GREGARIOUS IMMUNISATION IN THE MEALWORM BEETLE, *Tenebrio molitor*

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ABSTRACT

Investment in immunity is costly: one way in which hosts can ameliorate these costs is through immune priming, whereby hosts develop increased protection to future infection following previous exposure to a parasite or immune elicitor. Priming offers hosts a more efficient way of managing immune insult by allowing for a stronger and faster response to an immune insult. As well as investing in physiological immune defences, hosts can also leverage behavioural responses to reduce the costs of infection.

Group-living in insects offers several benefits, such as predator avoidance. However, it can be costly in terms of increasing the risks of exposure to parasites. Group facilitation of disease resistance through a variety of processes collectively known as 'social immunity' is well established in the eusocial insects. Many gregarious insects share several features of their ecology with eusocial species, and should thus be predisposed to many of the same risks of infection, and the same evolved processes that mitigate these risks. A form of immune priming known 'social immunisation' has recently been described in eusocial insects, whereby immunologically naïve individuals exhibit enhanced immunity against infection after being housed with infected nestmates. Whether similar mechanisms exist in gregarious but non-social insects is unknown, and it is this premise that forms the conceptual basis of this thesis.

I investigated whether a non-social but gregarious insect, the mealworm beetle (*Tenebrio molitor),* altered its immune investment following cohabitation with an immunestimulated conspecific. I examined the potential role of both physiological and behavioural defences in offering prophylactic protection against perceived pathogenic threat. I also investigated the potential mechanisms of such an form of immunisation by examining immune responses induced by cohabitation with conspecifics challenged by a live (and transmissible) bacterial infection and those challenged by either heat-killed bacteria or an artificial antigen (both non-transmissible). Finally, I examined the role of host behaviour in affecting immunisation, quantifying behavioural changes in immune-stimulated hosts (referred to as 'sickness behaviours') to try and identify visual or behavioural cues which may be utilised by naïve hosts to stimulate prophylactic defences,

There was no robust evidence for a parsimonious process of gregarious immunisation. However, there were differences between the sexes in their immune responses to infection threat, as well as in their induction of sickness behaviours

following infection. Whilst there was little evidence for an upregulation of immunity in naïve females, females appeared to exhibit enhanced tolerance of infection following cohabitation with a 'sick' conspecific, as they suffered no decrease in longevity despite the presence of relatively high parasite loads. Males showed the opposite pattern to that predicted by gregarious immunisation, decreasing their investment in physiological defence following exposure to 'sick' conspecifics.

Despite finding no clear evidence for enhanced resistance through a straightforward process of gregarious immunisation, these data suggest that naïve *T. mollitor* may be able detect social cues of infection produced by parasitised conspecifics. I propose that the immune responses displayed by both males and females constitute tolerance strategies which help hosts to minimise the costs of parasitism. Due to intrinsic differences in the life-history trajectories of the sexes, females are predicted to invest in immunological tolerance mechanisms aimed at self-preservation in order to preserve their capacity for future reproduction, whereas males are predicted to terminally invest in reproduction in order to maximise their fitness.

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Your poetry of thought and
warmth of heart are
life;
You're a rainbow in January,
and a beer garden in July.

I'm eternally grateful for and lucky to have my Mam and Dad; they've made me into the person that I am, and I can only hope that person ends up just like them. Their unconditional support – emotional, financial and most often culinary – has helped me to get here and to stay here, as has the support of all the other wonderful and generous members of my family, both blood and water.

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I dedicate this thesis to my Grandad.

TABLE OF CONTENTS

CHAPTER ONE:
INTRODUCTION

Parasites are the largest and most diverse group of living organisms on the planet (Schmid-Hempel, 2011). By definition, parasites are detrimental to hosts fitness, and have been a strong selective force favouring the evolution of a sophisticated immune system in almost all cellular organisms (Schmid-Hempel, 2011). The consequences of parasitism are seen at both the individual and population level and play an important role in shaping the ecological and evolutionary dynamics of natural host populations (Altizer *et al.* 2003; Anderson & May 1982; Hudson *et al.* 1998).

1.1. The costs of immunity

Whilst immunity provides a crucial defence against parasitism, immune defences can themselves be costly (Zuk & Stoehr, 2002; Schmid-Hempel, 2003). Hosts are predicted to optimise, rather than maximise, their investment in immunity in order to balance the costs of defence with the potential costs of parasitism (Sheldon & Verhulst, 1996). In brief, the costs of immunity are two-fold; firstly, there are evolutionary costs of immunity associated with negative genetic covariance between the immune system and other fitness components (discussed in detail in Moret & Schmid-Hempel, 2000), and secondly, there are use and maintenance costs incurred during the deployment and maintenance of an effective immune response (Kraaijeveld & Godfray, 1997).

Three classifications of use and maintenance costs have been defined (Zuk & Stoehr, 2002). Firstly, resource-based costs, which stem from energetic constraints resulting from hosts having access to only a finite amount of resources which must be partitioned to and traded off between all necessary physiologies, including immunity (Zuk & Stoehr, 2002; Schmid-Hempel, 2003). Immunity may also be constrained by option costs (Zuk & Stoehr, 2002) that are not paid in energetic currency but rather through the use of shared structural components or functional pathways in the host; for example, resistance against malarial infection leads to follicular apoptosis in *Anopheles* mosquitoes (Ahmed & Hurd, 2006). Together, resource-based costs and option costs account for many of the trade-offs commonly observed between immunity and various life-history traits, such as longevity, development rate, competitive ability and reproduction (Zuk & Stoehr, 2002; Schmid-Hempel, 2003). Trade-offs also occur within the immune system

itself, as investment in two different immune effectors can be antagonistic; for instance, a negative phenotypic relationship exists between constitutive phenoloxidase production and induced antimicrobial activity in honey bees (*Apis mellifera*) (Moret & Schmid-Hempel, 2001) and *Tenebrio molitor* (Moret & Siva-Jothy, 2003). Finally, hosts may incur costs of immunopathology through damaging their own cells and tissues during an immune response (Nappi *et al.*, 1995; Sadd & Siva-Jothy, 2006). Insects possess an arsenal of non-specific and cytotoxic effector systems, and are particularly susceptible to self-harm due to their relatively uncompartmentalised open haemocoel (Siva-Jothy *et al.*, 2005). Indeed, the effects of immunopathology have been demonstrated in *Tenebrio molitor* (Sadd & Siva-Jothy, 2006), where Malpighian tubules become melanised following encapsulation of a novel antigen, and lose a significant degree of osmoregulatory function (Sadd & Siva-Jothy, 2006). Immunopathology has been suggested to form as important a cost of immunity as pathological damage inflicted by parasites (Graham *et al.*, 2005), and it is likely an important contributor to the decline in longevity reported after immune stimulation in *Tenebrio molitor* (Armitage *et al.*, 2003).

1.2. Mechanisms of immunity

In order to infect an invertebrate, parasites must first successfully enter the host through ingestion by the host or by breaching the external barriers imposed by the host cuticle. Some host responses are rapidly induced following infection or wounding, such as haemolymph coagulation and wound repair, which limit the systemic spread of parasites throughout the haemocoel and reduce the risk of further infection through open wounds (Hajek & St. Leger, 1994; Lavine & Strand, 2002).

Upon parasite invasion, the host relies on the ability of the physiological immune system to distinguish self from non-self (Siva-Jothy *et al.*, 2005), through the recognition of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) or peptidoglycans, which are highly conserved features of bacteria, fungi and viruses (Janeway, 2001; Siva-Jothy *et al.*, 2005). Parasite recognition activates a suite of immune effectors which provide protection for the host. In invertebrates, these effectors are generally classified as either cellular or humoral in nature (Siva-Jothy *et al.*, 2005).

Cellular immunity is comprised by haemocytes which phagocytose or encapsulate parasites, and also function in coagulation and wound healing (Strand, 2008). Humoral immunity refers to soluble proteins, including antimicrobial peptides (AMPs) (Bulet & Stöcklin, 2004) and lysozymes, and various reactive cytotoxins, such as quinones, lectins and reactive oxygen species (Siva-Jothy *et al.*, 2005).

1.2.1. Constitutive versus inducible defence

Perhaps the two most important parameters characterising invertebrate immune effectors are their speed and specificity (Schmid-Hempel *et al.*, 2003; Figure 1.1). Invertebrate immune effectors are often classified as either constitutive, which are always ready to act, or inducible, which are expressed only following parasite recognition (Hamilton *et al.*, 2008).

