the rate of product formation and enzyme activity must be shown. To do this it is necessary to show the following:

a) The reaction is working at its maximum velocity (V_{max}) during the assay: At V_{max} all the available enzymatic sites are saturated with substrate. This ensures that the amount of enzyme, not the substrate concentration, limits the reaction.

b) The rate of product formation is linear with time: Enzyme activity must be measured while the change in optical density is linear with respect to time. When this is not the case there is no longer enough substrate to saturate the enzymatic sites.

c) The rate of conversion of the substrate into the product increases linearly with the amount of enzyme used: If the amount of product formed is linearly related to the amount of enzyme present (i.e. haemolymph extract), then quantifying product formation is equivalent to quantifying enzyme levels.

2.5.2. Assaying phenoloxidase - Finding V_{max}

2ml of haemolymph was extracted from 20 beetles, collected from the stock populations, using the method described above (section 2.3), using sodium cacodylate/$CaCl_2$ buffer (0.01M Na-coc, 0.005M $CaCl_2$) rather than anticoagulant. The extract was immediately frozen in liquid nitrogen, thawed and vortexed. The cellular

debris was pelleted by centrifugation (2800G, 15 minutes, 4°C). The resulting supernatants from the 20 beetles were pooled. 2ml aliquots of this pooled extract were added to 4ml of one of the substrate solutions, which were L-DOPA solutions (L-DOPA in Na-coc/$CaCl_2$ buffer) of between 0 and 15 mM. These were placed in a 30°C water bath for 20 minutes. After this time the absorbance of the sample at 490 nm was measured in a spectrophotometer (Guilford instruments 300-N), and enzyme activity expressed simply as the difference in optical density between the sample and the control (i.e. the sample run with a 0mM L-DOPA solution).

The values obtained were plotted on a Lineweaver-Burk plot (figure 2.2), and from this both Vmax and the Michaelis constant (K_m) of the reaction were calculated. The x intercept is equal to $-1/K_m$ and the y intercept represents 1/Vmax. This yields the following results:

$K_m = 9.11 \times 10^{-4}$ M

Vmax = 0.870 enzyme units

$Vmax = 2K_m = 1.822$ mM

2.5.3. Assaying phenoloxidase - Linearity with time

2ml extracts were taken from four beetles and not pooled. Each 2ml extract was assayed with 3mM L-DOPA, as described above, every 5 minutes over a period 3 hours. The resulting plots (figure 2.3 - graph only shows the first 60 minutes of assay, as the reaction continued in a similar pattern after this) all appear to be fairly steep at the fifteen minute mark, making this a good point to measure activity. A regression was done for each beetle for the first 5 readings (i.e. up to the twenty minute mark) and all four

recordings showed significant linearity with time.(r^2 = 0.955, p = .004; r^2 = 0.855, p = 0.024; r^2 = 0.982, p = 0.001; r^2 = 0.992, p < 0.001).

2.5.4. Assaying phenoloxidase - Linearity with extract

Haemolymph from 20 beetles was extracted and pooled using the same protocol as section 2.5.2. Aliquots of the extract were serially diluted with Na-coc/$CaCl_2$ buffer, so that a series of haemolymph solutions from 100% (2ml of extract) down to 0% (2ml of buffer, no extract added) were produced at intervals of 5%. To these was added 4ml of L-DOPA in Na-coc/$CaCl_2$ at a concentration of 10 mM, and they were placed in a 30°C waterbath for 20 minutes, before being read in a spectrophotometer as above (section 2.5.3), with the 0% extract serving as a control. Figure 2.4 shows the increase in enzyme activity as the concentration of extract is increased. This increase is linear and significant (r^2 = 0.985, p = 0.0001).

2.5.5. Assaying phenoloxidase - Repeatability

Sections 2.5.2 to 2.5.4 show that a measurement of PO activity within the first 15 minutes of reaction and using 4ml of L-DOPA solution of over 1.82 mM as substrate will accurately reflect the amount of enzyme present. In this section the repeatability of two measurements (a single point measurement of optical density at 15 minutes and a measure of the slope of reaction between 5 and 15 minutes) are calculated to see which gives the most consistent results. As with the haemocyte counts, it is not possible to test the repeatability of PO measurements within individual beetles, because the haemolymph

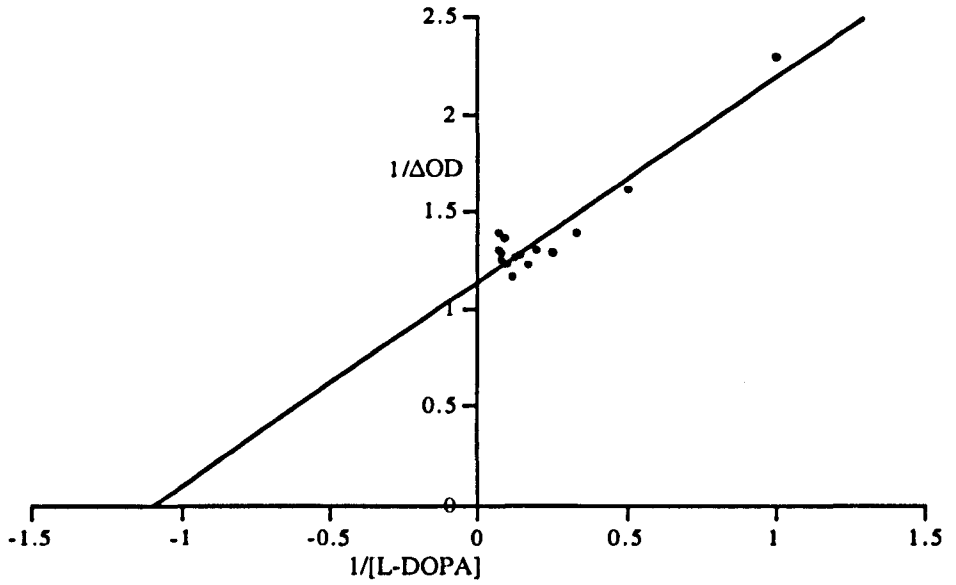

Figure 2.2: Lineweaver-Burk plot for the haemolymph phenoloxidase activity of *T.molitor*. Substrate concentrations ([L-DOPA]) were between 0 and 15mM at 1mM intervals (see section 2.4.2). The fitted line shows a significant positive relationship between substrate concentration and the rate of product formation (measured as a change in optical density in a spectrophotometer) ($r^2 = 0.891$, $p < 0.001$). See section 2.5.2 for calculations of V_{max}.

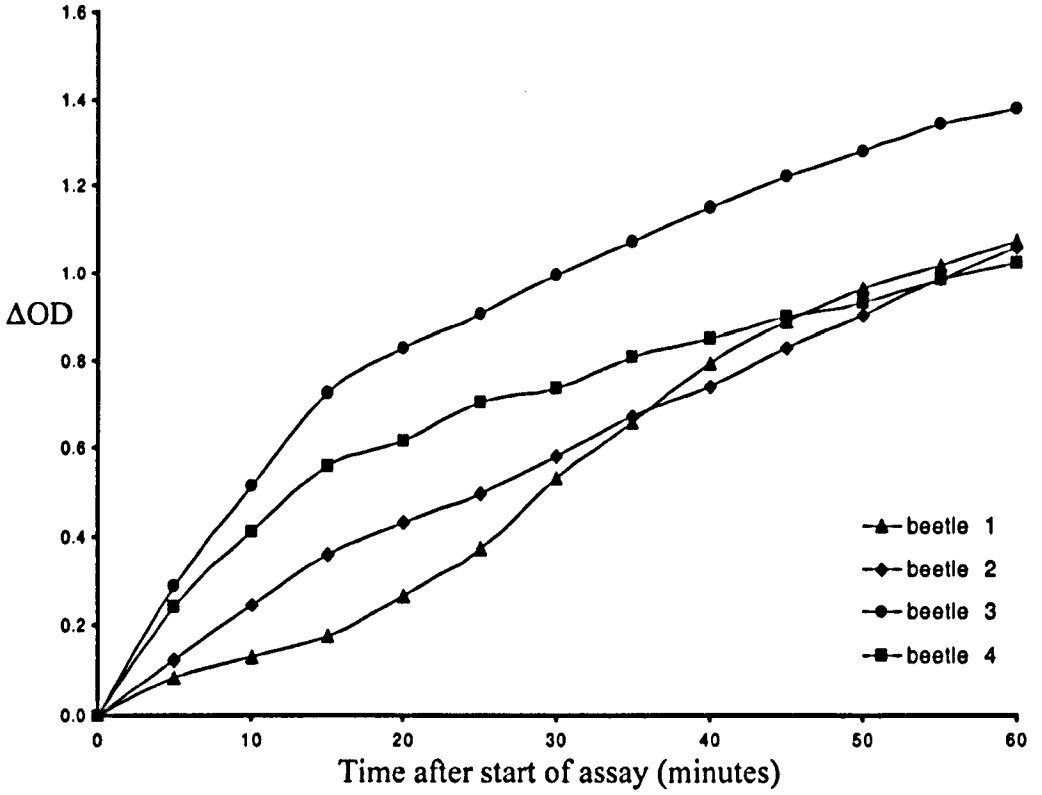

Figure 2.3: The activity of haemolymph phenoloxidase (measured as a change in optical density caused by the conversion of L-DOPA to melanin) over the first 60 minutes of reaction for four beetles. In order to measure phenoloxidase activity accurately, it is necessary to assay it while the rate of product formation is linearly related to time. This relationship was found during the first twenty minutes of reaction (see section 2.5.3).

extraction technique is destructive. It is still possible to test the repeatability of multiple measurements of a single extract.

20 Beetles were taken from the stock cultures and chilled on ice for 3 minutes before being bled with 2ml Na-coc buffer, as described above. Two 500µl aliquots were taken and transferred to separate centrifuge tubes. These were processed and analysed as in section 2.5.4., except that the Ultraspec 2000 spectrophotometer was used (Pharmacia Biotech) and data automatically recorded on Swift II software after every minute of the assay. Thus 2 readings per beetle per measurement (slope or point) were obtained. Repeatabilities were calculated from F-ratios using the formulae of Lessels and Boag (1987).

There was a significant effect of individual on both the slope and a point reading of the PO assay profile ($p < 0.0001$ in both cases). Repeatabilities were higher for slope readings ($r = 0.93$, $F = 29.449$) than for point readings ($r = 0.87$, $F = 13.010$).

2.5.6. Assaying phenoloxidase - Conclusions

It is possible to assay the levels of PO in *T. molitor* using spectrographic techniques. The conversion of L-DOPA to melanin by phenoloxidase contained within total haemolymph extracts of *T. molitor* proceeds at a maximum rate at L-DOPA concentrations above 1.82 mM. The reaction proceeds linearly with respect to time for at least the first 15 minutes of assay. Within this time the rate of conversion of L-DOPA to melanin is linearly related to the amount of enzyme present. Measurements of the slope of the increase in optical density between 5 and 15 minutes after the start of the reaction

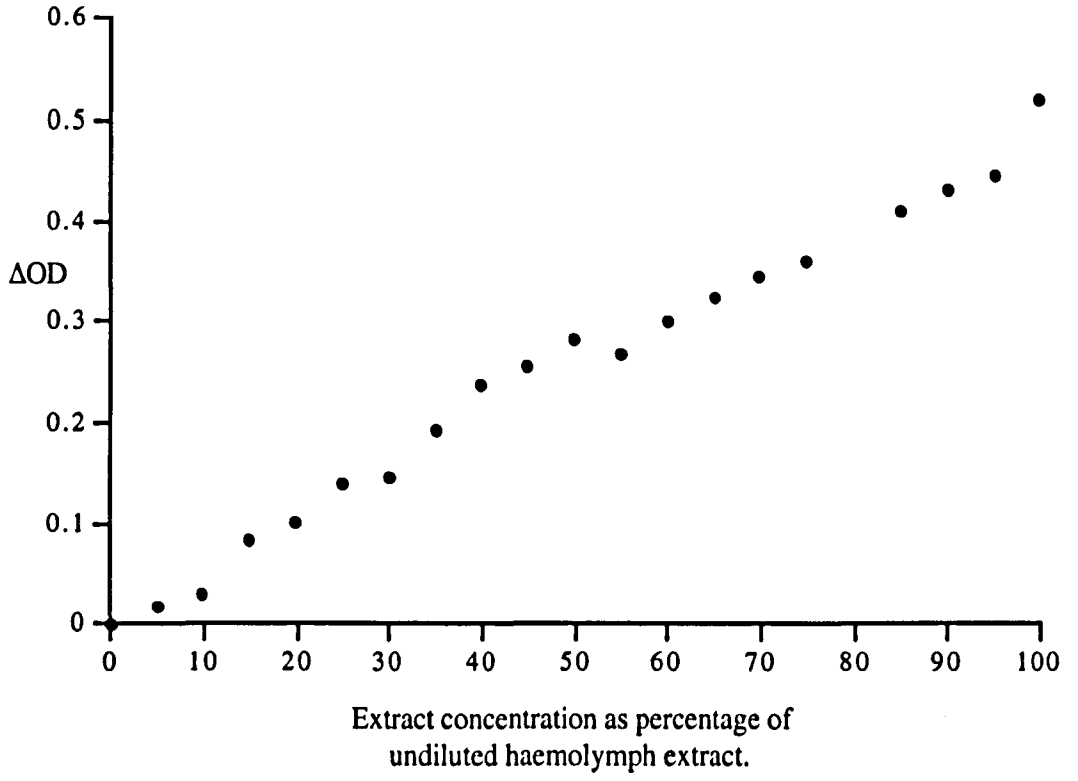

Figure 2.4: Phenoloxidase activity (measured as a change in optical density caused by the conversion of L-DOPA to melanin) of pooled haemolymph extracts from 20 adult *T.molitor*, after dilution with sodium cacodylate buffer. If the rate of substrate formation is to be used as an indicator of the amount of enzyme present, then it is necessary to show the nature of the relationship between the amount of enzyme present (represented by the amount of haemolyph present) and amount of product formed (change in optical density). The relationship in this case is strongly linear ($r^2 = 0.985$, $p = 0.0001$) (see section 2.5.4).

are highly repeatable (93%). Thus an assay using these parameters will reflect the amount of enzyme present.

2.6. Fungal bioassays

2.6.1. Fungal bioassays - Introduction

Sections 2.3 and 2.4 describe indirect assays of immune function which measure a component of the immune response. The third assay developed determines the susceptibility of *T. molitor* to a real pathogen, the hyphomycetic fungus *Metarhizium anisopliae* (strain F142). Prior exposure between host and pathogen can cause co-evolution between them, and this can result in greater specificity of the interactions between the two, even in invertebrate systems (Dularay & Lackie 1985; Huxham et al. 1988). *M. anisopliae* is a generalist entomopathogenic fungus and is a pathogen to which the study population of *T. molitor* is immunologically naïve. This ensures that levels of host resistance reflects investment in immunity to a general pathogen type, rather than a coevolved response to a specific population or species. Hyphae of *M. anisopliae* invade the haemocoel through the cuticle. They activate the host's PO when they enter, and are responded to by melanisation and encapsulation (Hajek & St. Leger 1994). Failure to resist the infection results in the death of the host.

2.6.2. Fungal bioassays - Culturing and inocula preparation

Spore preparations and assays were performed as described by Goettel and Inglis (1997). Fungus was cultured on 3% PDA plates and doses were prepared by scraping

fungal spores into a 0.05% Triton X-100 solution. Spore concentration was determined by counting spores with the aid of a compound microscope (Leitz Diaplan) using a haemocytometer. Only intact spores were counted. Inoculates were never older than 24 hours and were stored at 4°C when not in use.

2.6.3. Fungal bioassays - Inoculating adult beetles

Beetles taken from the stock population were dipped in 1 ml spore solutions for five seconds and allowed to crawl over tissue paper to remove excess moisture. The hydrophobic spores remain attached to the insect cuticle. Tables 2.1 and 2.2 show the percentage mortality of adult beetles exposed to different ranges of spore concentrations.

2.6.4. Fungal bioassays - Results and Conclusions

Table 2.1. indicates that mortality due to the process of dipping the beetles in Triton X-100 is negligible, because no deaths occurred with no spores present and only one death occurred at the six lowest spore concentrations. The more refined dataset in table 2.2 indicates that susceptibility is linearly related to dose between spore concentrations of $1x10^6$ and $1x10^7$ spores/ml. A range of doses between these values will therefore give a range of mortalities which can be used to assess overall susceptibility to the fungus, e.g. by assigning LD_{50} values. A suitable spread of mortalities over the doses used can be obtained by scoring the beetles for mortality 6 days post-inoculation.

Table 2.1: The number of adult beetles dying after exposure to *M.anisopliae* over a range of doses and at a range of time post-inoculation. Doses were increased by a factor of ten to determine the order of magnitude of a lethal dose. 20 beetles were infected at each dose and mortality scored cumulatively over 7 days. Doses were not scaled for beetle size and beetles were not controlled for age or sex. The table shows that mortality due to dipping the beetles in triton X-100 is negligible, because no deaths occurred in the absence of spores and there was only a single death in the 6 lowest fungal doses (120 beetles). The table indicates that doses between $1x10^6$ and $1x10^7$ spores/ml should produce a range of mortalities between 0% and nearly 100% if scored after 6-7 days post-infection (see section 2.6). This is refined in table 2.2.

Days after	Spore concentration (spores/ml)							
inoculation	$1x10^0$	$1x10^1$	$1x10^2$	$1x10^3$	$1x10^4$	$1x10^5$	$1x10^6$	$1x10^7$
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	7
5	0	0	0	0	0	0	0	13
6	0	0	0	1	0	0	0	15
7	0	0	0	1	0	0	0	17

Table 2.2: The number of adult beetles dying after exposure to *M.anisopliae* over a range of doses and at two different times post-inoculation. 20 beetles were infected at each dose, and the mortality scored over the two days is cumulative. Doses were not scaled for beetle size and beetles were not controlled for age or sex. This dataset indicates that mortality increases with the dose of fungus used between $1x10^6$ and $1x10^7$ spores/ml, making this a suitable range over which to assay *T.molitor* for susceptibility to *M.anisopliae*. Furthermore, it shows that a range of levels of mortality can be obtained by scoring for mortality 6 days post-inoculation (see section 2.6). The reason for the decrease in mortality at the highest dose is unknown.

Days after inoculation	Spore concentration (spores/ml)					
	$1x10^6$	$3x10^6$	$6x10^6$	$8x10^6$	$1x10^7$	$1.5x10^7$
6	4	6	6	11	17	0
7	6	8	8	14	19	6

2.7. Discussion

The main aim of this chapter was to develop a number of assays which potentially reflect the ability of *T. molitor* to resist pathogenic challenges. Three such assays were described: total haemocyte counts, measures of the levels of phenoloxidase (PO) and resistance to a generalist entomopathogenic fungus (*Metarhizium anisopliae* strain F142). This discussion attempts to address the question of what these assays reflect, specifically within the framework of 'ecological immunity'.

Section 2.3 shows that by washing the haemocoel through with 2ml of buffer, approximately 90% of the total haemolymph is consistently extracted, as indicated by haemocyte numbers. This is probably a conservative estimate because many haemocytes adhere to the haemocoel wall, rather than being free circulating in the haemolymph (Lackie 1988). This method of extraction means that subsequent assays using this total extraction technique (i.e. total haemocyte counts and PO assays) cannot be subject to spatial sampling bias, because all of a beetle's haemolymph is removed and homogenised before a sub-sample for assaying is removed. Spatial bias is potentially a big problem in *T. molitor* haemocoelic samples, because the extremely low water content (reflected by exceptionally high osmolalities) of the haemocoel means that haemolymph circulation is more limited than in other insects (Wigglesworth 1972). The high viscosity of the haemolymph also makes partial sampling difficult because consistently removing an assayable volume of liquid is impractical. Thus the 2ml bleed-through technique is favoured for acquiring samples for these assays.

Perfusing buffer through the haemocoel is, however, destructive, resulting in the death of the beetle. Thus, although the spatial repeatability of the extracted samples was high in both assays (sections 2.4.3 and 2.5.5), it was not possible to assess the temporal repeatability. Furthermore, it is not possible to assess the total volume of haemolymph collected because it represents a small fraction of the perfused liquid. All values are therefore absolute amounts, and not relative to the haemolymph volume. Previous studies have tended to look at relative levels of immune parameters (especially haemocytes) (Eslin & Prévost 1996; Eslin & Prévost 1998; Ryder 1999), probably because haemocyte proliferation per unit haemolymph has been used as an indicator of the activation of the immune system (Sequeira et al. 1996). However, absolute levels of immunity in an individual are probably better represented by absolute levels of immune parameters, rather than relative ones, so the inability to assess haemolymph volume is not considered a problem in this respect.

Total haemocyte counts reflect the levels of a parameter almost ubiquitous in insect immune responses. Haemocytes are responsible for the cellular immune responses within the haemocoel, which occur in response to metazoan, unicellular and even viral pathogens (Ratcliffe et al. 1985; Lackie 1988; Washburn et al. 1996), as well as being the media by which many immunochemicals are manufactured and transported to sites of infections (Locke & Krishnan 1971; Söderhäll et al. 1979; Söderhäll et al. 1984; Leonard et al. 1985; Söderhäll et al. 1985b; Nayar & Bradley 1994; Ashida & Brey 1995; Hyun et al. 1999). Immediately following the recognition of non-self material by the immune system, the haemocytes proliferate (Lackie 1988; Sequeira et al. 1996), indicating that a greater number of haemocytes is desirable in coping with pathogens. Both inter- and

intra-specific correlations between parasitoid resistance and haemocyte number have been observed in *Drosophila* (Eslin & Prévost 1996; Eslin & Prévost 1998), and a genetic correlation between haemocyte load and size of encapsulation response has been shown in the orthopteran *Acheta domesticus* (Ryder 1999). The size of a capsule is important in determining its efficacy in killing the encapsulated pathogen (Kuris et al. 1980). Thus determining the number of haemocytes within the haemocoel is likely to be an indicator of an organism's ability to respond to pathogens. However, the assay fails to distinguish haemocytes by type or function. Up to eight classes of such cells exist, separable by surface proteins they express, and many have specialised functions within the haemocoel (Lackie 1988). Despite this, all have functions or putative functions in immune responses (Lackie 1988), so a crude assay of haemocyte number which does not distinguish by class, is still likely to reflect the ability to respond to an immune challenge.

PO is similarly nearly ubiquitous in invertebrate immune responses. Levels of the enzyme have been postulated to reflect the level of the activation of the immune system, because the pathway by which it is activated may be part of the pathway by which the entirety of the immune system is activated following recognition of non-self material (Söderhäll 1982; Yoshida et al. 1986). Furthermore, cellular responses are almost always accompanied by melanisation (Ratcliffe et al. 1985), leading to speculation that melanin synthesis is a mechanism by which pathogens are killed within capsules (Nappi & Vass 1993). Despite much research, the specific role of PO in immune responses is not clear. However, its close association with them, and its suggested roles, indicate that PO levels may indicate the degree to which an individual is able to respond to non-self material.

There is, unfortunately, currently little evidence which shows that PO levels reflect resistance to pathogens, other than correlational data (Reeson et al. 1998).

PO is present in an inactive form (pro-PO) which is activated to PO by an enzyme cascade (Ashida & Brey 1998). Thus the level of active PO can be used as a measure of the size of an existing immune response, and the level of pro-PO as an indicator of the potential to respond. However, disruption of cellular integrity and physiological stress result in the activation of pro-PO (Dularay & Lackie 1985; Söderhäll et al. 1994), and these two conditions will occur during the extraction of haemolymph from live beetles. Thus the assay only reflects the levels of pro-PO, because it is probably not possible to extract only PO without activating at least some of its precursor.

Both haemocyte counts and PO assays are measures of aspects of the immune response that are thought to relate indirectly to pathogen resistance in general. The third assay developed is a direct assessment of resistance to a pathogen: the generalist entomopathogenic fungus *M. anisopliae* (strain F142). Using a real pathogen has the advantage over the previously described assays, in that there is no need to assume the link between the assay results and pathogen resistance: the results of the assay directly indicate the ability to resist *M. anisopliae*. However, haemocyte counts and PO assays reflect the levels of immune parameters known to be used in response to a variety of diverse pathogen types (see above), whereas an assay using a single pathogen type may not be particularly meaningful in terms of resistance to other pathogen types. Fungi such as *M. anisopliae* attach themselves as spores to the cuticle of potential hosts. Hyphae then extend and force their way through the cuticle, both by physical pressure and chemical digestion of tissues. Once inside the cuticle, the hyphae extract nutrients and liquid from

the host, resulting in host mortality (Hajek & St. Leger 1994). The host immune response includes thickening the cuticle at the site of penetration (Ashida & Brey 1995), encapsulation and melanisation of hyphae within the haemocoel (Ratcliffe et al. 1985), and the secretion of anti-fungal proteins (Young et al. 1995). The first and last of these will not be involved in (for example) a response to a bacterial pathogen ingested with food, and if these are the critical steps in fungal resistance then there is no apparent physiological reason that fungal resistance and resistance to other pathogen types will be correlated. However, *M. anisopliae* is a good model for fungal pathogens which invade through the cuticle. It is a generalist entomopathogen, to which the stock population of *T. molitor* has never been exposed (at least 60 generations). This means that there cannot have been any coevolution between the host and pathogen populations. If these are exposed to each other over a long period of time it is possible for hosts to become more immune reactive to that pathogen (Dularay & Lackie 1985) or for the pathogen to evolve a way of avoiding the host's immune response (Huxham & Lackie 1988). In this system there has been no opportunity to coevolve, and thus *M. anisopliae* remains a model generalist fungal pathogen to the stock population of beetles.

As intimated in the introduction (section 2.1.1), it is an objective for researchers in ecological immunity to develop a consistant and empirically testable definition of immunocompetence (Siva-Jothy 1995; Skarstein 1996; Svensson & Skarstein 1997). However, the idea that immunocompetence can be considered a life history trait, with certain individuals having more immunity in a general sense, is controversial, and evidence is lacking (Owens & Wilson 1999). Furthermore, even if immunocompetence is theoretically definable in a biologically realistic way, there is no consensus on the

methodology for empirically measuring this trait within an individual. Assays of individual components of the immune system can be misleading (Braude et al. 1999) and assays of multiple aspects of immunity (Lochmiller 1995) are impractical, and still suffer from the same problems of relating immune components to immunity itself.

Thus the assays developed in this chapter do not attempt to measure immunocompetence, but are designed instead to reflect specific aspects of immunity that may be important in a general sense. Haemocytes and PO are involved in immune responses to a variety of pathogens, and the levels of both increase in response to infection (Söderhäll 1982; Sequeira et al. 1996). The relationship between each of these and immunity is not clear. There is some evidence to suggest that absolute haemocyte numbers may reflect the efficacy of encapsulation responses against metazoan parasites (Eslin & Prévost 1996; Eslin & Prévost 1998; Ryder 1999), but the relationship of this to the other types of cellular responses (section 1.3.4) is unknown. There is even less evidence that absolute levels of PO reflect parasite resistance. Thus these two parameters are used as a reflection of the size of immune response without making assumptions about their relationship to immunocompetence. Fungal bioassays are likewise not used as an indicator of immunocompetence, because of the problems of relating fungal resistance to resistance to other pathogen types. Thus this assay is simply used as an indicator of resistance to a single general pathogen type.

2.8. Summary

In this chapter, three assays of immune function in *T. molitor* have been developed: a total haemocyte count, a measure of the activity of PO and a bioassay of resistance to a fungal pathogen. The first two of these require that the proportion of the total haemolymph volume being extracted is controlled for, and a technique for doing this is also developed. The repeatabilities of the assays have been assessed where possible, and these were high in all cases. The biological relevance of the assays are discussed, specifically their relationship to the ability to resist pathogens and the concept of immunocompetence.

Chapter 3

Density Dependent Prophylaxis

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3.1. Introduction

3.1.1. The expression of immunity is phenotypically plastic

Immune defences are not constitutively expressed at a maximum level, but are phenotypically plastic with respect to the environment (section 1.4). Expression is usually induced by the presence of pathogens (e.g. Söderhäll 1981; Ratner & Vinson 1983; Söderhäll 1983; Hultmark 1993; Nicolas et al. 1996; Sequeira et al. 1996), wounding of the cuticle (Nayar & Knight 1995) and the increased risk of encountering a pathogen (Reeson et al. 1998; Wilson & Reeson 1998). Thus the immune system constitutes an 'inducible defence' against pathogenesis (Harvell & Tollrian 1999). Phenotypic plasticity usually evolves when the optimal value for a phenotype varies with the environment. Any single phenotype is prevented from becoming fixed in the genome if the trait is subject to strong and varying selection, if the need for expression can be accurately assessed, and if there is a cost associated with at least some of the levels of expression of a trait, providing that the cost is counterbalanced by the benefits of expressing the trait in certain

environments (Harvell & Tollrian 1999). The interactions between immune systems and pathogens often fit these criteria (Frost 1999).

3.1.2. Density dependent prophylaxis

The expression of immune function is induced when a pathogen is encountered, because the cost of expression is ameliorated by the reduced cost of exposure to the pathogen. It is also possible that increased investment in immunity (and therefore increased costs of investment) may be advantageous if the risk of encountering pathogens is high, providing that accurate information about these risks is available. Such investment would be preventative (prophylactic) rather than responsive, and would greatly reduce the ability of the pathogen to incur costs on the host.

One situation predicted to raise the probability of pathogenesis, is a rise in population density. Many organisms experience irregular and unpredictable fluctuations in population density, and insects in particular are prone to such fluctuations because of their short generation times and large reproductive potential (Begon et al. 1990). Many insects are known to be able to assess population density during the larval stages and make subsequent adjustments to their phenotype (Dingle 1996). A wide range of insect traits show such 'density dependent phase polyphenism', including colouration, wing and sensilla morphology, developmental rate, hormone metabolism, pheromone production, reproductive potential and behaviour (Applebaum & Heifetz 1999).

For most organisms, conspecifics are the main source of disease (Freeland 1983), and contact with increasing numbers of conspecifics raises the probability of infection (Steinhaus 1958), and hence the likelihood of needing to mount an immune response.

Thus, underinvestment in immunity at high densities can lead to a loss of fitness due to the costs of pathogenesis. However, mechanisms by which organisms resist parasites and pathogens can also be costly to maintain and express (see section 1.4.2). Autoimmunity, immunopathology and reductions in investment in other aspects of life history all occur in response to the expression of immunity (e.g. Nappi & Vass 1993; Kraaijeveld & Godfray 1997; Råberg et al. 1998) and hence overexpression in immunity can lead to a loss of fitness due to the costs incurred by these processes. The ability of an organism to balance the costs and benefits associated with investment in immune function in the context of the probability of pathogenesis is therefore likely to be an important component of fitness. Consequently, insects that are able to assess population density should adjust their investment in immunity according to the perceived risk of infection. This is termed density dependent prophylaxis (DDP; Wilson & Reeson 1998).

Evidence that DDP occurs is found in interactions between baculoviruses and their lepidopteran hosts (Wilson & Reeson 1998). It has been demonstrated that larvae of the noctuid moth *Spodoptera exempta* are more resistant to a baculovirus when reared at high population densities (Reeson et al. 1998). A similar pattern was observed in another lepidopteran: *Mamestra brassicae* (Goulson & Cory 1995). In the former study, the levels of cuticular melanisation were positively correlated with resistance (black larvae were more resistant than green larvae) and more resistant individuals had higher levels of phenoloxidase (PO) in the haemolymph (Reeson et al. 1998). PO has been implicated as being central to both the recognition of foreign material within the haemocoel, and to the killing of pathogens in conjunction with the immune responses against them (see section 1.3). PO is activated from its zymogen if the threat of pathogenesis is perceived and is

intimately associated with cellular responses to a diversity of pathogen types (Söderhäll et al. 1994). Furthermore, the synthesis of melanin probably invokes a specific cost, because it involves the generation of reactive oxygen species as well as quinones in the haemocoel, both of which are harmful to the host's tissues (Nappi et al. 1995). Consequently, PO levels exhibit phenotypic plasticity dependent on the risk of parasite encounter and are therefore likely to show density dependent polyphenism in conjunction with prophylactic immune responses.

Density dependent polyphenisms involving cuticular melanisation are a well studied phenomenon in the Lepidoptera and Orthoptera, the gregarious forms of which frequently have more heavily melanised cuticles than the solitary forms (Applebaum & Heifetz 1999). The functions of cuticular melanisation are unclear, but it has been implicated in crypsis, aposematism, thermal regulation, blocking ultraviolet light and as a sexual signal (Kettlewell 1973; Majerus 1998). The results of Reeson et al. (1998) suggest that it may also have a function in prophylactic immune responses. If this is the case then it implies that this phenomenon may be widespread in these two insect orders. Density dependent polyphenism has been less well studied in other orders.

The aim of this chapter is to test for the existence of density dependent prophylaxis in *Tenebrio molitor*. Tenebrionids are stored product pests and frequently undergo changes in population density (Tschinkel & Willson 1971). Consequently, there are a number of traits which have been shown to respond to changes in population density. At high density, larvae have been observed to experience extra larval moults (Weaver & Macfarlane 1990), inhibition of pupation (Weaver & Macfarlane 1990; Connat et al. 1991; Kotaki & Fujii 1995), increased mortality and increased cannibalism

(Savvidou & Bell 1994). Immature tenebrionids are able to assess the density of conspecifics, apparently using both mechanical and chemical cues (Tschinkel & Willson 1971; Kotaki & Fujii 1995). Thus *T. molitor* are able to assess population density and adjust their phenotype accordingly, making them likely candidates for exhibiting density dependent prophylaxis. Furthermore, at high densities the larvae show increased levels of cannibalism (Savvidou & Bell 1994), and this raises the probability of acquiring a pathogen from conspecifics even further (Pfennig et al. 1998).

3.1.3. Assessing investment in immunity

In this study, investment in immunity was assessed using two of the assays developed in chapter 2: fungal bioassays (section 2.6) and measurement of PO (section 2.5). The fungus used is once again *Metarhizium anisopliae* strain F142. As a novel pathogen there is no *a priori* reason that a prophylactic response by *T. molitor* should be directed against it in particular, and so it is suited for testing for the presence of a prophylactic response, one of the elements of which is an increase in resistance to fungal pathogens. PO assays represent a more general measure of the level of an immune response (see section 2.7), although the results of the PO and fungal assays are expected to be correlated. The host's immune system responds to fungal infection by melanising the fungal hyphae (Hajek & St. Leger 1994), which invade the haemocoel through the cuticle (Goettel & Inglis 1997). Once inside, the hyphae activate the host's PO (Söderhäll et al. 1979). Hence PO is directly involved in resistance to *M. anisopliae*. Two measures of PO are used, one on the haemolymph of untreated adult beetles, the other using the haemolymph of beetles which have had an artificial immune challenge (a nylon implant).

These are used as a measure of the ability to respond and a measure of the size of response respectively.

As well as the two immune assays, the cuticular melanisation of the beetles will be assessed by subjectively assessing the colour (specifically lightness and darkness) of the elytra. Previous studies in Lepidoptera have shown a correlation between pathogen resistance and elytra darkness, with animals possessing darker cuticles being more resistant. Therefore, cuticular melanisation is a morphological correlate of and potential marker for immunity. Thus the following predictions are made. Firstly, that beetles raised gregariously as larvae will have greater resistance to a generalist pathogen than those reared at low density; secondly, that the former will have higher levels of PO compared to the latter, both with and without an immune challenge; and thirdly, that higher levels of immunity will be correlated with darker cuticles.

3.1.4. Aims of the chapter

In this chapter I address the following questions:

1) Are changes in investment in immunity, as indicated by resistance to a fungal pathogen, induced by the presence or absence of conspecifics?

2) Are differences in the ability to resist a fungal pathogen reflected by correlated differences in the amount of an immune parameter (PO)?

3) Is the level of a morphological character (cuticular melanisation) correlated with the ability to resist fungal pathogens?

3.2. Materials and methods

3.2.1. Beetle cultures

Beetles were taken from a single genetically depauperate inbred line maintained at the University of Sheffield. The line had been maintained by brother/sister mating for at least four years, after originally being derived from the stock population. Experimental cultures were set up using larvae less than a week old (and less than 4mm in length), which had hatched from a single cohort of eggs laid on or before Nov. 1st 1998. These were randomly assigned to either 'Solitary' or 'Gregarious' cultures. Solitary cultures (n = 215) consisted of a single animal in c.130 cm^3 of rat chow, whilst Gregarious cultures (n = 16) consisted of 15 individuals in c.400 cm^3 of rat chow. This amount of feed was effectively *ad libitum*. Half a gram of lettuce per beetle per culture was provided biweekly as a source of moisture.

3.2.2. Preparation of fungal inocula

M. anisopliae strain (F142) is pathogenic to a range of insects. Spore preparations and assays were performed as described by Goettel and Inglis (1997). Fungus was cultured on 3% PDA plates and doses were prepared by scraping fungal spores into a 0.05% Triton X-100 solution. Spore concentration was determined by counting spores with the aid of a compound microscope (Leitz Diaplan) and a haemocytometer. Only

intact spores were counted. Inoculates were never older than 24 hours and were stored at 4°C when not in use.

3.2.3. Assignment to treatments

Beetles were removed from their cultures upon pupation, at which point they were sexed. They were randomly assigned to either the fungal or PO assays. 60 males and 60 females from each rearing density were assigned for use in the fungal assay. 20 males and 20 females from solitary cultures and 2 beetles per sex per gregarious culture were randomly assigned to the phenoloxidase assay. On imaginal emergence, beetles were weighed and the date of emergence noted. Beetles failing to emerge properly (defined by failure of the elytra to meet posteriorly) were excluded from the study.

3.2.4. Phenoloxidase assay

Assays were performed as described in section 2.5.6. On the day of their emergence as adults, half the beetles of each sex had a nylon monofilament implanted in their haemocoel by forcing it between the abdominal sternites beneath the elytra. Control beetles had their elytra separated but no nylon implanted.