Constitutive defences include phagocytic engulfment by haemocytes, antibacterial activity of some lysozymes, and melanisation by phenoloxidase (PO) intermediates. These effectors provide the host with a fast and robust defence against a diverse range of parasitic threats, but can be coupled with immunopathological effects. For example, the PO cascade is a prominent component of constitutive defence, but releases a range of cytotoxic compounds which cause host immunopathology (e.g. Sadd & Siva-Jothy, 2006). Hosts may shield themselves from self-harm through protective barriers such as the basal lamina which line the haemocoel (Chapman, 1998) or temporally through the use of multi-level enzyme cascades which allow highly cytotoxic elements to be stored as inactive precursors until used in defence. For example, PO generally exists within the host in the form of an inactive zymogen, prophenoloxidase (proPO), whose activation is rapidly triggered via a serine protease cascade elicited by the recognition of basic pathogen markers (Cerenius *et al.*, 2010).

Inducible defences are only expressed by the host following parasite recognition. These defences include the production of antimicrobial peptides (AMPs; Bulet *et al.*, 1999), and inducible cellular responses such as the proliferation of haemocytes (Sequeira *et al.*, 1996) and the processes of phagocytosis, nodulation and encapsulation which act to smother invading parasites, starving them of oxygen and nutrients (Lavine & Strand, 2002). Due to their inducibility, these defences typically suffer from a lag phase; for example, it can take up to 48 h for AMP expression to reach peak levels in *Tenebrio molitor* following infection. However, inducible effectors are generally more specific than constitutive defences, with AMPs for example undertaking targeted action against narrow groups of parasites (e.g. Gram negative or Gram positive bacteria), and thus tending to cause less immunopathological damage to the host (Bulet *et al.*, 1999).

The constitutive and inducible arms of the invertebrate immune system work in tandem to provide effective and long-lasting protection for the host, and have been suggested to account for the success of insects due to their apparent ability to prevent the evolution of parasite resistance within the host and to manage persistent infections (Haine, 2008).

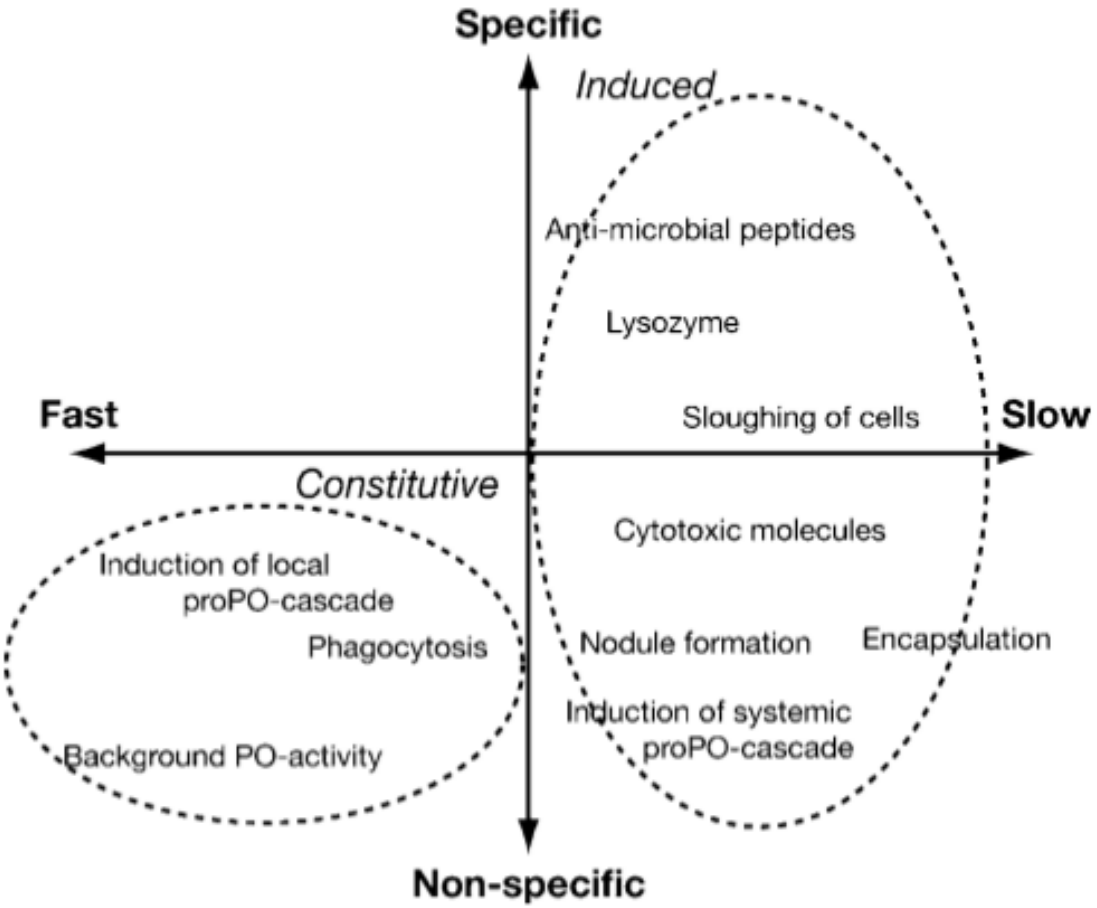


Figure 1.1. A summary of insect immune effectors, classified by their speed and specificity. Figure taken from Schmid-Hempel (2003).

1.3. Resistance versus tolerance

Host can undertake two types of strategy to preserve fitness during infection, either resisting the infection directly by attacking the parasite, or tolerating the negative effects of infection without targeting the parasite (Schneider & Ayres, 2008; Lazzaro & Rolff, 2011; Medzhitov *et al.*, 2012). Resistance responses are defined as host responses which reduce parasite fitness, generally by killing them, whereas tolerance responses are defined as host responses that allow the host to maintain health in the face of parasitic infection without attacking the parasite directly. Hosts may tolerate parasitism immunologically, through the use of physiological effectors which limit parasite growth or reproduction or otherwise tolerate pathogen-induced damage, or tolerate the negative effects of infection through non-immunological changes in their behaviour, ecology and life-history (see section 1.4.2.).

Resistance responses, particularly those provided by fast-acting constitutive immune effectors, are generally associated with immunopathology (Nappi *et al.*, 1995;

Siva-Jothy *et al.*, 2005). Immunopathology should only be beneficial for the host when the fitness preserved through parasite killing is greater than the costs of collateral self-harm, meaning that a trade-off should exist between resistance and tolerance strategies (Schneider & Ayres, 2008). Assuming the infective parasite load decreases during the course of an immune response, and assuming the rate of parasite killing to be proportional to the level of immunopathology, one may expect the host to shift from actively resisting the pathogen towards tolerating its negative effects as the infection progresses (Figure 1.2). At this point, the host may attempt to limit further tissue damage, resource expenditure or other costs in order to limit the negative impact of infection upon fitness, tolerating the remaining pathogen burden instead of trying to further deescalate it (Medzhitov *et al.*, 2012).

Essentially, there is an upper limit to which a host can invest in immune resistance, after which the damage caused by any residual pathogens in the host are actually less detrimental to host fitness than any further escalation of immunopathological self-harm. Tolerance may even provide a more economic defence in the case of highly virulent pathogens, or when the probability of reinfection is particularly high. Moreover, host tolerance strategies should be evolutionarily advantageous as they are not directly detrimental to parasite fitness and thus favour lower pathogenic virulence (Schneider & Ayres, 2008; Medzhitov *et al.*, 2012). Indeed, theoretical models suggest that costs of immunity and development of parasite virulence should favour the evolution of tolerance strategies in many hosts (Boots *et al.* 2009).