Haemolymph extracts were taken 24 hours later by washing the abdomen through with 2ml of ice cold sodium cacodylate buffer (0.01M Na-cacodylate, 0.005M $CaCl_2$). Samples were immediately frozen at -30°C to disrupt the haemocyte membranes. The frozen samples were thawed for 3 minutes at 30°C followed by centrifugation (4°C, 2800G, 15 minutes). Supernatants were removed and vortexed, after which 500µl of supernatant were mixed with 1 ml of 3mM L-DOPA in sodium cacodylate solution and

the reaction allowed to proceed at 30°C in a spectrophotometer (Pharmacia Biotech Ultraspec 2000) for 20 minutes. Readings were taken every minute at 490nm and analysed using Swift II software (Pharmacia Biotech). Enzyme activity was measured as the slope of the reaction curve during the linear phase of reaction (between 5 and 15 minutes after the reaction mix was made; section 2.5.3).

3.2.5. Fungal bioassay

Beetles were assayed on the second day after imaginal emergence. 20 beetles from each rearing density (10 males, 10 females) were assigned to each of one of six doses of fungus (0.0, $1.0x10^7$, $2.5x10^7$, $5.0x10^7$, $7.5x10^7$ and $1.0x10^8$ spores/ml). Doses were adjusted for beetle size by determining the surface area of the beetle from its weight using the following conversion:

Surface area (cm^2) = 10 [weight(g)]$^{0.67}$ (Schmidt-Nielsen 1984)

The fungal doses listed above were then defined as the doses for an average beetle (0.116g in weight, 2.38 cm^2 surface area: pers. obs.). Doses for beetles deviating from this measurement were scaled using the following formula:

$$\text{Actual dose} = \left(\frac{2.38}{s.a.}\right) \text{assigned dose}$$

Beetles were exposed to the fungus by dipping them in 1ml of spore solution for 5 seconds. These solutions were used on one beetle only. Control beetles were dipped in 0.05% Triton X-100 with no fungal spores. Beetles were individually incubated at 26°C, 70% RH for six days. After which mortality was scored.

3.2.6. Assessment of cuticular melanisation

On the fourth day after eclosion, the cuticular melanisation of beetles was assessed under an 11W florescent white light. Fully developed adult beetles were defined as either 'Black', 'Brown' or 'Tan' using the following subjective criteria. The elytra of 'Tan' beetles were light brown in colour and this was clearly visible under normal, diffuse lighting. The elytra of 'Brown' beetles were not easily distinguishable from 'Black' except under direct illumination, where they appeared dark brown. 'Black' beetles showed no obvious brown colouration to their elytra, even under strong, direct illumination. Elytra representative of each colour class were analysed using digitised images (collected using a Sony TR2000E camera under 11W florescent illumination) in Adobe Photoshop (v4.0). They differed in the 'intensity' of red colouration (Tan=165-200, Brown=133-156, Black=95-105; on a scale of 0 to 255, where 255 represents maximum intensity) and 'brightness' (Tan=62-78%, Brown=54-60%, Black=39-42%).

3.2.7. Analysis

Mortality and colour data were analysed using logistic regression analysis in Statview 5.0 (SAS). Phenoloxidase data were transformed to normality by the following formula: PO = log (slope + 0.0001). These data were then analysed using ANOVA in Statview 5.0 (SAS).

3.3. Results

3.3.1. Phenotypic plasticity in egg-to-adult development

Gregarious larvae reached adulthood significantly earlier (d.f. = 338, t = 27.78, p < 0.001) and gave rise to significantly heavier adults (Welch's unequal T-test: d.f. = 336, W = 53.91, p < 0.001) than solitary larvae (table 3.1). Gregarious larvae suffered significantly higher mortality (d.f. = 1, χ^2 = 26.42, p < 0.0001) than solitary larvae (table 3.1)

Gregarious beetles were also significantly darker than solitaries (d.f = 2, χ^2 = 14.21, p < 0.001) (table 3.1). When rearing density, sex, weight and age were included as terms in a logistic regression for the model 'colour', only rearing density contributed significantly to the fit of the model (d.f = 2 ,χ^2 = 14.41, p < 0.001) .

3.3.2. Fungal bioassay

It was not possible to assign specific LD50 values because the experiment produced very high mortality. However, susceptibility to M. anisopliae differed significantly between the two rearing densities (n = 20 / density / dose) across the range of doses (d.f. = 1, χ^2 = 5.88, p = 0.015) (figure 3.1). A logistic regression was performed in which the terms rearing density, colour, dose, sex and adult weight were included in the model for 'susceptibility', and terms were excluded if they did not contribute to the fit of the model at the p ≤ 0.10 level. The terms adult weight (d.f. = 1, χ^2 = 0.44, p = 0.506), sex (d.f. = 1, 1.19, p = 0.274) and rearing density (d.f. = 1, χ^2 = 2.74. p = 0.253) were

Table 3.1: Traits exhibiting density dependent phase polyphenism in *T.molitor* (see section 3.3.1). Errors (where given) are 1 standard deviation and numbers in brackets are sample sizes. * χ^2, $p<0.001$; ** t-test, $p<0.001$

Rearing Density	Elytra Colour *			Larval Period (days) **	Adult Weight (mg) **	Egg-to-Adult Mortality *
	Black	Brown	Tan			
Solitary	8%	43%	49%	140±7.7	103±14.2	3%
	(9)	(49)	(56)	(157)	(157)	(215)
Gregarious	15%	59%	26%	115±8.5	117±18.2	24%
	(17)	(67)	(30)	(175)	(175)	(240)

thus excluded. Only two terms contributed to the fit: dose (d.f. = 1, $\chi^2 = 7.23$, $p = 0.007$) and colour (d.f. = 1, $\chi^2 = 35.61$, $p < 0.001$). There was no effect of weight even when nested within colour classes (d.f. = 1, $\chi^2 = 5.70$, $p = 0.222$).

Percentage mortality (irrespective of dose) was significantly different between colour morphs (see table 3.2 for data, and see figure 3.2).

3.3.3. Phenoloxidase assay

There was no significant difference in the PO levels of beetles raised solitarily and gregariously (ANOVA; d.f. = 1, F = 2.32, p = 0.132), between challenged and non-challenged beetles (ANOVA; d.f. = 1, F = 1.22, p = 0.272) or between the sexes (ANOVA; d.f. = 1, F = 1,84, p = 0.177). Nor were there any significant interactions between these terms.

3.4. Discussion

Investment in immunity shows density dependent phase polyphenism in the mealworm beetle *Tenebrio molitor*: animals reared at higher density were more resistant to the entomopathogenic fungus *Metarhizium anisopliae* (figure 3.1). Resistance was related to the level of cuticular melanisation in the beetles, with darker beetles being more resistant (figure 3.2). Thus gregariously raised beetles tended to be darker than those raised solitarily, and darker beetles were more resistant than lighter ones. There was no difference in haemolymph PO activity between the two rearing densities.

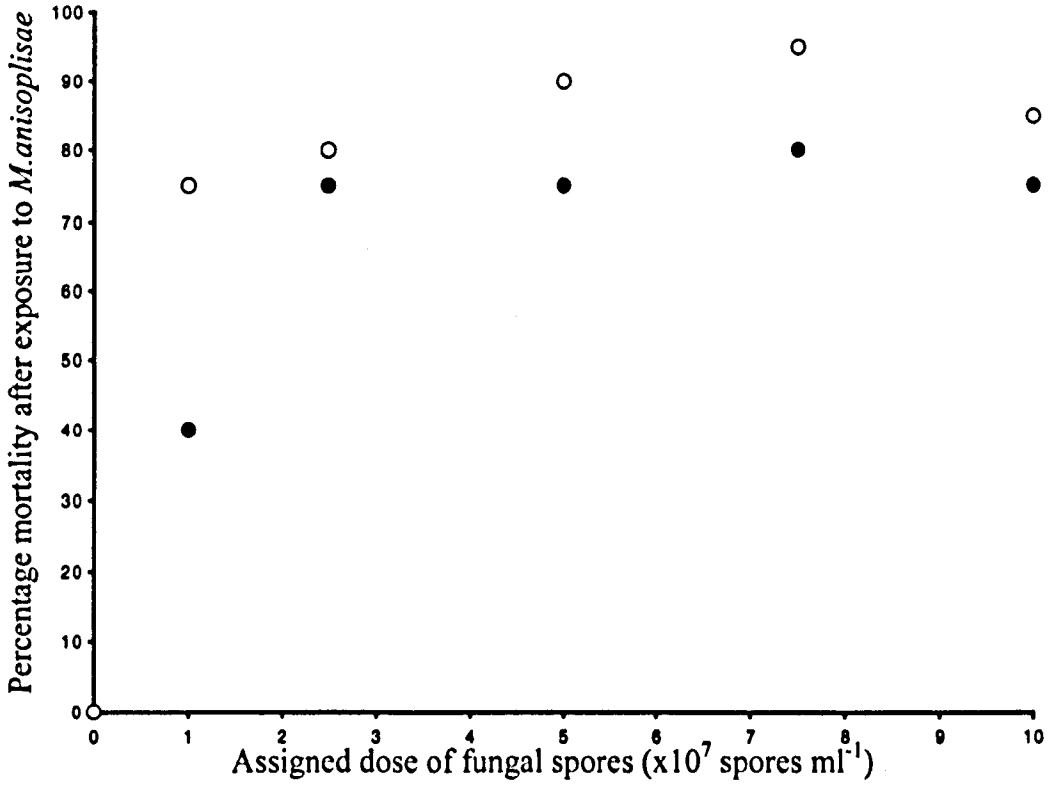

Figure 3.1: Percentage mortality of gregariously (closed circles) and solitarily (open circles) raised adult *T.molitor* exposed to *M.anisopliae* over a range of fungal doses. The mortality is significantly different between rearing densities ($\chi^2 = 5.88$, $p = 0.015$) (see section 3.3.2).

Table 3.2: Overall percentage mortality (regardless of fungal dose) of beetles in each category of cuticular melanisation, split by their rearing densities. Numbers in brackets are the total sample sizes for each cell of the table. Lighter beetles are significantly more susceptible to *M.anisopliae* than darker ones (d.f. = 1, $\chi^2 = 35.61$, $p < 0.001$). (see figure 3.2 for graphical representation).

	Total Percentage Mortality	
	Gregarious	Solitary
Black	29% (14)	29% (7)
Brown	69% (58)	84% (38)
Tan	89% (27)	95% (52)

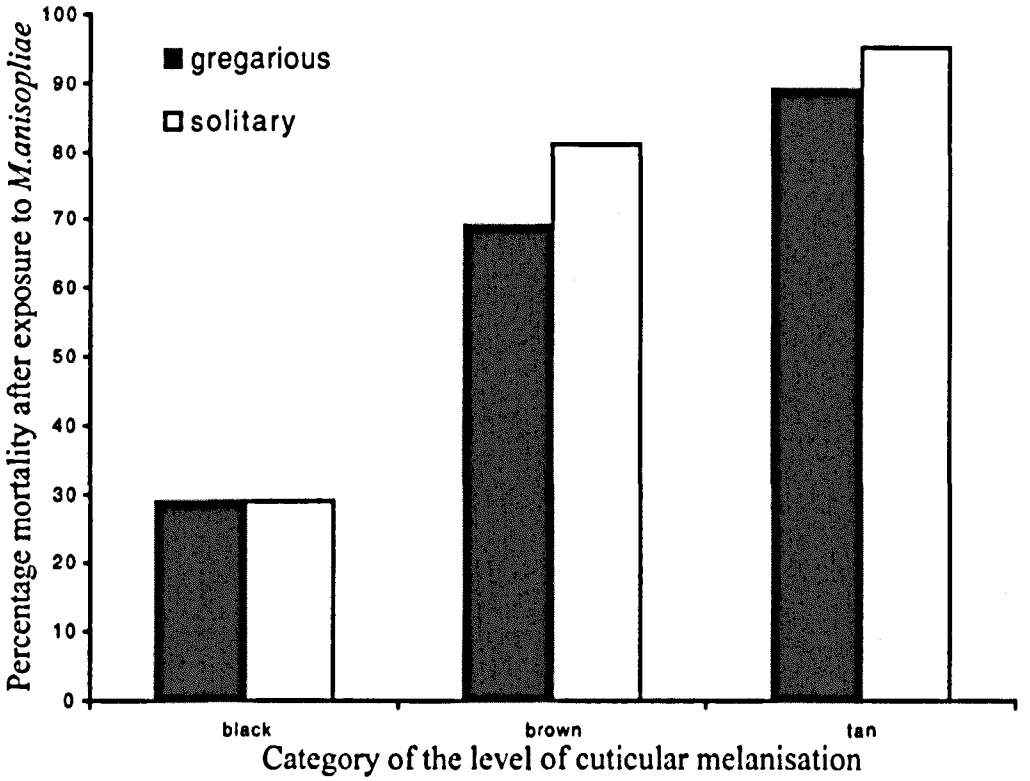

Figure 3.2: Overall percentage mortality (regardless of fungal dose) of beetles in each category of cuticular melanisation, split by their rearing densities. Open bars are solitarily raised and closed bars are gregariously raised beetles. Lighter beetles were more susceptible to *M.anisopliae* than darker ones (d.f. = 1, $\chi^2 = 35.61$, $p < 0.001$). Thus, because the gregarious beetles were significantly darker than the solitary ones (d.f. = 2, $\chi^2 = 14.21$, $p < 0.001$; section 3.3.1), mortality is significantly higher in beetles raised solitarily than those raised gregariously (d.f. = 1, $\chi^2 = 5.88$, $p = 0.015$; section 3.3.2).

These results support the theory that investment in innate immunity is mediated by the probability that an immune challenge will be encountered, specifically based on the number of conspecifics present (Wilson & Reeson 1998), and that this redistribution of resources is adaptive. An alternative non-adaptive explanation (that gregariously raised beetles are simply in better condition and have more total resources to invest in immunity) may seem to be supported by the fact that solitary beetles were smaller and took longer to develop (table 3.1). However, if size and development time were indicators of condition in the study population, then it would be predicted that they would contribute to the fit of the model 'mortality' in a logistic regression. This is not the case, either for the whole data set or when each colour morph is analysed separately. Thus a small black beetle is equally likely to be susceptible to the fungus as a large black beetle, and likewise for brown and tan. Secondly, both rearing densities had *ad libitum* food and liquid, so the only environmental difference was the presence or absence of conspecifics. Resistance to the fungus was not obligately dependent on conspecifics being present, because some solitary individuals showed high resistance to the fungus (i.e. belonged to the darker colour morphs). Thus, despite the fact that weight and development time differed significantly between rearing densities, rearing density itself was not a significant contributor to mortality. This means that at least some of the solitarily raised individuals were able to invest in immunity as highly as the gregarious, indicating that equal resources were available to both. Finally, differences in development time (Tschinkel & Willson 1971, Connat et al. 1991, Savvidou & Bell 1994, Kotaki & Fujii 1995) and weight (Weaver & Macfarlane 1990) are frequently observed in tenebrionids when

population density is manipulated. These have been interpreted as being adaptive for the avoidance of cannibalism (tenebrionids are highly cannibalistic in the immature stages), which occurs following larval moults and during pupation (see discussions in Tschinkel & Willson 1971, Weaver & Macfarlane 1990, Savvidou & Bell 1994). Thus differences in life history variations in tenebrionids are often due to alternative strategies for the minimisation of larval mortality, rather than being simply condition dependent, and an adaptive explanation (density dependent prophylaxis; [DDP]; Wilson & Reeson 1998) for the variation in fungal resistance found in this study is favoured.

Models of disease transmission tend to assume that the probability of infection is a simple function of population density (e.g. Anderson and May 1979; Anderson and May 1981). These results question the validity of that assumption, clearly showing that higher levels of investment in immunity can be induced by the threat of pathogenesis independent of the actual presence of a pathogen (Wilson & Reeson 1998). More specifically, investment in preventative immunity was greater in the presence of conspecifics (density dependent prophylaxis). Such phenotypic plasticity in the levels of investment also provides indirect support for the theory that there is a cost associated with the maintenance of immune function (e.g. Råberg et al. 1998) because if there were no such costs it would be possible to maintain immune function at the maximum level all of the time (Via et al. 1995). Density dependent prophylaxis has now been demonstrated in representatives of two insect orders: Lepidoptera (Reeson et al. 1998) and Coleoptera (this study). In both cases animals reared at higher densities were found to be more pathogen resistant and had more heavily melanised cuticles. This concord is interesting because the pathogens to which susceptibility was tested in each study were very

different. Reeson et al. (1998) used a baculovirus specific to *Spodoptera exempta*, which was administered *per os* and invaded the haemocoel via the mid-gut. This study used a generalist entomopathogenic fungus which adheres to, and invades through, exocuticle (Goettel & Inglis 1997). The fact that both insects reacted to high rearing density by increased investment in resistance to diverse pathogen types suggests that they may share a general prophylactic response to the threat of pathogenesis. This response is correlated with, among other things, a darkening of the cuticle.

Alteration in the level of cuticular melanisation is a commonly observed response to changes in rearing density in insects, particularly amongst the Orthoptera and some Lepidoptera (Applebaum & Heifetz 1999). Despite this, the function of cuticular melanisation is not clear. Putative functions include crypsis, aposematism, thermal regulation, blocking ultraviolet light and as a sexual signal (Kettlewell 1973; Majerus 1998). These are unlikely to be important in *T. molitor* because, as an inhabitant of grain stores, the chief mode of interaction between conspecifics is not visual but olfactory (Valentine 1931). Cuticular melanisation may occur because the cuticle is a sink for harmful quinones recruited for, but not used in, sclerotisation (Chapman 1982). Thus cuticular sclerotisation and melanisation are chemically related, but can have different functions. It is likely that cuticular sclerotisation is an important prophylactic barrier, because sclerotised proteins are impermeable to many fungal secretions, resistant to enzyme degradation and mechanically prevent hyphal penetration (Hajek & St. Leger 1994). It is therefore not clear if cuticular melanin is involved in prophylaxis (i.e. provides a preformed barrier which actively contributes to fungal resistance), if it is a by-product of increased sclerotisation or if it is a correlated response to changes in density

and not involved in immunity at all. (The genetic basis for this phenotypic correlation is addressed in chapter 5).

The fact that cuticular melanisation may be correlated with investment in fungal resistance, and is a commonly observed response to conspecific density in insects (Applebaum & Heifetz 1999), suggests that density dependent prophylactic measures may be common in this taxon. Further evidence that the degree of melanisation correlates with pathogen resistance in Lepidoptera comes from studies of *Ephestia kuhniella*, in which parasitoids develop more slowly in the melanic form (Verhoog et al. 1996), and *Antheraea pernyi*, in which the melanic form is more resistant to a nuclear polyhedrosis virus (Gershenzon 1994). However, in both cases, expression of melanism is genetically determined rather than a phenotypically plastic response.

The density dependent phase polyphenisms observed in this study included differences in length of larval period, adult weight and larval mortality (table 3.1). The last of these is interesting because the higher mortality in gregarious larvae compared to solitary larvae may be attributable to cannibalism. This could explain at least one of the selective forces behind the existence of density dependent prophylaxis in *T. molitor*. Cannibalism (or high mortality) is invariably observed at high larval densities (Tschinkel et al. 1967; Savvidou & Bell 1994) and increases the risk of pathogenesis (Pfennig et al. 1998). Polyphenisms in development time and inhibition of pupation in *T. molitor* have been interpreted as being adaptive for the avoidance of cannibalism (Weaver & Macfarlane 1990). Cannibalism may also explain the observation that gregariously raised larvae reached adulthood earlier and were larger than solitaries, though this has not always been found in other studies (Weaver & Macfarlane 1990).

There was no difference in the levels of PO in the haemolymph of beetles reared at different densities, or between beetles given an artificial immune challenge and those left as controls. This is surprising given that haemolymph PO activation is one of the primary responses when non-self material is encountered in the haemocoel (Söderhäll 1982). Nylon filaments of the type used in the experiment are known to cause an immune response in the stock population of *T. molitor* (personal observation), so some change in PO levels were expected, particularly as resistance to a pathogen was correlated with a melanic trait. However, haemolyph PO may not share a common origin with the enzyme responsible for cuticular melanisation (laccase PO), and hence may be regulated independently (Ashida & Brey 1998). However, once hyphae have penetrated the haemocoel it is the haemolymph PO which is responsible for resistance (Söderhäll 1981). These results suggest that the prophylactic response, at least as it applies to fungal pathogens, is manifested by greater measurable investment in barriers to penetration, rather than in defence within the haemocoel. On this point *T. molitor* seems to differ from *S. exempta* (Reeson et al. 1998), although in the latter case it is not clear if haemolymph PO levels are a causal factor of the prophylactic response. It should also be noted that, in this study, cuticular melanisation, and not rearing density, was the better predictor of susceptibility, and that the experimental design meant that assessing differences in PO between colour morphs was impossible, because PO was assessed one day after adult emergence and colour after four days. Such data would have helped to elucidate the relationship between haemolymph PO and pathogen resistance.

The relationship between haemolymph PO levels, melanisation ability and resistance to pathogenesis has yet to be experimentally demonstrated. This study failed to

find a significant relationship between haemolymph PO activity and resistance to the fungus *M. anisopliae* in *T. molitor*. This suggests that the measurement of PO levels used in this study may not reflect accurately a beetle's ability to resist fungal pathogens. More empirical evidence is required to elucidate the relationship between PO levels and resistance.

T. molitor is capable of mounting a density dependent prophylactic response, which increases resistance to a generalist entomopathogen (*M. anisopliae*). This is the first demonstration of this phenomenon outside the Lepidoptera, and is the first to use a generalist pathogen. The nature of the density dependent response suggests that there is a maintenance cost associated with insect immune function. The correlation between resistance and cuticular melanisation suggests a function in immunity for this trait, something not previously mooted as one of its putative functions. These results, coupled with the observation that phase polyphenism in cuticular melanisation is widespread, make it likely that density dependent prophylaxis is a common phenomenon in the Insecta.

3.5. Summary

In this chapter I have shown that investment in immunity (indicated by the level of mortality when exposed to a fungal pathogen) is increased in the presence of conspecifics. This supports the theory that the immune system can be induced by not just the presence of pathogens, but the presence of the threat of pathogenesis. This increased investment in immunity was not shown to correlate with an immune parameter known to

be involved in fungal resistance, the activity of PO. There was, however, a phenotypic correlation between fungal resistance and the level of adult cuticular melanisation, with darker beetles being produced more often at high density and showing greater levels of resistance.

Chapter 4
Heritability of Immune Function

4.1. Introduction

The work presented in chapter 3 showed that increased resistance to a fungal pathogen (*M. anisopliae*) and increased cuticular melanisation were both correlated adult responses of increased larval density in *T. molitor*. This pattern has also been observed in another insect taxon, the Lepidoptera (Goulson & Cory 1995; Reeson et al. 1998), suggesting that the level of cuticular melanisation may be an indicator of resistance to fungal pathogens, or may even be an immune trait in itself. The nature of this relationship is established more firmly in the next chapter. There is considerable variation within the stock cultures in the darkness of beetles' cuticles, and this is apparently independent of the larval density of the cultures (personal observation), suggesting that there are other causes of variation. This chapter aims to identify and quantify any genetic components to variability in cuticular melanisation, as well as any genetic correlation between cuticular melanisation and the total haemocyte count within the haemocoel (a trait also implicated as a determinant of immunity in invertebrates; Kuris et al. 1980; Eslin & Prévost 1996; Eslin & Prévost 1998; Ryder 1999), thereby determining the ability of immune-linked traits to respond to selection.

4.1.1. Heritability and additive genetic variance

One important measure of the genetic control of variability is the narrow sense heritability (h^2) of a trait. This is defined as the proportion of the total phenotypic variance (V_P) which is explained by additive genetic variance (V_A) (Falconer & Mackay 1996). The additive genetic variance, or 'breeding value', represents the average effect on a trait of all the alleles an individual has, summed over the loci involved in that trait. Thus heritability is not a measure of the genetic determination of a particular phenotype *per se*, but rather is a measure of the genetic determination of the variability of that phenotype in a population. This is an important value for evolutionary biologists because it determines the ability of a trait to respond to selection. The relationship between selection and response is directly mediated by the heritability. Thus, $R = h^2S$, where R is the response to selection and S is the strength of selection (Falconer & Mackay 1996). An h^2 of 0 implies that there is no variability under genetic control, and selection cannot affect the phenotypic value of a population, whereas a value of 1.00 implies that all observed differences in phenotypic values are due to genetic differences, and the population will be extremely amenable to selection.

4.1.2. Additive genetic variation in insect immune traits

Phenotypic traits that are closely connected to fitness should, theoretically, have low levels of additive genetic variation, because they are subject to strong selection, which drives optimal alleles to fixation (Fisher 1930). However, such traits are frequently shown to exhibit this type of variation in nature. Previous studies of insects have observed a high degree of phenotypic variation in the ability to respond to an immune

challenge, and that there is a genetic component to this variation. The mosquito *Anopheles gambiae* varies in its resistance (refractoriness) or susceptibility to the malaria causing agent: the sporozoan *Plasmodium*. Zheng et al. (1997) were able to generate lines of mosquitoes differing in their refractoriness, and were able to show that 70% of the variation in this trait was under the control of two loci, with susceptibility being recessive to refractoriness at each. Subsequent investigations identified two unlinked loci (Pif-B and Pif-C) involved in the determination of resistance (Vernick et al. 1989). In another dipteran, the fruit fly *Drosophila melanogaster*, levels of variation in susceptibility to hymenopteran parasitoids have been shown to be under genetic control. Iso-female lines showing differences in resistance to *Leptopilina boulardi* showed constancy within lines in the levels of resistance over several generations not subject to selection. This suggests an additive genetic element to this trait (Carton & Boulétreau 1985). The ability to melanotically encapsulate eggs of this parasitoid was found to be autosomally dominantly inherited (Carton et al. 1992). Resistance to another parasitoid, *Asobara tabida*, was also found to be autosomally dominant, at a loci independent of the *L. boulardi* system (Benassi et al. 1998; Hita et al. 1999). In a laboratory population of the orthopteran *Acheta domesticus*, two immune parameters (haemocyte load and capsule size) were found to have significant heritabilities (20% and 30% respectively), and therefore significant levels of additive genetic variance (Ryder 1999). Immune parameters in insects are therefore frequently heritable.

If a trait is able to respond to selection, then this is an indicator of additive genetic variance, because if h^2 is zero there can be no response to selection ($R = h^2S$; see above). Artificial selection for immune traits in insects has frequently produced large responses,

indicating high levels of additive genetic variance. Selection for resistance to granulosis virus in Indian meal moths resulted in a two-fold increase in levels of resistance within two years (Boots & Begon 1993). *A. gambiae* from a susceptible stock population were selected for refractoriness to *Plasmodium* species and achieved full refractoriness within a few generations (Collins et al. 1986). Likewise, *D. melanogaster* selected for resistance to three species of parasitoid (*A. tabida, L. boulardi* and *L. heterotoma*) showed a ten- to one hundred-fold increase in resistance after 5 generations of selection (Kraaijeveld & Godfray 1997; Fellowes et al. 1998; Fellowes et al. 1999b). This evidence indicates that, in these species at least, immune traits can retain a high heritability, and that optimal alleles (optimal in terms of parasite resistance) have not gone to fixation in the study populations.

4.1.3. Methods of assessing additive genetic variance

In this chapter, levels of additive genetic variance for immune traits in *T. molitor* are estimated by assessing the resemblance between relatives, using a nested full sib/half sib design (section 4.2.2), and partitioning the causes of this resemblance into additive genetic and non-additive effects (Lynch & Walsh 1998). In this design, larvae are reared at constant density, in an attempt to eliminate the environmental effects seen in chapter 3. This method allows the quantification of the total phenotypic variation of a sample and partitions it into its components, including the additive genetic variation, from which h^2 can be estimated (see, e.g., Stearns 1992; Falconer & Mackay 1996). Analyses are performed using both Henderson's ANOVA method (section 4.2.6.1; Henderson 1953; Stearns 1992) and, because of statistical problems with this, a maximum likelihood

procedure (section 4.6.2.2: dfREML; Meyer 1990). The statistical problems associated with the ANOVA method mean that estimates of heritability obtained using this technique are not meaningful, and so are not included here. The method is still presented, however, because the components of variance it estimates are informative about the regulation of the development of the traits.

4.1.4. Additive genetic correlations between traits

Data collected as outlined above can also be used to estimate genetic correlations between traits. Genetic correlations arise if the genotypes of two traits share genes in disequilibrium (e.g. due to linkage) or share genes with two distinct phenotypic effects (pleiotropy). Genetic correlations tend to be strongly positive between characters that are developmentally or functionally related, and negative between characters involved in trade-offs with each other (e.g. Windig 1994). This is particularly interesting in terms of the relationship between cuticular melanisation and the total haemocyte count. Both are implicated in immune function (see chapters 3 and 5 for cuticular melanisation, Kuris et al. 1980; Eslin & Prévost 1996; Eslin & Prévost 1998; Ryder 1999 for haemocyte numbers). There is currently much debate about 'immunocompetence', and whether this is a definable, assayable trait (Sheldon & Verhulst 1996) (see section 2.1.1). Ecological immunologists have used it to mean the relative ability of different individuals in a population to respond to pathogens (Sheldon & Verhulst 1996). This suggests that a 'highly immunocompetent' individual should be more resistant than a 'less immunocompetent' individual, regardless of the type of pathogenic challenge it is presented with. Different pathogen types are dealt with by different aspects of the

immune system, so assaying one aspect of immune function will not necessarily be informative about pathogen resistance in general (Braude et al. 1999). This has led to the suggestion that assays of immunocompetence should use a battery of immune traits (Lochmiller 1995). This chapter tests this idea by looking for genetic correlations between two putative immune traits (cuticular melanisation and total haemocyte count) that may be involved in resistance to diverse pathogen types (see above). If both these traits reflect an aspect of pathogen resistance then a positive correlation between them would support the idea that increased investment in immunity is global with respect to the immune system, and that these diverse aspects of immunity had a common genetic basis, implying the possibility for a genetic basis for immunocompetence as a whole.

4.1.5. Aims of the chapter

This chapter aims to quantify the level of genetic determination of the variability of two putative immune traits (cuticular melanisation and total haemocyte counts), by estimating the heritability of both using a full sib/half sib nested design to assess the resemblance between relatives. Genetic correlations between these traits are also investigated.

4.2. Materials and Methods

4.2.1. Beetle cultures

Beetles were taken from the University of Sheffield stock population, an outbred population maintained for several years . They were kept in a constant temperature room at 26±2 °c and maintained in *ad libitum* powdered rat chow, apple chunks and a water supplement.

4.2.2.The full sib/half sib design for assessing heritabilities

Heritabilities were estimated using a standard full sib/half sib design (Lynch & Walsh 1998). Beetles were removed from the stock population as pupae and sexed. They were then isolated to ensure that all individuals were virgins at the start of the experiment. When the adult beetles were 7-10 days old they were placed in a mating arena. A single male (sire) was placed into a plastic container (26 x 13 cm) with 20 females (dams). Brother/sister mating was avoided by taking the sires and dams from different stock boxes which had not been interbred for 1 generation prior to the experiment. The plastic container had approximately 5 pieces of cotton wool soaked in water, but no substrate for the dams to oviposit into. This procedure was replicated 23 times, and the dates on which each replicate was established was noted.

After 24 hours in the mating arena, the dams were removed and placed in individual boxes containing c.50 cm^3 of powdered rat feed and a 1/2 ml centrifuge tube of

water with a cotton wool plug. This allows the beetles to oviposit. Dams were left for 10 days in order to lay eggs and to allow those eggs to hatch.

At this time the offspring were sorted into containers so that they were at a constant larval density. The dam was removed from the culture and 10 offspring (all first instar larvae) were transferred to a plastic container containing c.150 cm^3 of rat feed. Rat feed was not renewed during the course of the experiment. Each culture had two 1/2 ml centrifuge tubes of water with cotton wool plugs added weekly. The larvae were allowed to develop to adulthood in this environment.

After the eclosion of the first pupa, cultures were sieved weekly and pupae were removed, sexed and isolated. The date of their imaginal eclosion was noted, and their larval development time calculated. On the fourth day after imaginal eclosion, the beetles were weighed, total haemocyte counts were performed (section 4.2.4) and both elytra were removed and frozen (-20°C) for later analysis of cuticular melanisation (section 4.2.5). Only the first four pupae to eclose into adults were analysed from each culture.

4.2.3. Sample size

Initially 23 males were each placed in a mating arena with 20 females. Not every female was successfully mated, and not all mated females produced 10 viable offspring. After data collection, the sample was balanced by only including the first 13 sib groups of a particular male in the analysis. This meant that the final analysed sample included 17 sires mated to 13 dams each, and 4 offspring per dam.

4.2.4. Haemocyte counts

Adult beetles were immobilised by chilling on ice for approximately ten minutes. Their genitals were extruded and severed to create a hole for haemolymph collection. Anti-coagulant (146mM NaOH, 216 mM NaCl, 25mM EDTA, 61 mM citric acid, pH=4.5, mOs/Kg=550) was then washed through the abdomen by injecting it anteriorly and collecting the perfused liquid through the posterior hole. 2ml were injected in total (see section 2.3). Haemocyte counts were performed using a haemocytometer (Weber Scientific) under phase contrast microscopy at x500 magnification (Leitz Diaplan).

4.2.5. Analysis of cuticular melanisation

Elytra were stored at -20°c until analysis. Cuticular melanisation was measured as a greyscale value from digitised images taken using a PULNiX TM-765 black and white camera and analysed using Optimas 6 software. The elytra were placed dorsal side down on a light box and the amount of light transmitted through the elytra measured. Higher greyscale values indicate more transmitted light and therefore lighter cuticles, whereas lower values indicate darker cuticles. All readings were calibrated to a single pale elytra arbitrarily designated as greyscale value 200. All other values are relative to this.

Because colour is measured as transmitted light in this chapter rather than reflected light (chapters 3 and 5), it is necessary to show that this measurement correlates to the *in situ* colour of beetles' elytra. This was done by taking 25 beetles at random from the stock population and ranking them visually according to the darkness of their cuticles, with 1 being the lightest and 25 the darkest. Elytra were then measured as above, and the

measurements again ranked 1 to 25. The two sets of ranks were then correlated against each other.

There was a significant correlation between the visual and measured rank (d.f. = 24, correlation coefficient = 0.971, $p < 0.0001$), indicating a strong degree of agreement between the visual colour of a beetle's elytra and their greyscale value measured using the above protocol. This means that the cuticular colour measures used in this chapter are compatible with those taken in chapters 3 and 5.

4.2.6. Statistical analysis of heritabilities

The heritabilities and genetic correlations of four traits were assessed (length of larval period [or 'age'], adult weight, cuticular melanisation and total haemocyte count). Heritabilities were originally analysed by Henderson's ANOVA method (Henderson 1953; Stearns 1992), but statistical problems (see section 4.2.6.1) meant that heritability estimates thus obtained were not meaningful. However, because the components of variance obtained using Henderson's ANOVA method are informative, the analysis is still presented below (section 4.2.6.1). In order to obtain meaningful estimates of heritabilities, a second analysis was performed using a maximum likelihood procedure (dfREML, Meyer 1990).

4.2.6.1. Henderson's ANOVA method

Henderson's ANOVA method uses a nested ANOVA to partition variance to sires, dams and offspring. The significance of the levels of variance partitioned between

sires, dams and offspring is determined by the F-statistic of the analysis. Mean square values have variance partitioned between them as follows:

Between sires (MS_S) = $\sigma^2_W + k\sigma^2_D + mk\sigma^2_S$

Between dams (MS_D) = $\sigma^2_W + k\sigma^2_D$

Between offspring (MS_W) = σ^2_W

(k = no. of offspring per dam, m = no. of dams)

From these the components of variation can be estimated:

Total phenotypic variation = $V_P = \sigma^2_T = \sigma^2_W + \sigma^2_D + \sigma^2_S$

Additive genetic variation = $V_A = 4\sigma^2_S$

Common environmental variation = $V_{EC} = \sigma^2_D - \sigma^2_S$

'Other' variation = $V_{EW} = \sigma^2_W - 2\sigma^2_S$

And finally these variance components can be used to estimate heritability. Two estimates are available: a sire estimate which comprises only the additive genetic variance and a dam component which includes additive genetic, non-additive genetic and maternal/common environment effects.

Sire component: $h^2 = \frac{4\sigma^2_S}{\sigma^2_T}$

Dam component: $h^2 = \frac{4\sigma^2_D}{\sigma^2_T}$ (Falconer & Mackay 1996)

Covariance between traits were to be estimated from the same data using a nested ANCOVA. Variance components can similarly be partitioned into components of

covariance, and the additive genetic correlations responsible for any observed phenotypic correlations can be estimated.

Henderson's ANOVA technique is however, susceptible to negative estimates of variance components (Khattree 1998). These lower the total phenotypic variance, artificially inflating the heritability and making the estimate obtained meaningless (Falconer & Mackay 1996). This was a problem with this data set for two of the measured traits (length of larval period and cuticular melanisation; see table 4.2). These negative estimates of environmental variance are, however, interesting in themselves and may be informative regarding the effects of the rearing environment on the affected traits. Because of this, results from this analysis are included in section 4.3.1 and discussed in section 4.4. The heritabilities obtained from this analysis, however, are not meaningful and so are not presented.

4.2.6.2. dfREML

A second analysis was performed on the collected data using a maximum likelihood technique. Maximum likelihood finds the estimate for a parameter which maximises the probability of observing the data given a specific model. The likelihood function is maximised iteratively, until the parameter estimates (in this case estimates of variance components) are most likely to yield the observed data set given the model of analysis. These iterations are performed using only non-negative estimates of variance components, precluding the problems with the ANOVA method (section 4.2.6.1). In addition, maximum likelihood techniques are more robust than Henderson's ANOVA technique (Smith & Savage 1992). The program used for the analysis was derivative free

maximum likelihood (dfREML; Meyer 1990), which yields a single estimate of heritability (the sire component estimate) for each trait, as well as estimates of genetic correlations between traits, and standard errors for these.

4.3. Results

4.3.1. Components of variance estimated using Henderson's ANOVA technique

There was a significant within sire component of variance for all four traits measured, indicating significant levels of additive genetic variance in each case (see table 4.1). However, for two of the traits (cuticle and age), there are highly negative environmental components of variance (table 4.2). This artificially reduces the total phenotypic variance (Vp) while leaving the additive genetic component (Va) unaffected. Since h^2 is a ratio of Va to Vp, this results in inflated heritability values. These negative environmental components of variance are informative with regards to the regulation of the trait during development, and the data in table 4.2 are included for discussion in section 4.4. However, they do render the data useless in estimating heritabilities and genetic correlations.