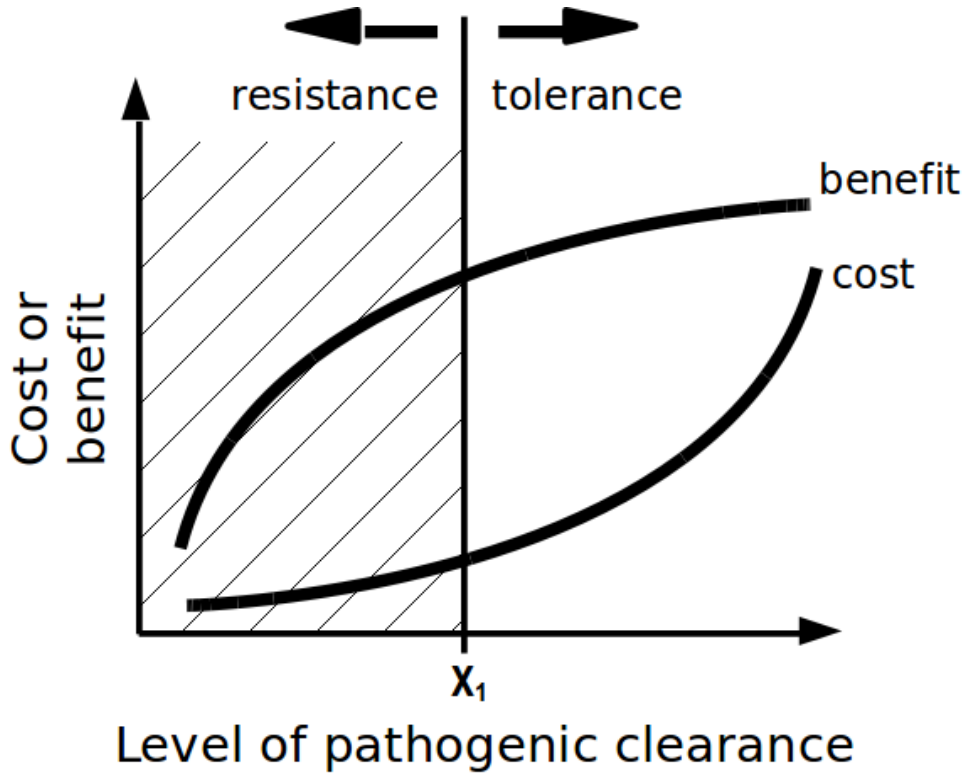


Figure 1.2. A theoretical function of the costs and benefits of pathogenic clearance upon the fitness of an infected host. The net benefit of immune investment is maximised at X_1. Below the X_1 threshold, ineffectual levels of host defence allow the pathogen to grow and multiply, inflicting damage upon the host and exploiting host resources. The use of resistance-based responses which actively attack or kill the parasite should be favoured at this stage. Above the X_1 threshold, escalating costs of immunopathology and resource expenditure resulting from immune defence start to outweigh damage caused by the residual pathogen population. At this stage, the host may benefit from switching to a tolerance-based response which is no longer directly aimed at killing the pathogen but does act to otherwise preserve host fitness; this may include the use of non-immunological life-history responses, such as terminal investment in reproduction. The threshold X_1 is expected to vary depending on the virulence of the pathogen in question (e.g. rapidly escalating costs of highly virulent pathogens may favour a faster switch to host tolerance).

1.4. Behavioural immunity

Although physiological immunity is a vital component of anti-parasitic defence, it can be thought of as an 'emergency service' which is launched only when the 'front-line' defences provided by morphology, behaviour and life-history have been breached (Hart, 1997; Siva-Jothy *et al.*, 2005). Behavioural defences are generally less costly than physiological defences (Siva-Jothy *et al.*, 2005; Schulenburg & Ewbank 2007), are faster to enact and have a greater degree of plasticity (West-Eberhard, 1989), and can be as effective as physiological immune responses at preserving host fitness in the face of parasitism (Moore, 2002; Schmid-Hempel *et al.*, 2003). Behavioural responses can complement, augment or even replace the need for physiological defences by reducing the risk of infection for the host or the risk of transmission to other susceptible conspecifics, or by influencing successful resistance or tolerance of infection by the host.

Both physiological and behavioural responses may thus be considered part of a single unified immune defence (Wilson-Rich *et al.*, 2009), and low levels of physiological immunity are not necessarily indicative of poor immunocompetence. On the contrary, effective use of behavioural strategies may circumvent the need for investment in costly physiological defences, and could offer a net fitness benefit to the host. Immunological studies should therefore benefit from accounting for behavioural immune responses, yet these have been largely overlooked in the invertebrate immunology literature, particularly in studies conducted on non-social species (Hart, 1994).

1.4.1. Sickness behaviours

Following infection, hosts may modify their behaviour to reduce the costs of parasitism by engaging in behaviours such as anorexia, self-grooming or behavioural thermoregulation (Johnson, 2002; Dantzer, 2009; Adamo, 2006). These behavioural changes are collectively termed 'sickness behaviours' (Hart, 1988; Moore, 2002). Behavioural sickness has been described as a 'motivational state' which allows infected hosts to re-prioritise their resource investment so as to minimise the costs of parasitism (Hart, 1988; Johnson, 2002; Dantzer, 2009). For example, reduced investment in reproduction may allow infected hosts to refocus their resources into immune defence by preventing trade-offs between the two physiologies (Sheldon & Verhulst, 1996; Zuk & Stoehr, 2002). Illness-induced anorexia is a common sickness behaviour which may facilitate host recovery and prevent the escalation of infection by limiting further parasite consumption from contaminated food sources (e.g. Pujol *et al.*, 2001; Schneider & Ayres, 2008; Adamo *et al.*, 2010). Although sickness behaviours are assumed to be less costly

than investment in physiological immune defences (Siva-Jothy *et al.*, 2005; Schulenburg & Ewbank 2007), hosts may still incur costs through missed opportunities (de Roode & Levefre, 2012), such as decreased mating success (e.g. Carver & Hurd, 1998; Adamo *et al.*, 1999; Pai & Yan, 2003; Shoemaker *et al.*, 2006). Correspondingly, some behavioural responses to infection are not intended to facilitate resistance against or recovery from infection, but rather aimed at tolerating the negative effects of infection through an increased investment in other (non-immunological) life-history traits, such as reproduction.

1.4.2. Behavioural tolerance

Behavioural tolerance strategies differ from immunological tolerance mechanisms as hosts do not seek to withstand physiological damage resulting from infection, but rather regain fitness by investing in reproduction. Such fecundity compensation has been described as a 'life-history escape attempt' (van Baalen, 1998; Minchella, 1985).

If the perceived threat to survival is great enough, infected individuals may seek to protect against fitness losses by undertaking a 'terminal investment' in reproduction (Clutton-Brock, 1984; Sheldon & Verhulst, 1996), redirecting resources away from immunity and into reproductive traits such as pheromone production, copulatory activity and sperm production. Due to intrinsic differences in life-history between the sexes, males stand to gain more from a terminal reproductive investment as females cannot linearly increase their reproductive success through increasing their number of matings. Females should instead be selected to invest more in immune defence in order to improve their chance of survival and so preserve their capacity for future reproduction (Bateman, 1948; Rolff, 2002). Indeed, most examples from the literature document terminal investment in males (Polak & Starmer, 1998; Adamo, 1999; Abbot & Dill, 2001; Shoemaker *et al.*, 2006; Sadd *et al.*, 2006; Krams *et al.*, 2011).

1.4.3. Behavioural avoidance

Behavioural responses can also provide a host with prophylactic protection against parasitism by reducing their risk of contracting an infection, thus increasing the chance of 'qualitative resistance' (i.e. complete avoidance of infection) (de Roode & Lefevre, 2012) and thus avoiding costs associated with physiological immune activation. Hygienic behaviours, such as grooming behaviour, undertaking behaviour and sharing or spreading of antimicrobials may offer qualitative resistance by discouraging the growth of pathogens in the environment (Cremer *et al.*, 2007; Otti *et al.*, 2014). Physical avoidance

of parasites in the environment through avoiding contact with, feeding on or oviposition in contaminated sites or resources can allow hosts to avoid infection in themselves or in their offspring (Tasin *et al.*, 2012; Hussain *et al.*, 2010; Yanagawa *et al.*, 2011; Ormond *et al.*, 2011; Mburu *et al.* 2012; Sun *et al.*, 2008; Stensmyr *et al.*, 2012; Lam *et al.*, 2010; Villani *et al.*, 1994; Myles, 2002; Zhang *et al.*, 2005). Furthermore, hosts may physically avoid infected conspecifics and reduce their contact rates with them in order to reduce the chance of disease transmission (Hamilton & Zuk, 1982; Hart, 1990; Moore, 2002). Effective use of parasite avoidance behaviours rely on the production of danger cues by immune-challenged individuals or pathogens in the environment, and on the ability of other hosts to be able to detect these cues and respond to them (see section 1.7.1).