4.3.2. Heritabilities estimated using dfREML

All four measured traits yielded heritabilities significantly different from zero (table 4.3) and less than 100% (age = 95.4±6.1%, weight = 53.1±9.0%, cuticle = 59.3±8.6%, haemocytes = 49.2±9.4%), indicating a significant contribution of additive genetic variance to the total phenotypic variance.

4.3.3. Genetic correlations estimated using dfREML

None of the four traits measured in the analysis were phenotypically or genetically correlated (table 4.4)

4.4. Discussion

Both the degree of cuticular melanisation of the beetles' elytra and the total number of haemocytes within the haemocoel show significant non-zero levels of additive genetic variance. This yielded estimates of heritability of 59.3±8.6% for the former and 49.2±9.4% for the latter, using a maximum likelihood method of estimation (dfREML; (Meyer 1990). Both traits therefore have part of their variability under genetic control, and will be amenable to selection. The two other (non-immune) traits measured (weight and length of larval period) were also significantly heritable (table 4.3). There were no phenotypic or additive genetic correlations between the two putative immune traits, indicating that they are not in disequilibrium and do not share a common genetic pathway (table 4.4).

Traits closely related to fitness are predicted to have low heritabilities because strong selection will reduce the genetic variability (Fisher 1930). Meta-analyses have shown that fitness components do have lower heritabilities than other types of traits (Mousseau & Roff 1987). Immune systems, however, frequently show significant levels of heritable variation. This is to be expected in vertebrate systems, where variability is intrinsic to immune function, and is probably maintained by coevolutionary cycles of host

Table 4.1: ANOVA tables for four traits assessed in the full sib/half sib design described in section 4.2. The mean squared values are used to estimate variance components (section 4.2.6.1) and heritabilities (shown in table 4.2). The significant p values indicate that there is a significant contribution of the variance on both the sire and dam levels to the total genotypic variance, indicating that there is a significant non-zero level of additive genetic variance, and therefore significant heritability, of all four traits.

Trait	Component	df	Mean Squares	F	p
Age	Sire	16	2967	47.48	< 0.001
	Dam	216	62	2.08	< 0.001
	Offspring	702	30	-	-
Weight	Sire	16	1860	6.55	< 0.001
	Dam	216	284	1.78	< 0.001
	Offspring	702	160	-	-
Cuticle	Sire	16	10027	12.48	< 0.001
	Dam	216	803	1.68	< 0.001
	Offspring	702	477	-	-
Haemocytes	Sire	16	3.23×10^{11}	2.91	< 0.001
	Dam	216	1.11×10^{11}	1.83	< 0.001
	Offspring	702	6.06×10^{10}	-	-

Table 4.2: Genetic components of variation of four traits estimated using Henderson's ANOVA method (section 4.2.6.1). (Degrees of freedom equal 16 in all cases). Two of the traits ('age' and 'cuticle') have negative environmental components of variance (V_{Ec}). These make the estimation of heritability for these two traits using Henderson's ANOVA method meaningless (section 4.3.1). Negative V_{Ec} may be indicative that competition between larvae is driving the phenotypic values of individuals within a culture apart (discussed in section 4.4). Heritabilities of all four traits are therefore estimated using a different method of analysis (section 4.2.6.2 and table 4.3).

Trait	Variance Components			
	V_P	V_A	V_{Ec}	V_{Ew}
Age	89.56	210.24	-45.88	-74.81
Weight	221.24	121.24	0.72	99.28
Cuticle	763.35	735.2	-96.85	125.3
Haemocytes	7.86×10^{10}	7.74×10^{10}	8.68×10^{9}	5.25×10^{10}

Table 4.3: Genetic components of variance of four traits and their heritabilities estimated using dfREML (Meyer 1990). Standard errors are provided by dfREML.

Trait	df	Variance Components		$h^2 \pm$ 1 s.e.
		V_P	V_A	
Age	16	22.38	21.35	95.4±6.1%
Weight	16	219.38	116.47	53.1±9.0%
Cuticle	16	698.08	413.97	59.3±8.6%
Haemocytes	16	2.06×10^{10}	1.01×10^{10}	49.2±9.4%

Table 4.4: The phenotypic and additive genetic correlations between the traits studied in chapter 4, calculated by dfREML (Meyer 1990). There were no significant additive genetic correlations between any combinations of the four traits. (thc = total haemocyte count).

Traits		Phenotypic Correlation			Additive Genetic Correlation		
		d.f.	r	p	d.f.	r	p
age	weight	883	0.237	< 0.01	16	0.130	> 0.05
age	cuticle	883	-0.038	> 0.05	16	-0.015	> 0.05
age	thc	883	0.010	> 0.05	16	-0.101	> 0.05
weight	cuticle	883	-0.120	< 0.01	16	0.037	> 0.05
weight	thc	883	0.111	< 0.05	16	-0.120	> 0.05
cuticle	thc	883	0.010	> 0.05	16	-0.073	> 0.05

and pathogen genotypes (Hamilton & Zuk 1982), but this is not necessarily the case in invertebrates. Nevertheless, heritable variation is often seen in invertebrate systems. The two heritability values obtained for putative immune function (59% for cuticle, 49% for haemocytes) are higher than those reported elsewhere in the literature. *Drosophila melanogaster* showed 43% heritability in their ability to encapsulate eggs of the parasitoid *Leptopilina boulardi* (Carton & Boulétreau 1985). Resistance of *D. melanogaster* to its parasitoids in general has been reported as being about 25% heritable in a natural population (Fellowes & Godfray 2000). In *Acheta domesticus*, haemocyte load and encapsulation were both significantly heritable (30% and 20% respectively) in a laboratory population (Ryder 1999).

Traits with heritabilities approaching 50% were described by Mousseau and Roff (1987), as 'morphological', although this does not preclude them having important functions. The heritability estimate obtained for cuticular melanisation is broadly in agreement with estimates for melanic traits in other insects, some of which have been shown to have specific functions capable of influencing fitness. Melanic wingspots in Lepidoptera have been shown to be highly heritable (Majerus 1998). In *Bicyclus anynana* (Nymphalidae) heritability of wing spots vary from 47% to 76%, depending on age and sex (Monteiro et al. 1994). Similar values (40% to 57%) have been reported in other butterfly species (Kingsolver & Wiernasz 1991; Windig 1999). In all these cases melanism has been shown to function in thermoregulation (Brakefield & Lees 1987; Windig 1994). Another lepidopteran, *Mamestra brassicae*, shows high heritabilities for larval melanisation (42% in the 5^{th} instar), which is also a thermoregulatory trait (Goulson 1994). In other insect orders (Homoptera: Marooka & Tojo 1992, Stewart &

Lees 1996; Dictyoptera: Appel & Tanley 1999; Coleoptera: De Jong et al. 1993, Majerus 1998), genetic polymorphisms exist for cuticular melanisation, even where melanisation has been shown to have a fitness related function. In the two-spot ladybird (*Adalia bipunctata*), aposematic spots show heritabilities of between 40% and 80% (Holloway et al. 1995). Thus cuticular melanisation of *T. molitor* shows heritability higher than those found for immune traits but similar to other selectively non-neutral melanic traits in insects.

The failure of the ANOVA method to produce meaningful estimates of heritability (section 4.2.6.1) is, in itself, interesting. The common environmental component of variation is calculated by subtracting the 'between sire' (σ^2_S) component of variance from the 'within sire, between dam' (σ^2_D) component (Stearns 1992). Negative values imply that there is more variation within the offspring of a single sire than there is between the offspring of multiple sires. This can occur if there is competition within cultures, causing phenotypic values to be driven apart (Falconer & Mackay 1996). However, mortality did not appear to be very high in the cultures, probably less than that in the gregarious cultures reported in chapter 3 (section 3.3.1) and the larval competitive ability cultures in chapter 5 (section 5.3.3) (personal observation), indicating that competition was probably not particularly intense. The two traits thus affected were development time and cuticular melanisation. The former may be explicable in terms of the avoidance of cannibalism. The pupal period is highly susceptible to cannibalism because it is immobile, and the majority of cannibalism in the later stages of development is by adults on pupae (Weaver & Macfarlane 1990). Thus, if a larva is able to perceive that it is 'losing' the race to adulthood, it may be beneficial to delay the pupal stage until

it no longer perceives a risk of cannibalism. Plasticity in development time in *T. molitor* has previously been attributed to cannibalism avoidance (Weaver & Macfarlane 1990; Savvidou & Bell 1994), and could, potentially, cause negative environmental variance. There is no evidence that competition, if it exists, is resource based, because there is actually a positive phenotypic correlation between weight and development time (table 4.4). Thus there may be alternative strategies available within a single culture: develop quickly to avoid cannibalism or develop slowly to accumulate resources. This may cause the observed high variance within cultures. The observed phenotypic correlations did not have an underlying additive genetic cause (table 4.4).

The negative environmental variance for cuticular melanisation is more difficult to explain. It is intriguing to think that in this case competition may be resource based. The potential costs of melanic traits have generated much recent interest, particularly because of the role of melanin in the insect immune system and the explosion of the field of ecological immunity. Most putative costs concentrate on the process of melanogenesis, which involves the production of toxic substances (Nappi & Vass 1993), but this is unlikely to be affected by competition. A potential cost of melanogenesis is the utilisation of resources in its production that are essential to other systems and environmentally limited. Interest in this regard has centred on the amino acid tyrosine. This is the substrate for melanogenesis and must either be acquired from the environment or manufactured from phenylalanine, itself an essential amino acid. Because of its role in melanin formation, tyrosine is required in all life stages and a number of processes, including cuticular tanning (Chapman 1982), immune responses (e.g. Nappi & Vass 1993), the formation of melanic signals (Majerus 1998) and egg production (Ferdig et al. 1993).

There is therefore a high demand for tyrosine and the potential for environmental limitation. However, no empirical evidence currently exists that the latter actually occurs. If it does, then resource competition within cultures may result in unequal distribution of tyrosine between individuals, causing the negative environmental variances seen in this chapter. The fact that there is a negative phenotypic correlation between cuticular melanisation and weight lends some support to this idea (darker cuticles had lower greyscale values, so a negative correlation means that darker individuals weighed more), because it implies that individuals that have accumulated more resources (i.e. were heavier) had darker cuticles. This idea is, however, speculative.

There was no correlation between cuticular melanisation and total haemocyte count, either on a phenotypic or genetic level. Current definitions of immunocompetence suggest that levels of one immune parameter should be correlated to levels of another, if an individual is to be designated as having a particular level of 'immunocompetence' (Sheldon & Verhulst 1996). This is clearly not the case here. There are good reasons for thinking that total haemocyte number (Kuris et al. 1980; Eslin & Prévost 1996; Sheldon & Verhulst 1996; Eslin & Prévost 1998; Ryder 1999; discussed in section 2.4.1) and the degree of cuticular melanisation (chapters 3 and 5) both reflect an aspect of pathogen resistance ability. A positive correlation between these traits would be consistent with the idea that individuals invest in 'immunity' at a particular level, and if this correlation was on the genetic level, it would suggest that 'immunocompetence' has a genetic component. Conversely, a negative relationship would be consistent with the 'immunoredistribution' hypothesis, in which resources are shunted around the components of the immune system as required, causing no overall change in the level of 'immunity' whilst altering the levels

of these different components (Braude et al. 1999). No correlations, positive or negative, phenotypic or genetic, were found, indicating that the two traits under discussion are effectively independently regulated.

4.5. Summary

In this chapter I have shown that additive genetic variance exists in four measured traits: length of larval development, adult weight, total haemocyte count and cuticular melanisation. Total haemocyte count and cuticular melanisation are both traits which potentially reflect a beetle's ability to resist pathogenesis and, as such, are closely related to fitness. The existence of additive genetic variability in both traits is indicative of an associated cost of immunity. None of the measured traits are genetically correlated to each other.

Chapter 5
Correlated Responses to Selection for Cuticular Melanisation

5.1. Introduction

Phenotypic plasticity in the level of cuticular melanisation is commonly seen in insects (Applebaum & Heifetz 1999). Darker cuticles have been shown to be correlated to pathogen resistance in two insect taxa: Lepidoptera (Goulson & Cory 1995; Reeson et al. 1998) and Coleoptera (chapter 3). In these studies, both cuticular melanisation and pathogen resistance are correlates of a third variable (larval density). This chapter aims to examine whether the former two traits are correlated independently of the latter, and whether this correlation is on a genetic, as well as phenotypic, level. A genetic correlation between cuticular melanisation and fungal resistance would show that the former could be used as a reliable indicator of the latter, making it an easily assayable immune trait. The chapter also investigates the maintenance of the observed additive genetic variance in this putative immune trait, by investigating a further correlated response to selection, namely the relative ability of selected lines to survive in conditions causing high levels of larval competition.

5.1.1. The meaning of correlated responses to selection

If genetic correlations exist between two traits then selecting for one will result in a correlated response in the other (Stearns 1992). This chapter aims to show the existence of a genetic correlation between cuticular melanisation and resistance to the generalist fungal entomopathogen *Metarhizium anisopliae* by selecting for the former and looking for a correlated response in the latter. The ability of a character to respond to selection is equivalent to its heritability ($R = h^2S$; Falconer & Mackay 1996; also see section 4.1.1). The stock population of *T. molitor* showed significant heritability in the level of cuticular melanisation (h^2 = 59.3±8.6%, see table 4.3), indicating that it will be amenable to selection. Any correlated response in other characters is indicative of a significant regression in the breeding values between selected and responding traits. Such regressions arise because of genetic correlations between traits, implying that there is either linkage (the genes involved in different traits being in disequilibrium) or pleiotropism (a single gene having distinct phenotypic effects in each trait) in genes contributing to the additive genetic variance of the two traits (Falconer & Mackay 1996). In this chapter, a positive genetic correlation is predicted, so that lines in which beetles have been selected for greater cuticular melanisation will also show greater levels of resistance to fungus, and conversely, lines selected for lighter cuticles will show lower levels of resistance. This will provide a genetic basis for the phenotypic correlation seen in chapter 3.

5.1.2. The maintenance of additive genetic variation in immune-linked traits

If cuticular melanisation is an immune-linked trait, then the large amount of additive genetic variance for this trait must be actively maintained, because the ability to resist disease is a trait closely allied to fitness, and should hence show low heritability (Fisher 1930). However, immune traits have frequently been shown to have large amounts of additive genetic variance (reviewed in section 4.1.2). Theory suggests that in order to maintain this variation there must be a cost associated with the expression and maintenance of immune function. There is much evidence that this is the case in invertebrates. Immune defences tend to be inducible, rather than constitutively maximally expressed, indicating that the benefit of expression is offset by a cost (Harvell & Tollrian 1999). Immune responses are implicated in the production of harmful substances involved in cellular damage (Nappi & Vass 1993; von Schantz et al. 1999) and the expression of immunity is associated with a reduction in other life-history traits (König & Schmidt-Hempel 1995; Siva-Jothy et al. 1998; Rigby & Jokela 2000) (see section 1.4.2). Identifying the specific costs of immunity is essential to understanding the maintenance of additive genetic variance in immune traits (Gemmill & Read 1998).

Previous selection experiments have indicated a negative genetic correlation between immune function and life-history traits. Selection resulting in increased resistance to granulosis virus in Indian meal moths also caused an increase in development time, pupal weight, and the proportion of eggs failing to hatch. This was calculated to equal a theoretical 15% drop in fitness (Boots & Begon 1993). *Drosophila melanogaster* selected for resistance to hymenopteran parasitoids showed a decreased ability to compete as larvae in conditions of food stress (Kraaijeveld & Godfray 1997),

due to a decrease in their feeding rates compared to non-selected stock (Fellowes et al. 1998; Fellowes et al. 1999a). The mechanisms of trade-offs between immunity and other life history traits are not known, although energetic limitation (König & Schmidt-Hempel 1995; Siva-Jothy et al. 1998), resource limitation (Sheldon & Verhulst 1996) and antagonistic pleiotropy (Kraaijeveld & Godfray 1997) have all been suggested.

5.1.3. Larval competitive ability as a potential trade-off with immunity

This chapter will also use selected lines to identify an associated cost of immunity, by looking for a negative correlated response to selection for cuticular melanisation in the ability of larvae to compete in conditions of nutritional stress. Similarly to *Drosophila* (Kraaijeveld & Godfray 1997), *T. molitor* show increased larval mortality at higher population densities (Weaver & Macfarlane 1990; Savvidou & Bell 1994), indicating that larval competition is an important determinant of fitness in both species. In *Drosophila*, the mechanism of competition was indirect, through relative feeding rates of the larvae (Fellowes et al. 1998; Fellowes et al. 1999a). *Tenebrio* are facultatively cannibalistic, and cannibalism increases at higher larval densities (Savvidou & Bell 1994; see section 3.3.1), indicating that the ability to survive in conditions causing higher larval competition may be directly linked to the ability to cannibalise and/or avoid cannibalism. Pre-adult mortality is likely to be an important determinant of fitness, and hence one of the traits which may possibly be traded-off against increased investment in immunity. Kraaijeveld and Godfray (1997) found that, in *Drosophila*, selection for increased immunity caused a correlated drop in larval competitive ability. A similar outcome is predicted in this chapter, i.e. that lines selected for increased melanisation

(and therefore, potentially, increased fungal resistance) will show a reduced ability to compete as larvae against lines selected for decreased cuticular melanisation.

5.1.4. Aims of the chapter

This chapter aims to show that selection for cuticular melanisation in *T. molitor* causes a correlated response in the level of susceptibility to the fungal pathogen *M. anisopliae*. This will provide an additive genetic basis for the phenotypic correlation shown in chapter 3, and will show that cuticular melanisation is a marker for fungal resistance. The maintenance of additive genetic variation in cuticular melanisation is also investigated, by looking for a negative genetic correlation (indicative of a trade-off) between cuticular melanisation and another aspect of life-history, the ability to compete as larvae.

5.2. Materials and methods

5.2.1. Selection procedure

Beetles used in this experiment were from the fifth generation of lines selected for their degree of cuticular melanisation. Lines were the result of selection for either very dark (BLACK or B) or very light (TAN or T) elytra. Selection was via an 'artificial selection' protocol in which the reproducing individuals were directly chosen by the experimenter on the basis of their phenotype. This contrasts the 'laboratory evolution' type experiment, in which a population of organisms are exposed to an environment in which the selecting agent is included. The former design excludes much of the normal selection which individuals undergo in culture, the latter does not (Rose et al 1996).

Because of this, artificial selection experiments do not require large populations in each generation whereas laboratory evolution studies do in order to maintain a normal response to selection (Rose et al 1996). Artificial selection for colour in *Tenebrio* is performed broadly following within-family selection protocols such as those employed in selection experiments on phenotypic traits in other insects (*Drosophila*: Partridge & Fowler 1993, Ritchie et al 1996; *Onthophagus*: Emlen 1996). In these experiments multiple pairs of individuals are used per generation (between 4 and 7), ostensibly to reduce the effects of inbreeding depression and spurious genetic correlations (Falconer 1996). However, small populations are still subject to these effects so multiple replication of lines (between 8 and 30 in the examples cited) is required, regardless of population size.

For the work presented in this chapter, lines were initiated by taking individuals with extreme phenotypes (light or dark elytra) from an outbred stock population and mating them in pairs. Subsequently, the offspring off each pair with the most extreme phenotype were mated to carry on the line. Cuticular colour was assessed by eye on the fourth day after emergence as adults in each generation. Hence after the initiation of the lines, each new generation was formed by the mating of a single brother/sister pair with the most extreme cuticular phenotype. This stringent selection was performed to produce the maximum possible response to selection given limited time. Because this design can give rise to spurious genetic correlations within lines, 10 replicate lines selected in each direction (BLACK and TAN) were set up, but not all lines survived to the fifth generation (see section 5.2.2.4).

Lines were maintained in powdered rat chow. Centrifuge tubes containing water were added approximately weekly. This was occasionally supplemented with apple chunks.

5.2.2. Assaying selected lines for susceptibility to fungus

5.2.2.1. Analysis of cuticular melanisation

Individuals from the fifth generation of the selected lines were removed from their cultures on pupation, sexed and isolated. On the fourth day after eclosion as adults the beetles were weighed on a balance (Mettler PM480) and had their cuticular melanisation evaluated *in situ*. This was done by placing the beetle in a box containing a single diffused constant light source. A hole in the top of the box allowed images of the beetle to be collected using a PULNiX TM-765 black and white camera without interference from ambient light. Images thus collected were digitised and analysed using Optimas 6 software. Elytra darknesses were measured as greyscale values. Because it was necessary to measure elytra *in situ* in this chapter (they were removed in chapter 4; section 4.2.5), values are not comparable between chapters. The measurements in this chapter were calibrated using a paint sampler as a standard.

5.2.2.2. Preparation of fungal inocula

Spore preparations and assays were performed as described by Goettel and Inglis (1997). Fungus was cultured on PDA plates and doses were prepared by scraping fungal spores into a 0.05% Triton X-100 solution. Spore concentration was determined by

counting spores with the aid of a compound microscope (Leitz Diaplan) using a haemocytometer. Only intact spores were counted. Inoculates were never older than 24 hours and were stored at 4°C when not in use.

5.2.2.3. Fungal bioassay

Beetles were assayed immediately after weighing and melanisation analysis (section 5.2.2.1). Beetles from each selected line were each assigned to one of six doses of fungus (0.0, $1.0x10^7$, $2.5x10^7$, $5.0x10^7$, $7.5x10^7$, $1x10^8$ spores/ml). They were assigned to a particular dose by order of eclosion, with the first beetle assigned to $1.0x10^8$ spores/ml, the second to $7.5x10^7$, etc., repeating the series after 6 beetles. Beetles were exposed to the fungus by dipping them in 1ml of spore solution for 5 seconds. Control beetles were dipped in 0.05% Triton X-100 with no fungal spores. They were incubated individually at 26°C, 70% RH for six days, after which mortality was scored.

5.2.2.4. Sample sizes for the fungal bioassay

A total of 9 of the selected lines were used in the fungal bioassay: 5 BLACK (lines B3, B5, B6, B7, B9) and 4 TAN (T2, T5, T6, T7). Initially, the intention was to use four beetles per line per dose, but logistics dictated that this was not possible. Therefore, the lines had a variable number of animals assigned as described above (n = 24, 24, 24, 12, 24, 16, 10, 16, 24 respectively, or a total sample of 108 individuals from BLACK lines and 66 from TAN lines).

5.2.3. Assaying selected lines for their larval competitive ability

5.2.3.1. Genotyping individuals

Individuals from the fourth generation of the selected lines were removed from their cultures on pupation, sexed and isolated. On the second day after imaginal eclosion they had a middle limb removed for allozyme analysis. This was homogenised in 35 µl of extraction buffer (100µl Triton X-100, 0.13mM NADP, 6mM DTT, in 100 ml dH_2O). All samples were frozen at -80°C before electrophoresis.

Genotyping was done by visualising the phosphoglucose isomerase (PGI) locus using cellulose acetate gels. Gels were soaked in Tris glycine buffer (17mM Trizma base, 192mM glycine, 1 L dH_2O) for 20 minutes before loading. The gel was run on tris maleate buffer (100mM Trizma base, 40mM Maleic acid, 1L dH_2O) at 200 volts (1-2 amps) for 18-20 minutes.

Bands were visualised by developing the gel for 2-5 minutes using PGI stain [3ml tris-HCl (92mM Trizma base, 400 ml dH_2O, pH = 8.0), 200 µl NAD (30mM), 100 µl fructose-6-phosphate (50mM), 100 µl MTT (24mM), 100 µl PMS (7mM), 5 µl glucose-6-phosphate dehydrogenase, 3 ml agar (2.0 g in 125 ml dH_2O)]. Two alleles were present in the population, designated slow (S) and fast (F) depending on their mobility on the gels.

5.2.3.2. Assessing larval competitive ability

By knowing the genotype at the PGI locus of individuals within selected lines, it was possible to produce offspring with known genotype from controlled matings. Four

males and four females (all virgin) with known genotypes from a single line were placed in a mating arena (a petri dish containing cotton wool soaked in water but no oviposition substrate) and left overnight to mate freely. The females were removed 24 hours later and placed in a petri dish containing flour and allowed to oviposit. After one week the flour was sifted for the presence of eggs. These were allowed to hatch, producing larvae of known genotype.

Selected lines which were complimentary with respect to colour (i.e. a BLACK with a TAN) and genotype (e.g. one set of larvae being SS and the other being FF and/or SF) were paired together. Ten first instar larvae from each line were put together in one of three rearing conditions: 'Full food' (c.150 ml of rat feed, 1/2 ml of water weekly), 'half food' (c.50 ml of rat feed, 1/2 ml of water weekly) and 'no water' (c.150 ml of rat feed with no liquid supplement).

At the end of the experiment (see section 5.2.3.3), viable individuals were collected and immediately frozen at –80°C. Whole individuals were homogenised in 0.5 ml of extraction buffer, pending genotyping at the PGI locus as above

5.2.3.3. Sample sizes for larval competitive ability

Six pairs of lines (T2xB5, T5xB7, T1xB6, T7xB3, T3xB2, T6xB9) were set up under all three culture conditions (full food, half food and no water) for sampling as adults. In addition, the whole set up was replicated twice, so that survival could also be assessed early (after one month). There were insufficient larvae produced to use six pairs of lines per treatment for the one month sample, so this included 5 full food (T2xB5,

T5xB7, T1xB6, T7xB3, T3xB2), 4 half food (T5xB7, T1xB6, T7xB3, T3xB2) and 4 no water (T2xB5, T5xB7, T7xB3, T3xB2) pairings.

5.2.4. Analyses

The data from the fungal assays were analysed using logistic regressions in Statview version 5.0 (SAS) for the Macintosh. Larval competitive abilities were also analysed in Statview.

5.3. Results

5.3.1. Correlated responses to selection of weight and cuticular melanisation

There were significant differences between the lines with respect to weight (ANOVA; d.f = 8, F = 6.385, $p < 0.0001$; see figure 5.1). However, these differences were not systematic with respect to the direction of selection (ANOVA; d.f. = 1, F = 0.082, p = 0.7754), and there was no significant correlation between cuticular colour and weight within individual beetles (correlation; d.f = 176, r = 0.038, $p > 0.05$).

There were significant differences between the selected lines in their degree of cuticular melanisation (ANOVA; d.f = 8, F = 30.808, $p < 0.0001$). Cuticles were significantly darker in the BLACK lines than in the TAN lines (Tukey-Kramer multiple comparison test; $p < 0.05$) and there were no significant differences between lines selected in the same direction, with the exception of T6 being significantly darker than T2 (see figure 5.2).

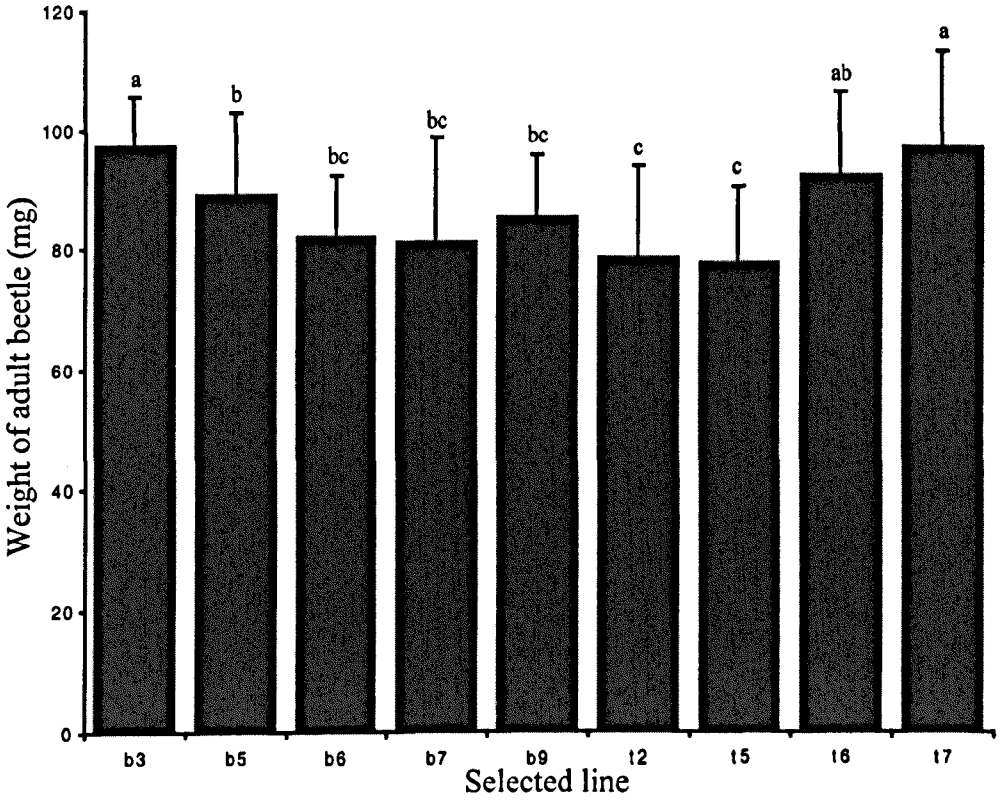

Figure 5.1: Mean weights of adults from the nine seleted lines used in chapter 5. Bars show one standard deviation. Selected lines sharing letters above the error bars are not significantly different (Fisher's PLSD, $p > 0.05$). Although lines do differ in their adult weight (ANOVA; d.f = 8, $F = 6.385$, $p < 0.0001$), these differences are not systematic with respect to the direction of selection, i.e. BLACK lines are not consistantly heavier or lighter than TAN lines (see section 5.3.1).

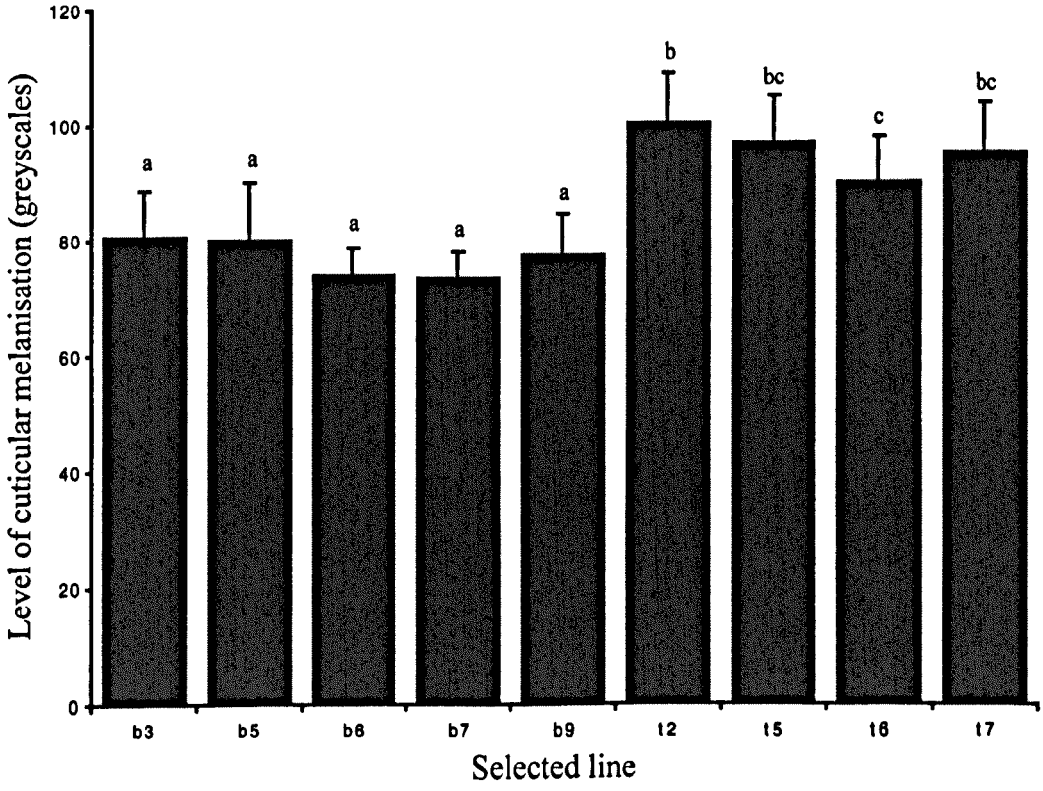

Figure 5.2: Mean level of cuticular melanisation of adults from the nine seleted lines used in chapter 5. Bars show one standard deviation. Selected lines sharing letters above the error bars are not significantly different (Fisher's PLSD, $p > 0.05$). Lines differ in their level of cuticular melanisation (ANOVA; d.f = 8, $F = 30.808$, $p < 0.0001$), and this difference is systematic with respect to the direction of selection, i.e. BLACK lines are consistantly darker than TAN lines, indicating a successful response to the selection regime (see section 5.3.1).

5.3.2. Correlated response to selection of fungal resistance

It was not possible to assign specific LD_{50} values to each line because overall mortality was too high. A logistic regression was performed in which the terms 'direction of selection', 'dose' and 'sex of individual' were included in the model for 'mortality', and terms were excluded if they did not contribute to the fit of the model at the $p \leq 0.10$ level. The term 'sex...' (d.f. = 1, $\chi^2 = 0.03$, $p = 0.855$) was thus excluded. Only one term contributed significantly to the fit at the $p < 0.05$ level: dose (d.f. = 5, $\chi^2 = 7.23$, $p = 0.007$). 'Direction of selection' was marginally non-significant (d.f. = 1, $\chi^2 = 3.212$, $p = 0.073$), although there was a trend towards lines selected for darker cuticles showing lower susceptibility to the fungus (figure 5.3) . If, however, the analysis is repeated using the factors 'dose', 'sex', 'mean weight of beetles in a selected line' and 'mean cuticle of beetles in a selected line', then 'sex' (d.f. = 1, $\chi^2 = 0.126$, $p = 0.778$) and 'mean weight...' (d.f. = 8, $\chi^2 = 0.469$, $p = 0.844$) drop out as not contributing significantly to the fit of the model. Two terms contributed significantly: dose (d.f. = 5, $\chi^2 = 18.244$, $p < 0.0001$) and 'mean cuticle...' (d.f. = 8, $\chi^2 = 6.349$, $p = 0.021$) (see table 5.1).

5.3.3. Differences between treatments in mortality

There were no significant differences in mortality between the one month and adult samples in mortality (ANOVA; d.f. = 1, $F = 0.247$, $p = 0.624$), although the power of this test was low (8%). In an attempt to increase this power, a paired t-test was done on

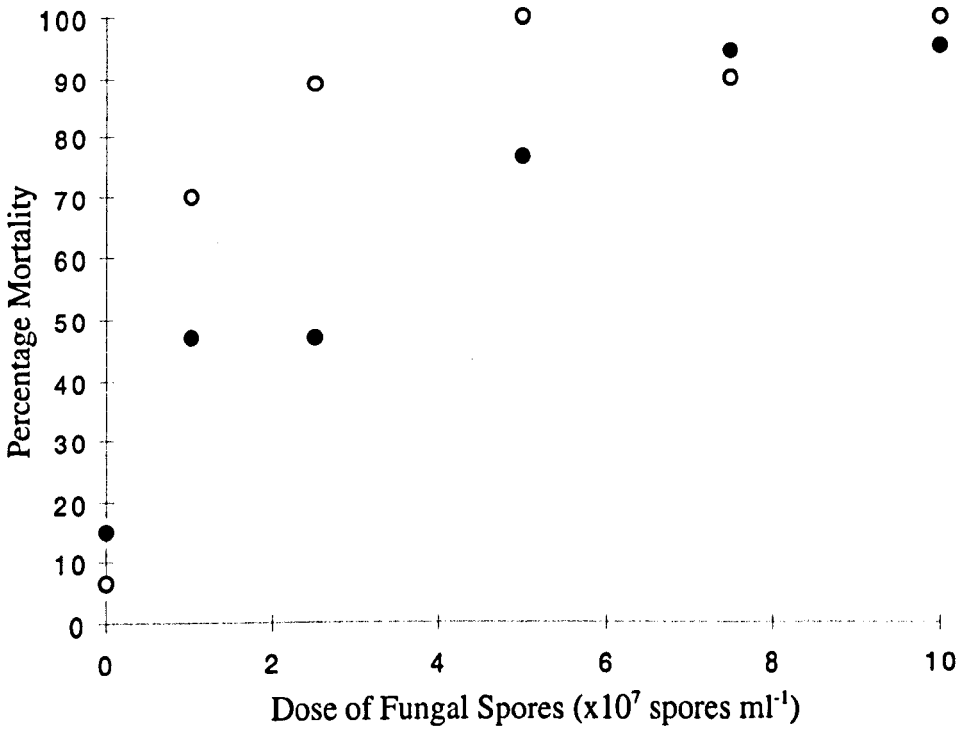

Figure 5.3: The percentage mortality of beetles from line selected to have either darker (closed circles) or lighter (open circles) cuticles. Fungal dose contributed significantly to the probability of mortality (d.f. = 5, χ^2 = 7.23, p = 0.007), with higher doses of fungus resulting in more dead beetles. Direction of selection did not contribute significantly to the probability of mortality, though this non-significance was marginal (d.f. = 1, χ^2 = 3.373, p = 0.066), and there appears to be a strong trend in which lines selected for darker cuticles are less likely to suffer mortality.

Table 5.1: The overall percentage mortality of the selected lines used in this experiment, after exposure to *M.anisopliae*. Lines prefixed with a B have been selected to have darker cuticles, lines prefixed with a T have been selected for lighter cuticles The efficacy of selection of a line (represented by its mean cuticular colour in the experimental generation) contributes significantly to the probability of mortality (logistic regression: d.f. = 8, χ^2 = 6.349, p = 0.021; see section 5.3.2).

Line Name	N	Mean Cuticular Colour (greyscales)	Percentage Mortality
B3	20	80.5	65%
B5	20	80.0	90%
B6	20	73.6	70%
B7	8	73.0	50%
B9	20	77.1	75%
T2	12	100.2	92%
T5	8	96.9	88%
T6	13	90.1	92%
T7	20	92.3	90%

the lines and treatments that were present in both the one month and adult samples. This was also non-significant (paired t-test; d.f. = 12, t = 0.985, p = 0.344).