1.5. Immune priming

Although encounters with parasites are often unpredictable (Combes, 1995; Schmid-Hempel, 1998), being infected once with a parasite increases the probability of a reinfection for the host as the parasites are now present in the environment and also are likely to increase in abundance due to host-to-host transmission (e.g. Moret & Siva-Jothy, 2003). Selection should therefore favour the evolution of an immune memory which provides the host with a stronger and more rapid immune response upon secondary exposure to the same parasite (Kurtz, 2005).

Immunological memory is well understood in vertebrates, where the adaptive immune system augments phenotypic resistance over the lifespan of individuals and even allows for the communication of specific resistance to offspring via the maternal transfer of antibodies (Janeway, 2001). Whilst such adaptive immunity was long thought to be absent in invertebrates due to their lack of specific B-cell–mediated and T-cell–mediated immune memory, a growing body of evidence has demonstrated the existence of specific immune memory in invertebrates, also known as 'immune priming' (reviewed in Little & Kraaijeveld, 2004; Kurtz, 2005; Schulenburg *et al.*, 2009).

Invertebrates can become primed against a wide range of live pathogens, including parasitic tapeworms (Kurtz & Franz, 2003), Gram-positive (Pham *et al.*, 2007) and Gram-negative (Cong *et al.*, 2008) bacteria, and viruses (Tidbury *et al.*, 2011), as well as heat-killed pathogens (Sadd & Schmid-Hempel, 2006; Roth *et al.*, 2009a) and synthetic immune elicitors (Moret & Siva-Jothy, 2003). Immune priming can augment general defence against pathogens, and a priming state can persist over the lifespan of an individual (Haine *et al.*, 2008; Thomas & Rudolf, 2010), and can even be transmitted transgenerationally, priming offspring immunity (e.g. Little *et al.*, 2003; Kurtz, 2004;

Sadd *et al.*, 2005; Moret, 2006; Roth *et al.*, 2009b). Different forms of immune priming may be non-specific, providing an host augmented general defence against a wide range of parasites (e.g. Faulhaber & Karp, 1992; Lowenberger *et al.*, 1999; Moret & Siva-Jothy, 2003; Roth *et al.*, 2009a), or highly specific, priming the host against one particular strain, species or group of parasites (e.g. Sadd & Schmid-Hempel, 2006; Roth *et al.*, 2009a).

1.6. Immune prophylaxis

Whilst inducible defences and long-lasting priming, which are both activated post-infection, allow the host to ameliorate some of the costs of infection in the event of a secondary encounter with the same pathogen, hosts may still suffer considerable pathogenic damage between the time of primary infection and the time it takes them to induce an effective immune defence, especially in the case of highly virulent pathogens. Anticipatory immune priming can bolster host defences in advance of infection, reducing the costs associated with their delayed induction.

Hosts benefit from initiating immune prophylaxes in response to environmental cues that provide a reliable indicator of increased pathogenic threat. Bacteria, fungi and yeast produce a range of volatile organic compounds (referred to as microbial VOCs, or MVOCs) during various metabolic functions. MVOCs are of ecological relevance to insects, as they can provide a reliable indicator regarding the quality of food sources or ovipoisition sites, and may signal pathogenic hazards in the environment (reviewed in Davis *et al.*, 2013). Insects have evolved various neural pathways for the detection of such olfactory cues (Stensmyr *et al*., 2012; Farag *et al.*, 2013), allowing for many MVOCs to serve as semiochemicals which induce a range of behavioural responses in the host, such as the behavioural avoidance, reduced feeding or reduced oviposition on sites contaminated with bacterial or fungal pathogens (Tasin *et al.*, 2012; Hussain *et al.*, 2010; Yanagawa *et al.*, 2011; Ormond *et al.*, 2011; Mburu *et al.* 2012; Stensmyr *et al.*, 2012; Lam *et al.*, 2010; Villani *et al.*, 1994; Myles, 2002; Zhang *et al.*, 2005) (see section 1.7.1.2).

1.6.1. Density Dependent Prophylaxis (DDP)

Density-dependent prophylaxis (DDP) is a form of socially-induced immune priming, which has been observed in gregarious coleopterans, lepidopterans and orthopterans, whereby individuals reared at high population densities tend to develop more heavily melanised cuticles (Wilson & Reeson, 1998; Applebaum & Heifetz 1999; Barnes & Siva-Jothy, 2000; Cotter *et al.*, 2004). In Coleoptera, the cuticle may form a sink for harmful

quinones recruited for but not used in sclerotisation (Chapman, 1998), providing the host with a frontline defence against infection (Siva-Jothy *et al.*, 2005). Cuticle darkness and thickness are both positively correlated with disease resistance in many species (Gershenzon 1994; Verhoog *et al.* 1996; Reeson *et al.*, 1998; Barnes & Siva-Jothy, 2000), as thicker, more melanised cuticles are more able to resist enzyme degradation and hyphal penetration by fungal pathogens (Chapman 1982; Hajek & St Leger 1994).

DDP is a morphological immune prophylaxis which protects against the increased risk of pathogenesis present at higher host population densities (Reeson *et al.*, 1998; Wilson & Reeson, 1998; Schmid-Hempel, 1998; Barnes & Siva-Jothy, 2000), particularly in cannibalistic species which have an increased threat of wounding and subsequent opportunistic infection at high population densities (Barnes & Siva-Jothy, 2000). DDP appears to be stimulated by socially-derived mechanical cues which provide a reliable indicator of population size (i.e. number or frequency of physical contacts with conspecifics) (Barnes & Siva-Jothy, 2000). However, DDP is an inflexible defence, as the level of cuticular melanisation becomes fixed in early adulthood in tenebrionids (Thompson, 2002; Armitage *et al.*, 2003), and may have costs for the host. Darker, and therefore thicker, cuticles are less porous (Armitage *et al.*, 2003), which may limit the production of sex pheromones or cuticular hydrocarbons (CHCs) involved in sexual signalling and decrease the perceived attractiveness of the host (Armitage, 2002). Given that pathogenesis is often unpredictable (Combes, 1995; Schmid-Hempel, 1998), we may expect selection to favour the development of phenotypically plastic immune prophylaxes.

1.6.2. Mechanisms of immune priming

The molecular mechanisms underlying immune priming in invertebrates are largely unknown (Schulenburg *et al.*, 2007), but similar selection pressures have likely favoured the convergent evolution of immune prophylaxes in plants (Ausubel, 2005). Some plants induce immune prophylaxes in response to volatiles produced by nearby conspecifics experiencing infection or herbivory, enabling them to produce a more rapid and potentiated immune response in the event of a subsequent attack (Bate & Rothstein, 1998; Karban *et al.*, 2000; Arimura *et al.* 2002; Engelberth *et al.*, 2004; Karban *et al.*, 2006; Heil & Ton, 2008; Erb *et al.*, 2015). However, the primed state is, physiologically, relatively modest compared to that during actual parasite attack (Bate & Rothstein, 1998; Arimura *et al.* 2002; Engelberth *et al.*, 2004), sensitising the host towards future attack without suffering the costs associated with full-scale immune activation during actual

attack. The constitutive expression of inducible defences in plants has been shown to negatively affect several key aspects of fitness, including size, growth and seed production (Heil, 2002; van Hulten *et al.*, 2006), similarly to invertebrates (e.g. Kraaijeveld & Godfray, 1997; Moret & Schmid-Hempel, 2000).

Whilst the underlying mechanisms of immune priming differ greatly, it is likely that the primed state of immunity in invertebrates is also relatively dampened compared to that induced by actual infection, particularly considering the immunopathological potential of immune activation in insects (Nappi *et al.*, 1995; Sadd & Siva-Jothy, 2006). Intrinsic costs of immunity necessitate energetic, molecular and life-history trade-offs in all living hosts, suggesting there are inherent biological constraints to how an immune system can be constructed (Ausubel, 2005). In both plants and animals, immunological priming appears to provide a common adaptive solution to this problem, sensitising the host for a faster and stronger response towards future infection whilst reducing the costs of immediate biochemical investment and immunopathology in the meantime.

1.7. Social immunisation

A form of socially-induced immune prophylaxis has recently been described in two groups of eusocial insects, a termite, *Zootermopsis angusticollis* (Traniello *et al.*, 2002), and two ant species, *Lasius neglectus* (Ugelvig & Cremer, 2007; Konrad *et al.*, 2012) *a*nd *Camponotus pennsylvanicus* (Hamilton *et al.*, 2010). In these studies, immunologically naïve individuals that were housed with immune-stimulated nestmates exhibited greater levels of resistance in response to a subsequent infection. This phenomenon has been referred to as variously 'social immunisation', 'social vaccination', or a 'social transfer of immunity', and has been likened to the process of vaccination in humans (Konrad *et al.*, 2012). Essentially, it appears that naïve individuals become primed, or 'immunised', against future infection through the social environment without suffering the same fitness costs associated with a previous, full-scale infection.

1.7.1. Mechanisms of social immunisation

Konrad *et al.* (2012) propose two potential routes of social immunisation, active immunisation and passive immunisation, which need not be mutually exclusive. Essentially, active immunisation requires personal investment by a naïve host following contact with an infected conspecific (either through the personal induction of physiological immunity or behavioural responses), whereas in passive immunisation, defence is augmented in the naïve host by the transfer of immune factors, but not

pathogens, from an challenged conspecific (Rosengaus *et al.* 1999; Traniello *et al.* 2002; Feffermann *et al.* 2007; Konrad *et al.*, 2012).

It should be noted that each of the mechanisms discussed below are distinct from the process of herd immunity, whereby susceptible individuals are passively protected from disease by the presence of resistant individuals in the population, who act to reduce the risk of disease transmission (Anderson & May, 1982). In contrast, social immunisation relies on susceptible individuals actively augmenting their protection against pathogenesis through the induction of a personal response (e.g. upregulation of physiological immunity or behavioural defences), regardless of whether this physiological change is initiated by another conspecific or *de novo*.

1.7.1.1. Active immunisation following direct parasite contact

Physical interactions between hosts may result in the transfer of low-level infections (Yamada *et al.* 1992), causing naïve hosts to acquire a pathogenic load which is insufficient to cause systemic infection but large enough to provide long-lasting protection against the pathogen in question by priming the immune system (Rosengaus *et al.*, 1998; Konrad *et al.*, 2012). This process has been likened to inoculation (also known as 'variolation') against smallpox in human medicine (Konrad *et al.*, 2012).

Three of the four studies on social immunisation used a live entomopathogenic fungus, *Metarhizium anisopliae* (Traniello *et al.*, 2002; Ugelvig & Cremer, 2007; Konrad *et al.*, 2012). Entomopathogenic fungi are generally obligate killers and are highly transmissible; furthermore, they typically infect the host by penetration, as opposed to via an oral route, meaning that the physical removal of spores through self-grooming and allogrooming forms an effective defence (Schmid-Hempel, 1998). Most insects tend to ingest the fungal conidia they remove through grooming, as they are typically inactivated in the midgut (Schmid-Hempel, 1998). Ingestion of these spores may provide the host with a controlled primary exposure to the fungus (Hart, 1990), allowing the host immune system to recognise specifics PAMPs and possibly induce a long-lasting and specific form of immune priming akin to secondary mode prophylaxis (Moret & Siva-Jothy, 2003).

However, one study found that carpenter ants given a haemocoelic (and thus internalised) injection of the bacteria *Serratia marcescens* stimulated increased immune investment in the naïve individuals they were housed with (Hamilton *et al.*, 2010), suggesting that other processes besides allogrooming may be responsible for social immune transfer.

1.7.1.2. Active immunisation following indirect parasite recognition

Immune priming may also be stimulated without direct parasite contact if naïve hosts are able to detect and respond to pathogenic cues, such as compounds released by parasites or compounds released by infected hosts.

For example, cuticular hydrocarbons (CHCs) are compounds present on the outer integument of insects which are essential to intraspecific communication in insects (Singer 1998; Wyatt 2003), and have been shown to become modified following immune stimulation in both social and non-social insects (Trabalon *et al.* 1999; Salvy *et al.*, 2001; Richard *et al.*, 2008; Nielson & Holman, 2011; Baracchi *et al.*, 2012). Whilst pathogenic damage may be partially responsible for such changes – for example, fungal pathogens have also been shown to directly degrade host CHCs (Lecuona *et al.* 1991; Napolitano & Juarez, 1997) – similar changes in the chemosensory profile have been observed in response to non-pathogenic immune elicitors (Richard *et al.*, 2008; Nielson & Holman, 2011), suggesting changes may also be induced by the host. Immune factors induced during immune stimulation can be transported from the haemolymph to the epicuticular surface (Schal *et al.* 1998), and studies have shown that *Drosophila* can induce expression of AMPs on their surface epithelium (Tzou *et al.*, 2002), suggesting that the externalisation of induced immune factors during challenge may form a communicable immune cue for conspecifics.

Alternatively, hosts may detect pathogenic cues that are released into the environment through excretion by infected hosts. Many bacteria and fungi release low molecular weight compounds, known as microbial volatile organic compounds (MVOCs), that can diffuse readily through the environment (Davis et al., 2012). Whilst these volatiles primarily evolved to communicate information between bacteria and fungi themselves, they are also a reliable indicator of microbial presence in the environment, and a diverse range of host species have evolved the ability to detect them (Farag *et al.*, 2013). Many insect hosts, including ladybirds, moths, fruit flies, house flies and termites, exhibit behavioural avoidance, reduced feeding and reduced oviposition of sites containing MVOCs of pathogenic bacteria and entomopathogenic fungi (Tasin *et al.*, 2012; Hussain *et al.*, 2010; Yanagawa *et al.*, 2011; Ormond *et al.*, 2011; Mburu *et al.* 2012; Sun *et al.*, 2008; Stensmyr *et al.*, 2012; Lam *et al.*, 2010; Villani *et al.*, 1994; Myles, 2002; Zhang *et al.*, 2005). Some MVOCs, termed 'necromones' (Yao *et al.*, 2009), are chemicals which are passively produced by the metabolism of amino acids which occurs during putrefaction of animal tissues (Medzhitov *et al.*, 2012). Whilst necromones may mediate corpse management behaviours in eusocial species (e.g. Wilson *et al.*,

1958), they tend to induce avoidance of conspecific corpses in non-social insects, such as cockroaches (Rollo *et al.*, 1994), springtails (Yao *et al.*, 2009) and solitary bees (Abbott, 2006).

Termites (*Coptotermes formosanus*) with experimentally-removed antennae engaged in more frequent grooming of nestmates with cuticle-attached fungal spores, but also experienced higher mortality in response to experimental infection with the same fungus (Yanagawa *et al.*, 2009). Termites are able to distinguish between the fungal strains of different virulence, removing (and ingesting) more conidia with low virulence (Mburu *et al.*, 2005; Hussain *et al.*, 2010; Yanagawa *et al.*, 2011). This suggests that chemosensation plays an important role in coordinating grooming behaviour and social immunisation in termites, even though the mechanism of social transfer appears to be via mutual grooming (Yanagawa *et al.*, 2009). Indeed, naïve termites exposed to an aqueous solution containing *M. anisopliae* conidia also increase their levels of mutual grooming and attack behaviours, suggesting that chemosensory cues alone are sufficient to produce certain prophylactic responses (Yanagawa *et al.*, 2011).

Infected hosts may excrete pathogenic materials into the environment (live pathogens, dead pathogens or pathogen-derived compounds), but could also excrete immune factors or immune-related metabolites which could act as a semiochemical cue of pathogenic threat to nearby conspecifics, in a similar way to alarm pheromones (e.g. Schal *et al.*, 1998; Tzou *et al.*, 2002). For example, immunopathological damage incurred during an immune response in *Tenebrio molitor* can cause significant damage to the Malpighian tubules (Sadd & Siva-Jothy, 2006). These structures are essential regulators of osmoregulation and a loss of function could cause a greater build up of waste products within immune-challenged hosts, having the potential to alter their chemosensory profile of the challenged host directly (e.g. their CHCs, pheromones, antimicrobial secretions) or indirectly (e.g. their excreta).

Finally, mechanical, visual and behavioural changes during infection may provide an infection cue to nearby conspecifics. For example, upon detection of a fungal pathogen in the nest, termites use a vibratory display as an alarm signal which induces an immune response in nearby nestmates (Rosengaus *et al.*, 1999). Workers of the ant *Formica rufa*, which typically feed on nestmates carcasses, are able to discriminate and avoid nestmates killed by an entomopathogenic fungus when their carcasses are visibly covered with infectious (mature) conidia (Marikovsky, 1962). Interestingly, the visual perception of infectious disease by humans stimulates an upregulation of the proinflammatory immune molecule interleukin (IL)-6 (Schaller *et al.*, 2010). Sickness behaviours exhibited by

immune-challenged hosts, such as reduced locomotion or reduced mating effort, may also provide nearby conspecifics with a reliable cue of pathogenic threat, and stimulate their investment in behavioural or physiological prophylaxes. However, sickness behaviours in non-social hosts are more likely to be an incidental byproduct of infection which is intercepted, or 'eavesdropped' on (Stowe *et al.*, 1995), by naïve hosts, as opposed to a purposeful signal intended for communication, such as vibration in termites (Rosengaus *et al.*, 1999).