There were, however, significant differences between the treatment groups (d.f. = 2, F = 9.847, p = 0.0007). Mortality was higher in the no water group than in the full food (Fisher's PLSD; p = 0.0002) and half food (Fisher's PLSD; p = 0.0094) treatments. There was no significant difference between the latter two treatments (Fisher's PLSD; p = 0.138) (see figure 5.4).

5.3.4. Correlated response to selection of larval competitive ability

There were no significant differences in larval mortality between the offspring of parents from lines selected in the TAN and BLACK directions in any of the treatment groups. The proportion of surviving beetles that came from BLACK lines did not differ significantly from the starting proportion of 0.5 in the full food (one-group t-test; d.f = 5, t = 0.86, p = 0.935), half food (one-group t-test; d.f = 5, t = -0.527, p = 0.621) or no water (one-group t-test; d.f = 5, t = -0.107, p = 0.919) treatments. Only data from the 'adult' time group is included in this analysis, because the '1 month' samples were small and the two cannot be combined because of pseudoreplicative effects.

5.4. Discussion

The level of cuticular melanisation is positively genetically correlated with the level of resistance to the generalist entomopathogenic fungus *Metarhizium anisopliae* in the mealworm beetle *T. molitor*. Selection for cuticular colour caused significant

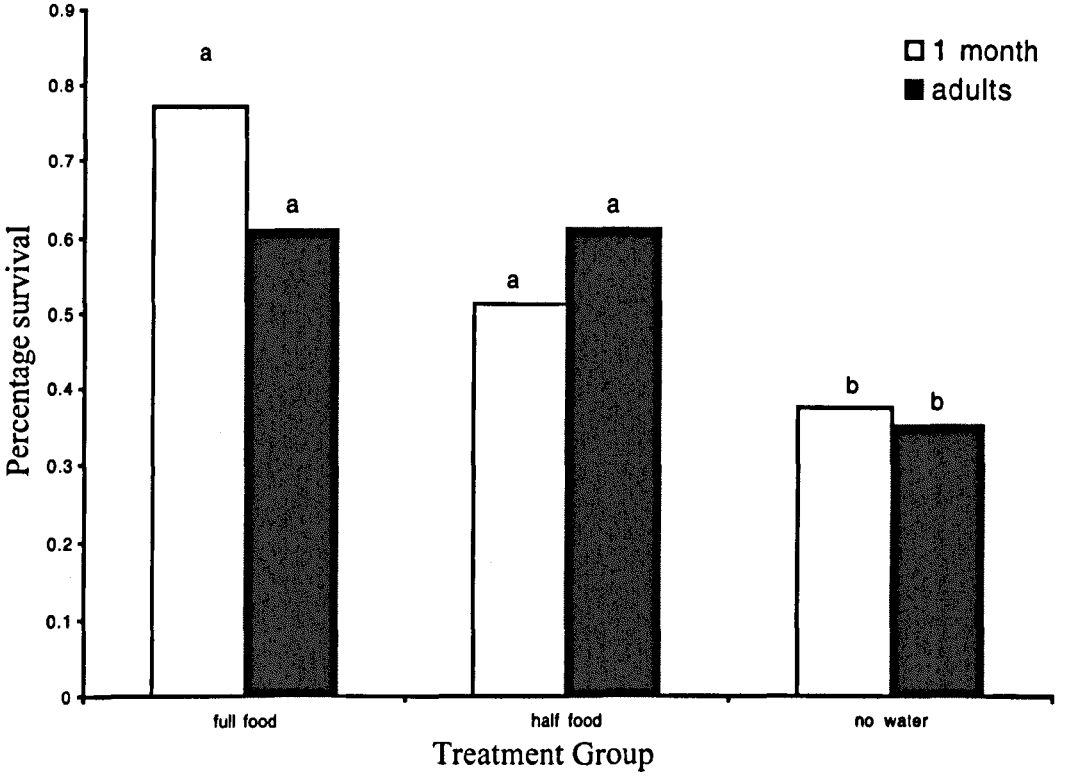

Figure 5.4: Percentage survival of beetles within cultures given three different feeding regimes (see section 5.2.3). Cultures were assessed for mortality either one month after the eggs first hatched (open bars) or when all the larvae reached adulthood (closed bars). Bars sharing letters are not significantly different (Fishers PLSD, $p > 0.05$). There were no differences between the one month and adult cultures in mortality (ANOVA; d.f. = 1, $F = 0.247$, $p = 0.624$), but there were significant differences between the treatments (d.f. = 2, $F = 9.847$, $p = 0.0007$), with the 'no water' culture showing higher mortality than the other two (see section 5.3.3).

phenotypic changes in only 5 generations (figure 5.2), confirming the presence of large amounts of additive genetic variance for this trait in the population ($h^2 = 59.3 \pm 8.6\%$; chapter 4). The efficacy of selection for cuticular melanisation, as represented by the mean level of cuticular melanisation within a selection line, significantly explained mortality on exposure to *M.anisopliae*. This shows that selection was able to effect changes in the levels of fungal resistance, and indicates that this too has additive genetic variance, and that this variance must covary with that for cuticular melanisation, because selection for the latter caused a correlated response in the former.

There is no evidence that genetic variation in the level of cuticular melanisation (and therefore the degree of fungal resistance) is maintained by a trade-off with larval competitive ability, unlike the case of *Drosophila melanogaster* (Kraaijeveld & Godfray 1997). Even under conditions of increased competition (as evidenced by increased larval mortality in the 'no water' treatment; figure 5.4) the proportion of surviving larvae from BLACK and TAN lines was not affected (section 5.3.4).

These results clarify the phenotypic relationships seen in chapter 3, in which beetles reared at higher densities showed increased weight, increased cuticular melanisation and increased fungal resistance. In chapter 3, weight was not correlated with cuticular melanisation or fungal resistance (once density was taken into account). In this chapter selection for cuticular melanisation did not cause a systematic correlated response in weight (figure 5.1), and weight was not a significant factor in the logistic regression for mortality (section 5.3.2). Hence it seems that the differences in weight reported in chapter 3 were responses to density that are independent of fungal resistance. The differences between lines in adult weight (figure 5.1) are probably the result of the significant

heritability of this trait (53.1 ± 9%; chapter 4) causing similarity within lines to be greater than that between lines.

Chapter 3 also showed a phenotypic correlation between cuticular melanisation and fungal resistance. This chapter shows that this relationship is at least partly genetic, because selection for the former trait causes a correlated response in the latter. However, there were no significant relationships between the direction of selection of the line an individual comes from and the probability of mortality (section 5.3.2), although this non-significance was marginal. There is a relationship between the efficacy of selection on a particular line (as represented as the mean level of cuticular melanisation of individuals from that line) and the probability of mortality. The reason why there is no significant effect of the direction of selection of the line an individual came from on the probability of mortality when exposed to fungus is probably because of variation around the mean cuticular darkness causing noise within lines. The very high mortalities at higher doses of fungus (almost 100% in the top 2 doses) also reduces the effect of differences in cuticular melanisation and masks differences between lines. Nevertheless, the evidence suggests that selection for cuticular melanisation can bring about correlated changes in fungal resistance. The strength of a correlated response to selection is given by the equation:

$$CR_Y = ih_x h_y r_a \sigma_{py}$$

where:

CR_Y = correlated response in trait y

i = the intensity of selection on x

h_x, h_y = the heritabilities of x and y

r_a = the genetic correlation of x and y

σ_{py} = the phenotypic standard deviation of y (Stearns 1992)

Thus it can be seen that for fungal resistance to respond to selection for cuticular melanisation, the former must have non-zero heritability and the two must have a non-zero genetic correlation. There is, therefore, additive genetic variance in the ability of *T. molitor* to resist *M. anisopliae*. Genetic correlations are the result of either linkage between genes involved in two traits, or of pleiotropic effects of genes involved in both traits (Falconer & Mackay 1996). If the genetic correlation shown in this chapter is also the cause of the phenotypic correlation in chapter 3, then it is unlikely that this is a result of linkage, because linkage can only explain the simultaneous expression of the alleles of two genes and not, as was the case in chapter 3, correlated phenotypic plasticities. This suggests that pleiotropic effects are causing genetic and phenotypic correlations between the two traits. Thus cuticular melanisation is an indicator of fungal resistance in *T. molitor*, probably because both traits share the phenotypic effects of one or more genes.

Chapter 3 and the current chapter both provide evidence for correlations between cuticular melanisation and fungal resistance on the phenotypic and genotypic levels. It is possible that cuticular melanisation may be more than a phenotypic marker for fungal resistance, i.e. that it may be causal in it. Cuticular melanisation has many putative functions: thermoregulation, protection against ultraviolet light, injury repair, crypsis and aposematism (Kettlewell 1973; Majerus 1998). There is some evidence that increased melanised makes cuticle harder: melanised feathers in birds are less prone to abrasion (Burtt 1981), and because wound sites in insect cuticles become more heavily melanised, implying that this has a protective function (Ashida & Brey 1998). Furthermore, lighter cuticles are more permeable to water in the German cockroach (*Blatella germanica*),

suggesting that more melanised cuticles are less penetrable (Appel & Tanley 1999). This is directly relevant to resistance to *M. anisopliae*, because it invades the haemocoel by secreting digestive enzymes onto the cuticle and exploiting the spaces thus created with its hyphae (Hajek & St. Leger 1994). More penetrable cuticles may therefore be more susceptible to this specific mode of invasion. Once again, however, it is not known if melanisation is causal in this hardening or a correlate of it. Sclerotisation of proteins is one method of hardening cuticle, and this results in the overproduction of quinones in the cuticle. These can be harmful if not removed, and one way of removing them is cross-linking them to form melanin (e.g. Nappi & Vass 1993). Therefore, melanisation of the cuticle may be a sink for the removal of harmful quinones produced in sclerotisation, and not causal in cuticular thickening (Chapman 1982). The exact nature of the relationship between cuticular melanisation and fungal resistance is still unclear.

The presence of additive genetic variance in fungal resistance implies that there is a cost associated with this trait. Kraaijeveld and Godfray (1997) found that lines of fruit flies which encapsulated parasitoids suffered higher larval mortality than those lines which did not encapsulate. Larval competitive ability is a potentially important determinant of fitness in *T. molitor* because the larvae are facultatively cannibalistic, with the levels of cannibalism rising under competitive conditions (Weaver & Macfarlane 1990; Savvidou & Bell 1994). No such relationship between larval competitive ability and cuticular melanisation was found in this study (section 5.3.4). Conditions in the experiment were conducive to competition, because survival from first instars was less than 100% in all but one culture, and one treatment group ('no water') suffered significantly higher mortality than the other treatments (section 5.3.3), although there is

no evidence of differences in competition between the two food treatments. (*T. molitor* are adapted to live in dry environments and absorb atmospheric water for their moisture. The 'no water' treatment is therefore not as harsh as may be supposed because atmospheric water was still available). Cannibalism tends to occur in two phases in *T. molitor*: during the early larval instars and during the pupal stage (Savvidou & Bell 1994). In this experiment there was no significant difference in mortality between the 1 month and adult samples, indicating that almost all mortality occurred in the first 4 weeks of the experiment. This may mean that a major component of pre-adult mortality (pupal cannibalism; Weaver & Macfarlane 1990) was absent from the samples. Thus if adult cuticular melanisation was only correlated with competition at the later stages of pupal development, then the experiment would fail to detect it. Why the later stages of development did not produce much mortality is not clear. Nevertheless, the work in this chapter shows that larval mortality under conditions of competition is effectively random with respect to adult cuticular melanisation, indicating that there are no genetic correlations between these traits. If there is an effect on the larvae of selecting for cuticular melanisation, then it is too subtle to be detected in this experiment.

5.5. Summary

Selection for cuticular melanisation in *T. molitor* caused a correlated change in resistance to the fungal pathogen *M. anisopliae*. Selection for darker cuticle resulted in greater fungal resistance, and selection for lighter cuticle resulted in decreased fungal

resistance. This shows that there is a significant additive genetic correlation between these two traits, providing a genetic basis for the phenotypic correlation seen in chapter 3. It also means that cuticular melanisation is a reliable indicator of fungal resistance ability. Selection for cuticular melanisation did not have a correlated effect on the competitive ability of larvae from selected lines. Therefore the maintenance of variation in cuticular melanisation and fungal resistance is not caused by a trade-off with larval competitive ability.

Chapter 6
General Discussion

6.1. Summary of the thesis

6.1.1. Synopsis of the thesis

This thesis has attempted to identify a specific situation in which the pattern of the expression of immune function is consistent with the hypothesis that immunity has an associated cost (density dependent prophylaxis), using the mealworm beetle *Tenebrio molitor* as a model system. The relationship between a morphological character (cuticular melanisation) and an aspect of the prophylactic response (fungal resistance) was subsequently investigated, with both phenotypic and genetic correlations being shown to exist between the two. Both phenotypic plasticity and additive genetic variance were shown to exist in immune parameters, indicating sub-maximal levels of immune expression both within individuals and within the population as a whole. This chapter reviews and synthesises the main findings from the preceding thesis, discusses the significance of the results with respect to current thinking on ecological immunology, and points out potential areas for future work.

6.1.2. Summary of the chapters

Chapter 1 provides a brief review of the field of ecological immunity, and illustrates the unique evolutionary paradigm represented by the interactions between parasites and hosts. These ideas are then discussed with specific reference to invertebrates and invertebrate immunity, demonstrating the applicability of explicitly vertebrate ideas to these taxa, and reviewing the evidence for the existence of costs of immunity in insects.

In the subsequent thesis, patterns of the expression of immunity were assessed by quantifying a number of parameters relating to immune function. The development and reliability of assays aimed at doing this, namely the number of haemocytes within the haemocoel (section 2.4), the levels of phenoloxidase (PO) activity in the haemocoel (section 2.5), and the response to a fungal pathogen (section 2.6), are reported in chapter 2. The relationship between these assays and the ability to resist parasites, or with definitions of 'immunocompetence' are discussed. None of the assays measure 'immunocompetence' *per se*, but all are considered to reflect factors which may reduce the costs of pathogenesis. Measurements of haemocyte numbers indicate the ability to mount cellular immune responses (Kuris et al. 1980; Eslin & Prévost 1996; Eslin & Prévost 1998; Ryder 1999), which make up a large proportion of invertebrate immune reactions (Ratcliffe et al. 1985). Measurements of PO levels may reflect a more general level of the activation of the immune system, because it is intimately involved with the activation of immune responses (Söderhäll et al. 1994). The level of mortality when exposed to an actual fungal pathogen is a direct measure of the level of resistance to a single pathogen type, so the link between this and immune function does not needs to be

assumed. However, its applicability as a measure of resistance to other pathogen types is questionable.

The level of resistance to fungal pathogens was shown to be phenotypically plastic with respect to population density in chapter 3. High larval densities can be used by insects to predict high adult population densities (Dingle 1996), and therefore a higher probability of disease transmission (Steinhaus 1958). Theory suggests that insects reared at high larval densities should show greater investment in immunity as adults (density dependent prophylaxis; Wilson & Reeson 1998). Chapter 3 shows that this is true of *T. molitor*, with gregariously reared beetles showing a lower level of mortality when exposed to the fungal pathogen *Metarhizium anisopliae* as adults compared to beetles that were reared solitarily as larvae (figure 3.1). There was no such phenotypic plasticity in the levels of PO, and no correlation between levels of PO and beetle mortality (section 3.3.3). There was a morphological correlate of mortality, namely the degree of cuticular melanisation, reflected by the darkness of the elytra. Darker beetles were less likely to die on exposure to fungus than light beetles (figure 3.2), and this effect was stronger in explaining mortality than rearing density. Elytra darknesses were also phenotypically plastic with respect to rearing density. Thus adults from the gregarious cultures had darker elytra than those from the solitary cultures, and darker beetles were less likely to die on exposure to fungus.

The close phenotypic association between cuticular melanisation and fungal resistance shown in chapter 3 implies that the former may be a phenotypic marker for the latter. Chapters 4 and 5 investigate the quantitative genetics of cuticular melanisation and look for a genetic link between this and fungal resistance. Chapter 4 assesses the

heritability of cuticular melanisation and a second measure of immunity, total haemocyte numbers. An ANOVA method of assessing the heritability of cuticular melanisation indicated the existence of large negative environmental components of variance, which artificially raise heritability estimates (Falconer & Mackay 1996). Negative environmental variances are interesting because they indicate that the expression of cuticular melanisation may be affected by competition within cultures (Falconer & Mackay 1996), suggesting that the process of cuticular melanisation is resource limited. However, estimates of heritability obtained from data which produces negative components of variance are unreliable. This necessitated the use of a second method of analysis: restricted maximum likelihood (dfREML; Meyer 1990). Maximum likelihood methods of analysis are not subject to problems caused by negative variances. This analysis showed that both cuticular melanisation and total haemocyte numbers were significantly heritable (59% and 49% respectively; section 4.3), indicating high levels of additive genetic variance in both. There is no genetic correlation between the two immune traits. Both immune traits have thus been shown to have variation at the genetic level within the study population of *T. molitor*.

A phenotypic correlation between cuticular melanisation and susceptibility to fungus was shown in chapter 3. Chapter 5 shows that this correlation has a genetic basis. Selection for cuticular melanisation over 5 generations results in significant changes in this trait (figure 5.2), as its high heritability would predict. This selection causes a correlated response in susceptibility to the fungus, with lines selected for darker cuticles having more resistant beetles than those selected for lighter cuticles, showing that fungal susceptibility also has underlying additive genetic variance (section 5.3.2). Furthermore,

the results show that fungal resistance and cuticular melanisation are genetically correlated to each other, and, therefore, that the latter is a reliable indicator of the former. Because they are both genetically correlated and covary in their phenotypic plasticity, the two traits are probably pleiotropically linked. Chapter 5 also investigates the maintenance of variation in immune function by looking for a further correlated response to selection: changes in the ability of the offspring of selected lines to compete as larvae. No correlation (negative or positive) was found between the level of cuticular melanisation of the parental line and the ability of larvae to compete (measured by their mortality) in conditions of food and water stress (section 5.3.4).

6.2. Discussions arising from the thesis

6.2.1. The role of cuticular melanisation in insects

A novel and interesting aspect of this thesis is the close relationship between cuticular melanisation of *T. molitor* and resistance to *M. anisopliae*. Although it is not possible to demonstrate a causal relationship between these two traits based on the data presented in the thesis, the probable pleiotropic nature of the genetic correlation between them indicates some sharing of the genetic pathways governing their expression. The identification of a highly visual indicator of an aspect of pathogen resistance gives a useful tool for empirical studies, namely an easy, non-destructive method of assessing resistance to a specific pathogen type, as well as suggesting a previously undescribed immune function for cuticular melanisation. Assessing investment in immunity to a pathogen without exposing an individual to that pathogen is often a complex and

controversial task (see section 2.1.1). This is no longer true in the *T. molitor/M. anisopliae* system.