Nevertheless, despite the potential for many different types of pathogenic cues to be produced, there has as yet been no experimental evidence of a signal-induced form of social immunisation (Konrad *et al.*, 2012; Masri & Cremer, 2014).

1.7.1.3. Passive immunisation

Social immunisation in termites and ants has been attributed to the transfer of immune factors from exposed individuals to naïve nestmates through social processes of grooming and mutual feeding (Traniello *et al.*, 2002; Ugelvig & Cremer, 2007; Hamilton *et al.*, 2010). During stomodeal trophallaxis, liquid nutrients are shared throughout the insect colony (Hölldobler & Wilson, 1990), along with hydrocarbons important in nest-mate recognition (Dahbi *et al.*, 1999) and potentially other compounds present in bodily fluids. Transfer may also occur over externalised routes which do not involve direct host-host contact. For example, gut microbiota in bumble bees (*Bombus terrestris*) appear to be socially transmitted throughout the colony via a faecal-oral route between individuals (Koch & Schmid-Hempel, 2011). Individuals prevented from feeding on faeces developed higher parasite loads when infected with a trypanosomatid gut parasite (Koch & Schmid-Hempel, 2011), suggesting that gut microbiota may be involved in the social transfer of immunity. The authors suggest that gut microbiota may reduce growth of pathogenic species indirectly by increasing the level of competition for resources in the host gut and/or directly through stimulated host production of antimicrobials (Koch & Schmid-Hempel, 2011).

In carpenter ants (*Camponotus pennsylvanicus*), immune-challenged individuals increase their frequency of trophallaxis with naïve nestmates (Hamilton *et al.*, 2010). The trophallactic droplets from challenged hosts also have increased antimicrobial activity (Hamilton *et al.*, 2010), which appears to be due to increased concentrations of a cathepsin D-like lysozymal protease, which directly exhibits antibacterial activity and catalyses the production of AMPs (Thorne *et al.*, 1976; Hamilton *et al.*, 2010). Trophallaxis appears to be essential for social immune transfer in this species, as naïve

ants kept near, but physically separated from, challenged nestmates did not display increased resistance to subsequent immune challenge, suggesting that chemosensory or visual cues are insufficient to stimulate a priming response (Hamilton *et al.*, 2010).

Whilst the transfer of immune factors has been suggested to be a ubiquitous immune defence across the social insects (Rosengaus, 2010), social behaviours like trophallaxis and allogrooming are absent in non-social insects. However, active immunisation routes, which could involve the direct transfer of low-level infection between hosts or the detection of conspecific cues of infection, are possible as they do not predicate upon sociality. It is also possible that immune effectors produced by challenged hosts may break down pathogens in the environment, creating elicitors (which may be host and/or pathogen derived) that prime other individuals that contact them (Bulmer *et al.*, 2010).

1.8. Importance of social immunity in eusocial insects

Gregariousness has often been associated with a higher risk of pathogenesis (Schmid-Hempel, 1998), and this has been suggested to be a selective force favouring the evolution of sociality in group-living species (Elliot & Hart, 2010). The principle of 'mass-action' assumes that the per capita risk of infection increases with population density, and is implemented in most standard epidemiological models (Elliot & Hart, 2010). However, this assumption is also dependent upon the specific host and pathogen in question, as not all parasites exhibit density-dependent transmission, and the relationship between contact rate between infected and susceptible individuals may be asymptotic with regards to host density (McCallum *et al.* 2001).

In fact, the risk of between-group disease transmission has been suggested by some models to be lower in more aggregated populations (Wilson *et al.* 2003; van Baalen & Beekman 2006). Indeed, many social insects tend to exhibit greater resistance when in dense groups than when alone (Rosengaus *et al.* 1998, Traniello *et al.* 2002; Calleri *et al.*, 2006; Hughes *et al.*, 2002), and per capita transmission has actually been found to decrease at higher densities, perhaps due to a saturating effect (Reeson *et al.*, 2000; Hughes *et al.*, 2002; Wilson & Cotter, 2008). Elliot & Hart (2010) suggest that host population density is not the most important determinant of the likelihood of disease, and that disease transmission is a function of both frequency and density dependency (Antonovics *et al.* 1995; Begon *et al.* 1999). They argue that levels of 'connectivity' (or contact rates) are a greater influence upon pathogenic susceptibility and transmission rates, and that behavioural defences in particular should become cheaper in more

connected populations (Elliot & Hart, 2010). Nevertheless, a meta-analysis has reported consistent positive correlations between group size and infection rates in the cases of parasites transmitted by close contact or via a faecal–oral route (Coté & Poulin 1995).

Kin selection is likely to be a stronger selective force in highly related populations, favouring group-level defences that reduce the risk of pathogenesis for all members of the group and should yield high inclusive fitness benefits. This has likely allowed for the evolution of complex 'social' immune defences in the eusocial insects, such as self-grooming, allogrooming and antimicrobial secretion (e.g., Hughes *et al.* 2002, Traniello *et al.* 2002, Cremer *et al.* 2007, Cremer and Sixt 2009, Jackson and Hart 2009). Individual-level behavioural and physiological immune responses (i.e. personal immunity) are also more likely to provide pleiotropic benefits in highly connected populations (Elliot & Hart 2010), and may provide an additional 'social barrier' against pathogenesis when expressed at the group level (Cremer *et al.*, 2007).

Social insects stand to gain indirect fitness benefits from acting to reduce the risk of pathogenic transmission to nestmates when infected. For instance, sick carpenter ants (*Camponotus aethiops*) and honey bees become ‘unsociable’, reducing the amount of time spent in the nest and reducing their frequency of social interactions, particularly with brood and the queen (Richard *et al.* 2008; Bos *et al.*, 2012). Conversely, healthy carpenter ants and termites have been shown to increase their contact rate with parasitised conspecifics, engaged in more frequent hygienic behaviours like allogrooming antimicrobial gland secretion which reduce their susceptibility to disease (Hughes *et al.* 2002; Traniello *et al.* 2002; Calleri *et al.* 2006).

Although increased rates of social contact with infected individuals could easily facilitate disease transmission in dense populations, social insect societies are highly structured, and adaptive modifications of spatial distributions and social interactions during pathogenesis can help to limit the spread of disease (Ugelvig & Cremer 2007). Furthermore, processes of social immunisation (e.g. Traniello *et al.*, 2002; Hamilton *et al.*, 2010; Ugelvig & Cremer, 2007; Konrad *et al.*, 2012) may provide a net benefit to the colony of increased contact with infected individuals, and it has been suggested that this could account for the relative rarity of contact limitation and social exclusion towards infected conspecifics in eusocial insects (Cremer *et al.* 2007).

1.9. Potential for 'social' defences in non-social insects

1.9.1. Selection for group-level defences

Although 'social immunity' is classified as any defence which increases the fitness of the

challenged individual and one or more conspecifics (Cotter & Kilner, 2010), some seemingly altruistic traits can be the product of selfish selective pressures. Kin-selection frameworks suggest that altruism in social insect colonies may be 'enforced' rather than voluntary, as it seems that social sanctions taken against 'cheaters' (e.g. reproductive workers) in the population may select for continuing cooperation in the population, rather than the inclusive fitness benefits of altruism (Wenseelers & Ratnieks 2006; Brown & Taylor 2010). Although high relatedness was probably initially required for sociality to evolve in insects (Brown & Taylor 2010), it has been suggested that the development of affordable punishment mechanisms could even permit levels of cooperation to increase as population relatedness decreases (Frank 2003; Brown & Taylor 2010).

Although immunity may confer pleiotropic benefits to indirect (kin) fitness in highly related populations, sociality need not be a prerequisite for the evolution of group-level defences which benefit multiple members of the population (Elliot & Hart 2010). Immune responses which increase the fitness of the individual, such as physiological resistance, behavioural avoidance or antimicrobial secretions, can become co-opted to act at the group-level, even if the group-level benefits they offer (i.e. to other conspecifics) are of secondary importance in terms of selection pressure (Elliot & Hart, 2010; Brown & Taylor, 2010).