Cuticular melanism is a frequently observed and well studied phenomenon in insects, with a number of demonstrated and putative functions. Levels of cuticular melanisation can vary due to phenotypic plasticity (e.g. Applebaum & Heifetz 1999) and genetic polymorphism within a population (e.g. Majerus 1998). The evolutionary causes of cuticular melanisation are not entirely clear, although it has been shown be involved in crypsis, aposematism, thermal regulation, blocking ultraviolet light and as a sexual signal (Kettlewell 1973; Majerus 1998). This thesis has not set out to explicitly test the role of any of these in *T. molitor*, and it is possible that one or more of these functions, or one not previously mentioned, may indirectly cause the correlations seen. However, it is difficult to explain the existence of a high degree of cuticular melanism in *T. molitor* using these functions. *Tenebrio* lives in grain stores (or in the laboratory population, in boxes of rat chow), spending much of their time below the surface. They are also negatively phototactic (Hurd & Fogo 1991). Thus they do not inhabit a visual medium, and the chief mode of interaction between conspecifics is olfactory (Valentine 1931; Happ 1969). Therefore crypsis, aposematism and sexual signalling are unlikely to be important because of the lack of visual acuity amongst beetles, and thermal regulation and UV protection likewise because of the lack of direct exposure to light.

None of the above functions are directly involved in immunity. This thesis suggests a novel role for cuticular melanisation: one that is intimately associated with an aspect of immune function, and which has the potential to explain its existence in *T. molitor*.

Genetic correlations have previously been observed between melanism and immunity in the Lepidoptera, in which higher levels of resistance or slower pathogen development are correlated with a greater degree of melanism (Gershenzon 1994; Verhoog et al. 1996). However, in the lepidopteran studies the degree of melanism is genetically determined, not phenotypically plastic, so the relationship between the two could be due to linkage rather than pleiotropy, meaning that there is no evidence of a causal component of their co-expression. The suggestion that cuticular melanisation may have a role in immunity, or be an indicator of it, is one only recently proposed (Reeson et al. 1998), and this was based on a phenotypic correlation between baculovirus resistance and cuticular melanisation in *Spodoptera exempta*, another lepidopteran. Reeson et al. (1998) suggested that the degree of melanisation of the cuticle may reflect the amount of PO in the haemocoel, and therefore the ability to respond to infection. This thesis failed to demonstrate a relationship between rearing density and PO levels, and failed to provide support for a relationship between PO levels, colour and fungal immunity (section 3.3.3), though these latter were not directly testable using the data collected. This suggests either that the prophylactic response differs in the two species, or that plasticity in PO levels is not part of that response.

In the specific case of the interaction between *T. molitor* and *M. anisopliae*, it is possible to postulate a prophylactic response which does not necessarily need to be mediated by haemocoelic immune functions such as PO. The cuticle, melanised or not, is an external barrier to infection through which most parasites are unable to pass (Ratcliffe et al. 1985). The outer cuticle is mechanically difficult to penetrate (Hajek & St. Leger 1994) and the inner cuticle consists of polymerised ('sclerotised') lipoproteins stabilised

by quinones (Dennell 1946), which makes it difficult to break down, other than enzymatically. A few fungi, including *M. anisopliae*, are able to do this, invading directly through the cuticle by secreting digestive enzymes into it and exploiting the gaps thus created (Goettel et al. 1989). The degree of resistance depends on the cuticle's thickness, its tensile strength and the degree of sclerotisation (Hajek & St. Leger 1994). Sclerotisation is a result of the polymerisation of quinones recruited to the cuticle (Chapman 1982). Quinones are toxic to insect tissues (Nappi & Vass 1996) and disrupt cellular integrity (von Schantz et al. 1999). Melanisation of the cuticle may provide a sink for these toxic substances, because melanisation also involves the polymerisation of quinones (Chapman 1982). If cuticular melanisation is a sink for quinones recruited for, but not used in, sclerotisation, then the degree of cuticular melanisation may accurately indicate the degree of sclerotisation, which is a determinant of resistance to fungal pathogens. Thus the prophylactic response in *T. molitor* may involve a thickening of the cuticle and a correlated response in cuticular melanisation.

Although melanisation is a visual indicator of resistance to fungi, it is unlikely to be used as an indicator by the beetles themselves, because, as mentioned above, the medium of communication between individuals is olfactory (Valentine 1931). However, visual melanistic sexual signals do exist in some insects which are used to attract mates, such as the wing spots of calopterygid damselflies (Hooper et al. 1999; Siva-Jothy 2000) and the cuticular colour of ladybirds (Ueno et al. 1998). If the degree of cuticular melanisation does reflect the thickness of the cuticle, and therefore the degree of resistance to fungi, then such sexual signals may honestly advertise an individual's ability

to resist fungal pathogens, providing a mechanistic link between immune function and sexual selection in some invertebrate species.

6.2.2. Potential costs of cuticular melanisation

Immune function in general tends to be inducible, only being maximally expressed in the presence of pathogens or when the risk of pathogenesis is high (section 1.4.1). This is true of fungal resistance in *T. molitor*, which is expressed to a greater degree in the presence of conspecifics than it is in their absence (chapter 3). Harvell & Tollrian (1999) give the following conditions under which 'inducible defences' (which include the immune system; Frost 1999) evolve:

1) The selection pressure of the inducing agent is variable and unpredictable, but sometimes strong
2) A cue which reliably signals the proximity of a threat is available.
3) The defence is effective
4) The inducibility is cost saving, i.e. the expression of the defence in the absence of a threat incurs a significant fitness cost. Without this cost the trait will become fixed in the genome.

All these criteria are met in the study population of *T. molitor*. While it is true that the populations are immunologically naive to *M. anisopliae*, signs of other diseases have been observed. At least two other fungal pathogens have been seen, and pathological signs of infection such as brittle, discoloured larvae and pupae are frequently observed. It is debatable as to whether or not the same level of immune challenge is present in culture as it is in a wild population, but the beetle cultures have been exposed to pathogenesis in

their recent evolutionary history. Therefore a strong, variable and unpredictable selection pressure from pathogenesis exists, and immune function is unlikely to be a selectively neutral trait. This suggests that the inducibility of fungal resistance is maintained because of an associated cost.

Further evidence that immune function has a cost comes from the observation that there is additive genetic variability for cuticular melanisation, haemocyte numbers (chapter 4) and fungal resistance ability (chapter 5). Variability in vertebrate systems is probably generated because cycles of host/parasite co-evolution result in fluctuating fitness values for alleles involved in resistance, preventing a single 'best' allele from reaching fixation (Hamilton & Zuk 1982). Invertebrates do not necessarily undergo such cycles, because the specific antibody/antigen based system of immunity is absent (Roitt 1988), and broad categories of pathogens are dealt with by stereotypical responses (Ratcliffe et al. 1985). However, if there is a cost to the expression of immunity, then possessing the alleles which minimise the costs of pathogenesis will only be desirable if such potential costs exist. Otherwise possessing the 'best' alleles for dealing with pathogens will actually lower fitness due to the costs of immunity. Once again, this assumes that immunity is not a neutral trait in the stock populations of *T. molitor*.

Although this thesis has identified patterns of immune expression (phenotypic and genotypic variability) which strongly suggest that immunity has an associated cost, it has failed to identify what the cost(s) is. Chapter 5, following the work of Kraaijeveld and Godfray (1997), failed to find a decrease in the larval competitive ability of lines selected for increased cuticular melanisation. This was chosen because of the high degree of larval competition and cannibalism that occurs in *Tenebrio* cultures, but was not a prediction

based on a specific mechanistic or physiological trade-off. There are as many possible life-history trade-offs as there are life-history traits. Previous studies have found trade-offs between invertebrate immunity and foraging ability (König & Schmidt-Hempel 1995), reproduction (Boots & Begon 1993; Ferdig et al. 1993; Siva-Jothy et al. 1998), predator avoidance (Rigby & Jokela 2000), larval competitive ability (Kraaijeveld & Godfray 1997) and development (Boots & Begon 1993). It is difficult to predict which of these, if any, would be applicable to *Tenebrio*.

Potential proximate costs of cuticular melanisation are perhaps easier to identify. Tyrosine is a precursor of the quinones used in melanin formation, and is therefore used in many processes at all stages of life-history (Chapman 1982). It is an amino acid which must either be environmentally acquired or formed from phenylalanine, which is itself an essential amino acid (Stryer 1988). Because of the restricted number of potential sources, tyrosine acquisition may be environmentally limited. The negative environmental variance for cuticular melanisation obtained in chapter 4 (section 4.2.6.1) indicates that some component of the cuticle is affected by competition between individuals. It is intriguing to think that this resource may be tyrosine, although the thesis provides no evidence to support this. If this was the case, then it may result in a possible trade-off with fecundity in female beetles. Tyrosine is required for tanning the egg chorion of insects, and a drop in fecundity in mosquitoes infected with filarial worms was correlated with a drop in tyrosine levels in the haemocoel (Ferdig et al. 1993), although this may have been an affect of parasitism rather than a cost of immunity, because changes in the amino acid content of hosts can be caused by parasitism (e.g. Hurd & Arme 1984). Another potential mediator of a trade-off with reproduction is N-acetyldopamine

(NADA). This is a substrate for cuticular sclerotisation, melanisation and egg pod formation (Hopkins et al. 1999; Nappi & Vass 1993), and therefore provides a direct link between fungal resistance and fecundity. Once again, however, it is not known if there is a limit to the levels of this in the beetle. Levels of investment in fungal resistance may also be restricted because of the sheer volume of cytotoxic compounds involved in the formation of cuticle (Ashida & Brey 1995). Detoxification mechanisms are known to extend insect longevities if overexpressed (Mockett et al. 1999; Seslija et al. 1999), so it may be that longevity is traded-off with immunity due to the action of ageing compounds produced by the latter.

Energetic costs of immunity are easy to postulate but difficult to make predictions from. The activation of the insect's immune system involves the simultaneous transcription of many genes, which is energetically costly (Harshman & James 1998). Haemocyte production is not known to be costly, but on the activation of an immune response the number of circulating haemocytes can increase six-fold (Sequeira et al. 1996), suggesting a high energetic demand on the haemopoeitic organs. Encapsulation responses have also shown to be reduced as a result of energetic stress, suggesting a cost of haemocyte production (König & Schmid-Hempel 1995, Siva-Jothy et al. 1998, Ryder 1999). In general, however, specific costs of insect immunity have yet to be identified, and this thesis, whilst indicating the probable existence of costs, failed to find any effect of investment in prophylaxis on life history.

6.2.3. The existence and measurement of immunocompetence in T. molitor

The thesis failed to provide any support for the notion that 'immunocompetence' is a definable and assayable life history trait in *T. molitor*. The term immunocompetence was introduced into evolutionary ecology by Følstad and Karter (1992), and it referred to the ability of an individual to respond to pathogenic challenge. Subsequently, however, a more specific, ecological definition has been used: the relative ability of different individuals within a population to respond to pathogens (Siva-Jothy 1995; Sheldon & Verhulst 1996; Svensson & Skarstein 1997). This broader definition suggests that immunity as a whole should be co-ordinately expressed, so that the relative levels of resistance between individuals should be the same with different types of pathogenic challenge and in different environments. Furthermore, this immunity should be quantifiable by assaying immune parameters which relate to pathogen resistance. This thesis consistently failed to produce results in the pattern predicted by these statements.

Three immune assays were developed in chapter 2, one of which, the resistance to *M. anisopliae*, is a direct reflection of pathogen resistance ability. No phenotypic correlation between the activity of PO and fungal resistance was detected (section 3.3.3). Although it was not possible to directly assess the correlation between cuticular melanisation and PO levels, no trend towards the gregarious beetles having more PO was detected and this would surely have been found if there was a correlation between PO and the cuticle. There was also no phenotypic or genetic correlation between the total haemocyte count and cuticular melanisation (i.e. fungal resistance) (table 4.4), despite the fact that both had additive genetic variation. This indicates that there is no common

genetic pathway controlling levels of variation in these two traits. Such pathways must exist if a small number of genes controlling immunocompetence are to be assumed.

The lack of any sort of correlation between immune parameters does not support the idea that an individual has a single level of immunocompetence. If, however, the assays do not reflect pathogen resistance ability, then this is not necessarily the case. Resistance to *M. anisopliae* is clearly a direct measure of the resistance of pathogenesis. Total haemocyte count has been shown to correlate with capsule size in house crickets (Ryder 1999) and the ability to kill encapsulated pathogens in shore crabs (Kuris et al. 1980). Haemocyte numbers also correlate with the ability to resist parasitoid attack both within and between species of *Drosophila* (Eslin & Prévost 1996; Eslin & Prévost 1998). No such relationship has ever been demonstrated in a coleopteran, but, because there are no known qualitative differences in the immune responses of the two orders, there seems to be no reason why a similar relationship will not exist. The relationship between PO and pathogen resistance is less clear, with only correlational evidence from Lepidoptera (Reeson et al. 1998) supporting the idea that high PO activity is equivalent to high levels of immunity. If these assays do not reflect pathogen resistance ability, then it could be argued that the data in this thesis does not refute the existence of immunocompetence as a definable trait in *T. molitor*. However, if this is the case, then a second problem with immunocompetence arises: that of how to assay it. Assaying partial aspects of immunity thought to reflect the ability to resist pathogens has been used as being synonymous to assaying immunocompetence (Sheldon & Verhulst 1996; Skarstein 1996; Owens & Wilson 1999). Many of the assays used, including the ones in this thesis, are assumed to have a link to pathogen resistance ability without this ever having been demonstrated. If

no such relationship exists, then immunocompetence will be impossible to look for empirically, no matter if single or multiple assays of immunity (as suggested by Lochmiller; 1995) are used.

Finally, if the assays do reflect pathogen resistance ability, and there is no simple way of defining or assaying immunocompetence, then the question of why we should expect immunocompetence to exist in these systems must be asked. The immune system is thought of as being a single system because it is responsible for protection against what can be phenomenologically labelled as a single threat: pathogenesis. However, the grouping of all pathogens under a single heading is a conceptual device, rather than a biological reality, as the methods by which pathogens invade and cause disease are diverse, and pathogens represent almost the full range of biodiversity, rather than being a single taxon in themselves. If there are no physiological or genetic reasons for believing that the systems that resist these diverse challenges are connected, then there is no reason why they should be expressed at similar levels. The concept of immunocompetence may be the result of treating a series of systems which can be conceptually linked as a single system that is biologically coherent. These results suggest that this idea is questionable, at least in *T. molitor.*

6.3. Future research

6.3.1. The relationship of cuticular melanisation to fungal resistance

An important finding of this thesis is the existence of a genetic correlation between resistance to *M. anisopliae* and the degree of cuticular melanisation in *T.*

molitor. The subsequent discussions have concentrated on the intimacy of this relationship, and even the possibility that the latter is causal in the former. Elucidating the nature of this relationship is important because it suggests a novel function of cuticular melanisation, a common trait among insects. Manipulating the level of cuticular melanisation whilst holding all other factors constant may prove to be difficult. It is, however, possible to examine the degree of permeability of the cuticle, both gravimetrically, using a balance to assess rates of water loss (Appel & Tanley 1999), and visually, looking at cuticle structure under an electron microscope (Tu & Singh 1993; Ishay et al. 1999; Gorb 2000). These could determine if cuticular melanisation is correlated to thicker cuticles, or if the relationship of the former to fungal resistance is through a more indirect route.

6.3.2. Other potential functions of cuticular melanisation

Cuticular melanisation has a number of demonstrable functions in a variety of insect taxa. Much of this thesis was involved in establishing an association between cuticular melanisation and immune function. Other potential functions for cuticular melanisation were ignored. Although they seem to be unlikely to be major factors in the evolution of melanism in *T. molitor* given its habitat (see section 6.2.1), it has not been shown that these other factors have no role to play in the expression of melanism. Before cuticular melanisation can be treated as solely an immune linked trait in *T. molitor*, it should be shown that there are no other demonstrable functions, especially considering the varied roles that melanism plays in other taxa (Kettlewell 1973; Majerus 1998).

6.3.3. Assessing immunocompetence in T. molitor

The existence of 'immunocompetence' in *T. molitor* has been questioned in this thesis (section 6.2.3), based on the absence of any correlations between putative measures of immunity (sections 3.3.3 and 4.2.6.2). If immunocompetence can be indicated by a battery of assays, as suggested by (Lochmiller 1995), then these assays must all be shown to indicate levels of pathogen resistance. This has not been done in this thesis, with assays such as PO activity and total haemocyte count only connected to resistance by theoretical arguments. This thesis identified the link between cuticular melanisation and fungal resistance by looking for phenotypic correlates of survival after exposure to fungus, identifying and selecting for one (cuticular melanisation) and looking for a correlated response in the other. This should be repeated, not only looking for correlates of fungal resistance among the other assays developed in chapter 2, but also using a variety of generalist pathogen types (a bacterium, a virus, a parasitoid, a nematode, etc.). This will establish the credibility of these assays in assessing immunity, as well as identifying common processes involved in resistance to one or more pathogen types. This may begin to test definitions of immunocompetence in *T. molitor* in the way envisioned by Lochmiller (1995), by providing a more comprehensive survey of immunity as a whole.

6.3.4. Identifying costs of fungal resistance

This thesis has provided much circumstantial evidence that fungal resistance has an associated cost in *T. molitor*, but it has not identified these costs. Section 6.2.2 outlines potential costs at the life-history and proximate levels. Only by identifying these costs can the maintenance of variation in immune function be understood. As mentioned in that

section, it is difficult to identify potential life history costs without a mechanistic basis for doing so. However, some aspects of life history will be a more pervasive force in selection than others in this particular taxa and these are more likely to be traded-off against immunity, and therefore should be concentrated on. Larval competitive ability (Weaver & Macfarlane 1990; Savvidou & Bell 1994) was an example of such a trait. Sperm competition is a strong selective force in insects (Simmons & Siva-Jothy 1998), and has been shown to be so in *T. molitor* specifically (Thompson 1998), and is therefore a candidate for being involved in a trade-off with immune function. Another candidate is olfactory communication. As well as being the chief mode of communication in *Tenebrio* (Valentine 1931), it is the medium of sexual attraction (Happ 1969), and, if it is an honest signal, is likely to have an associated cost (Zahavi 1977). Furthermore, it is known that pheromone production can drop as a result of pathogenesis (Hurd & Parry 1991). These are considered to be possible life-history traits against which immunity may be traded-off, and the best candidates for future study.

Tyrosine levels may also be important, being involved in both fecundity and immunity (section 6.2.2). The hypothesis that tyrosine is a limiting factor could be investigated by raising beetles on supplemented or depleted diets and looking for responses in cuticular melanisation, fungal resistance, haemolymph tyrosine levels and egg mortality (brought about by failure to melanise the chorion). These may identify costs of immune investment.

6.4. General Conclusions

Even the 'simple' immune systems possessed by invertebrates are complex interactions between many systems. In the mealworm beetle *Tenebrio molitor*, one aspect of the expression of immunity, resistance to the entomopathogenic fungus *Metarhizium anisopliae*, shows both phenotypic and genetic variability. The expression of this variability suggests that there is a cost associated with fungal resistance. Costly immunity is an important idea amongst ecological immunologists, and one supported in this thesis, though specific costs are not identified. Despite the fact that a simple and novel marker for fungal resistance was identified (cuticular melanisation), no correlates of this marker relating to other aspects of immunity were identified. This thesis has taken the view that this is evidence against the existence of immunocompetence as being a definable and measurable trait, although it is clearly not definitive. The postulated existence of immunocompetence is considered to ignore the complexity and diversity of pathogens and the immune responses raised against them, and the approach taken in this thesis, that of looking at specific aspects of pathogen ability, is advocated.

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