1.9.2. Antimicrobial secretions

Larvae of the *Chrysomela* leaf beetle, and the more distantly related brassy willow leaf beetle, *Phratora vitellinae*, constitutively secrete a volatile compound, salicylaldehyde, which has repellent effects on generalist predators but also attracts some specialist pathogens and parasitoids (Pasteels *et al.*, 1988; Gross *et al.*, 2008). It has recently been shown that salicylaldehyde also has potent antimicrobial effects, and that the release of even small amounts can create an antimicrobial fumigant cloud around larvae which can provide effective protection from fungal and bacterial pathogens in the microenvironment (Gross *et al.*, 2008; Gross *et al.*, 1998). Groups of six individuals can create a significantly greater bacterial zone of inhibition than solitary individuals (Gross *et al.*, 2008). Furthermore, leaf beetle larvae engage in a specialised group behaviour known as cycloalexy, where larvae feed together in closely aggregated groups of 10 to 30 individuals (Pasteels *et al.*, 1988). Whilst this behaviour has been explained as providing increased protection against predation, it may also offer a secondary benefit of augmenting antimicrobial defence through synergistically amplifying the salicylaldehyde fumigant cloud (Gross *et al.*, 2008).

Such fumigants which provide disinfection of the local environment have been suggested to be a common form of immune defence in non-social insects (Gross *et al.*, 2008). The evolution of externalised immune defences may be favoured in species with have large group sizes, use permanent and confined nests, and keep more permanent stores of food (Otti *et al.*, 2014). Larvae of the sawfly (Family Tenthridinoidea) and adult harlequin ladybirds (*Harmonia axyridis*) emit similar antimicrobial compounds to leaf beetles, whilst glandular secretions in water beetles (Family Hydradephaga) have also been found to inhibit bacterial growth on their cuticle (Gross *et al.*, 2008). Tenebrionid beetles also produce externalised secretions, such as volatile benzoquinones, which have antimicrobial action (Tschinkel, 1975; Yezerski *et al.*, 2000). Benzoquinone secretion is upregulated during tapeworm parasitism in *T. molitor* (Yan & Phillips, 1993), and the compounds have been shown to have a repellent effect on conspecifics and predators in *Tribolium* (Suzuki, 1980), suggesting they may have some function as a group-level defence.

1.9.3. Pathogen pressures

Non-social group-living insects are likely to face many of the same pathogen threats as eusocial species, having high population densities and often higher levels of genetic homozygosity, two factors which are both associated with an increased risk of pathogenesis (Schmid-Hempel, 1998). However, non-social gregarious species lack the specialised forms of spatial and behavioural compartmentalisation which have been key to the success of the eusocial insects (Fefferman *et al.*, 2007). Furthermore, although group living is predicted to cheapen the relative costs of behavioural defences (Elliot & Hart 2010), comparatively lower levels of relatedness may mean that selfish selective pressures in non-social species favour immune strategies which benefit personal fitness of the acting individual at the expense of increasing disease transmission in the population. For instance, in many non-social species, infected individuals (particularly males) terminally invest in reproduction, increasing their reproductive activity to try and counter the costs of parasitism (Knell & Webberley, 2004; Sadd *et al.*, 2006; Krams *et al.*, 2011; Goodacre & Martin, 2012; Nielson & Holman, 2011). Sexual dimorphism in life-history investment and immunocompetence (Bateman, 1948; Rolff, 2002) can lead to certain individuals becoming 'superspreaders' of disease (Galvani & May, 2005), with hosts actively driving parasite transmission in the population. Furthermore, hosts which tolerate infection are more likely to maintain within themselves a pathogenic reservoir which can infect other conspecifics. Whilst resistance may therefore be more strongly selected for in

social species (e.g. Stow *et al.*, 2007; Turnbull *et al.*, 2010), less highly related species are expected to favour more tolerance-based responses to infection or infection threat (Figure 1.3).

The relative costs of behavioural versus physiological immune defences have been predicted to decrease with increasing levels of population connectivity in non-social species, and even more so with the level of sociality (Elliot & Hart, 2010). However, hosts living in homogenous environments at high population densities with low dispersal rates are more likely to succumb to reinfection by the same pathogen (van Baalen, 1998). Long-lasting forms of physiological immune priming may therefore be less costly to hosts in terms of missed opportunities incurred through continual expression of behavioural defences, such as conspecific avoidance or anorexia.

It therefore stands to reason that non-social but gregarious species should benefit from responding to environmental cues of infection provided by conspecifics, which should be plentiful in their production, and from utilising these cues in order to modulate investment in both physiological and behavioural immune strategies in order to form an effective defence against parasitism.

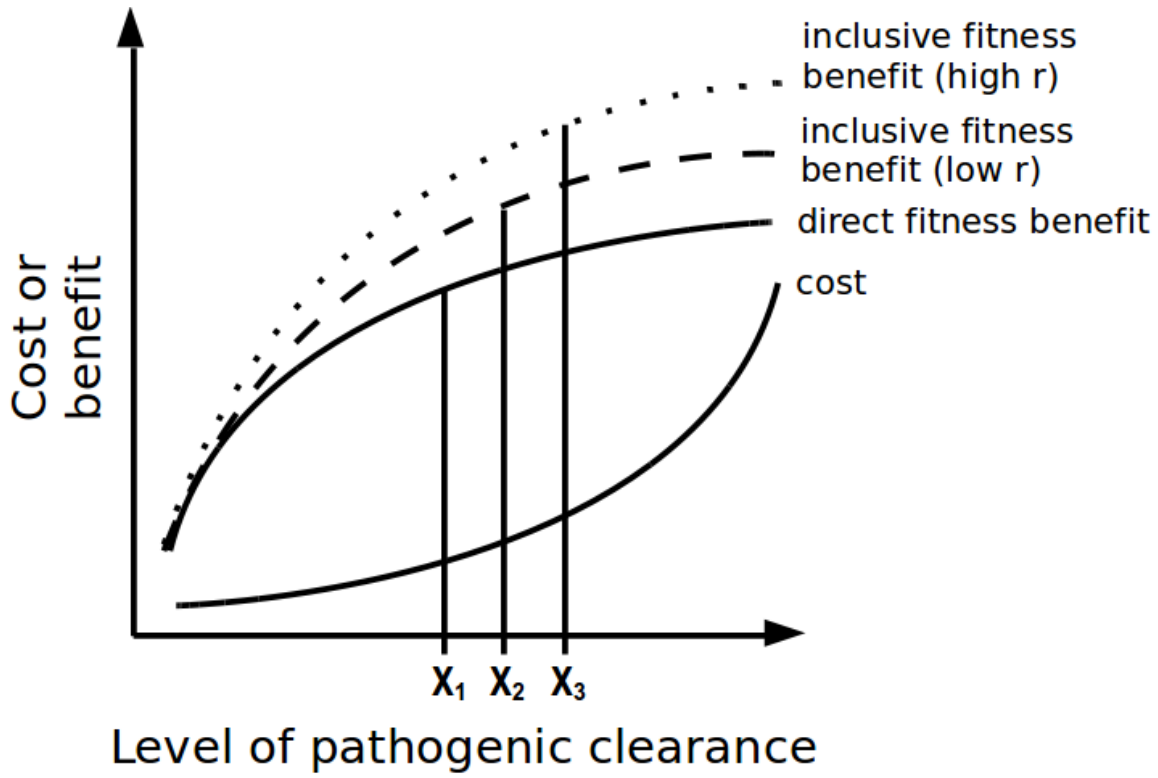


Figure 1.3. The effect of population relatedness, which is often used as a proxy for sociality, upon the costs and benefits of pathogenic clearance by an infected host. Resistance-based responses reduce the parasite load of infected hosts and thus lower their potential for disease transmission to other conspecifics. On the other hand, hosts which tolerate infection are more likely to maintain within themselves a pathogenic reservoir, increasing their potential for disease transmission, especially if these hosts also increase their reproductive activity to try and counter the costs of infection. When considering the detrimental effects of increased transmission upon indirect (kin) fitness in related populations, hosts may be expected to favour resistance-based responses which are more likely to eliminate the pathogen, despite inflicting greater costs upon personal fitness. The optimal level of pathogen clearance should increase as the level of relatedness within the host population increases; for example, from moderate levels of relatedness (dashed line; maximum inclusive fitness benefit at X_2) to high relatedness (dotted line; maximum benefit at X_3).

1.10. Study organism

The yellow mealworm beetle, *Tenebrio molitor* (Coleoptera: Tenebrionidae) has been used as a model organism for immunity for over a century (e.g. Gortner, 1910). Its relatively short generation time (ca. 8 weeks), ease of culturing and relatively large body size make it amenable to laboratory studies, and a significant body of literature exists concerning their immunological and reproductive biology (Barnes & Siva-Jothy, 2000; Moret & Siva-Jothy, 2003; Armitage *et al.*, 2003; Moret, 2006; Sadd & Siva-Jothy, 2006; Sadd *et al.*, 2006; Haine *et al.*, 2008; Johnston *et al.*, 2014). Furthermore, the beetle is a stored product pest which causes agricultural damage to stored food sources and endangers public health through food contamination (Stejskal & Hubert 2008).

As a gregarious stored product pest, the beetle shares several ecological factors with social insect species, such as high population density, the use of permanent nest sites with stored resources, and high pathogen presence in environment (e.g. Yezerski *et al.*, 2005). These factors should increase the vulnerability of the species to pathogenesis (Schmid-Hempel, 1998) and have been predicted to favour the development of group-level immune responses (Otti *et al.*, 2014). Indeed, many tenebrionids, including *T. molitor* produce externalised secretions with antimicrobial activity, such as benzoquinones (Tschinkel, 1975). Whilst the primary purpose of these secretions appears to be to manage unwanted microbial growth in food stores (Yezzerski *et al.*, 2000; Otti *et al.*, 2014), they may also function as a group-level immune defence by reducing the risk of pathogenesis for surrounding conspecifics (e.g. Yan & Phillips, 1993; Zanchi, 2011), as well as acting as a predator deterrent (e.g. Suzuki, 1980).

T. molitor females are polyandrous (Drnevich *et al.*, 2001; Worden & Parker, 2005), a trait that benefits eusocial insects by increasing allelic diversity (Baer & Schmid-Hempel, 1999; Hughes & Boomsma, 2004; Liersch & Schmid-Hempel, 1998; Seeley & Tarpy, 2007). Indeed, multiple mating in *T. molitor* has been found to increase larval viability of offspring (Pai *et al.*, 2005) and egg viability in F1 offspring (Pai & Yan, 2003). Owing to generational overlap, relatively low dispersal rates and a homogeneous environment, parents and offspring should tend to face the same pathogenic pressures (Schmid-Hempel, 2005); correspondingly, transgenerational immune priming has also been documented in this species, as well as other closely related tenebrionids (Kurtz, 2004; Sadd *et al.*, 2005; Moret, 2006; Roth *et al.*, 2009b).

All of these factors are therefore predicted to predispose *T. molitor* to the development of group-level immune responses through use of 'social' cues (i.e. information derived from conspecifics). In this thesis, I explore the idea of 'gregarious immunisation' in *T. molitor,* whereby uninfected individuals are predicted to reduce the costs of infection by physiological and/or behavioural responses to environmental cues of infection provided by immune-challenged conspecifics.

1.10. Thesis outline

The work conducted in this thesis investigates the potential for a form of socially-induced immune priming, which is hereafter referred to as 'gregarious immunisation', in the mealworm beetle, *Tenebrio molitor*. I examined the role that both physiological and behavioural immune responses may play in the horizontal transfer of immunity.

Chapter 2 investigates the potential for physiological immune priming in *T. molitor* via an active route of gregarious immunisation. Immunologically naïve females were housed with a same-sex conspecific suffering from a live, and potentially transmissible, bacterial infection, and the antibacterial activity and survival rates of naïve cohabitants are measured in response to subsequent bacterial challenge.

Chapter 3 further investigates a physiological form of gregarious immunisation, this time pairing naïve individuals with a conspecific challenge by an artificial immune elicitor to eliminate the potential for pathogen transmission. The mechanistic basis of priming was examined by measuring levels of phenoloxidase activity and encapsulation in naïve cohabitants both before and after a subsequent immune challenge. This aimed to determine whether exposure to a potential pathogenic threat upregulates constitutive immune defences in the naïve host or rather sensitises inducible effectors in order to allow for a potentiated, but not immediate, immune response reserved for future attack. I used both male/male and female/female pairs of challenged/unchallenged individuals in order to investigate for potential gender effects in priming.

Chapter 4 details the development of an automated tracking system which is capable of recording the movement of insects and extracting a range of metrics which allow for a quantitative description of locomotion.

Chapter 5 used the software developed in Chapter 4 to determine whether *T. molitor*

males and females exhibit sickness behaviours following immune challenge, in order to determine whether behavioural cues of infection produced by infected individuals play a role in immune priming for other conspecifics, and whether gender differences exist in behavioural responses to infection.

Chapter 6 brings together the main findings of the thesis and their implications, and discusses potential avenues for future research.

CHAPTER TWO:

COHABITATION WITH AN INFECTED CONSPECIFIC PROMOTES IMMUNE TOLERANCE IN IMMUNOLOGICALLY NAÏVE *Tenebrio molitor*

2.1. Introduction

Immunological memory is the ability of the immune system to respond more rapidly and effectively to pathogens that have been encountered previously. Whilst immune memory is well understood in vertebrates, it was long thought to be absent in invertebrates due to their lack of specific B-cell–mediated and T-cell–mediated immune memory. However, recent work has documented a long-lasting and sometimes specific form of immune memory in invertebrates, known as 'immune priming' (Schmid-Hempel, 2005b; Schulenburg *et al.*, 2007). Insects can become primed against a wide range of live pathogens, including parasitic tapeworms (Kurtz & Franz, 2003), Gram-positive (Pham *et al.*, 2007) and Gram-negative (Cong *et al.*, 2008) bacteria, and viruses (Tidbury *et al.*, 2011), as well as heat-killed pathogens (Sadd & Schmid-Hempel, 2006; Roth *et al.*, 2009a) and synthetic immune elicitors (Moret & Siva-Jothy, 2003).

Perhaps the most well-understood form of immune priming in insects is secondary-mode prophylaxis, which is analogous to adaptive immunity in vertebrates; i.e. a primary infection augments host resistance upon secondary exposure to the same pathogen (Moret *et al.*, 2006). Prophylactic defences are enacted before full-scale infection as a form of preventative defence, and may be initiated in response to environmental cues indicative of increased pathogenic threat, such as increased host population density (Wilson & Reeson, 1998; Barnes & Siva-Jothy, 2000), environmental pathogen presence (Pujol *et al.*, 2001; Yanagawa *et al.*, 2011), or conspecific infection status (Carver & Hurd, 1998; Worden *et al.*, 2000; Worden & Parker, 2005; Vainikka *et al.*, 2007).

The ability to discriminate infected from healthy conspecifics and subsequently induce a prophylactic immune response has recently been discovered in the eusocial insects, where it has been referred to as 'social immunisation' (Konrad *et al*., 2012). It has been shown in both termites (Traniello *et al.*, 2002) and ants (Ugelvig & Cremer, 2007; Hamilton *et al.*, 2010; Konrad *et al.*, 2012) that healthy individuals cohabiting with immune-stimulated nestmates can enhance their resistance and subsequently increase their survival in response to an infection to the same pathogen as that which infected their

nestmates.

There are two hypothesised (and non-exclusive) routes of social immunisation; active and passive (Konrad *et al.*, 2012). Active immunisation involves the *de novo* production of a personal immune response by a naïve host following exposure to an infected nestmate, either through the direct transmission of low-level infection between hosts (Konrad *et al.*, 2012) or through the detection of infection cues by the infected host (e.g. chemosensory, visual or behavioural modifications) (Rosengaus *et al.* 1999; Konrad *et al.*, 2012). Passive immunisation need not require the activation of the host's own immune system, as protection may be afforded by the direct transfer of (as yet unknown) immune factors from a challenged conspecific to naïve host, possibly through behaviours like social feeding (Rosengaus *et al.* 1999; Traniello *et al.* 2002; Feffermann *et al.* 2007). The exposure of a host to an artificially immune-challenged conspecific with a non-transmissible 'infection' may therefore be distinct to exposure to a conspecific with a live, potentially transmissible pathogenic infection.

Although social immunisation is a part of a suite of complex social immune defences in the social insects, eusociality need not be an essential prerequisite for the evolution of group-level immune defences (Elliot & Hart, 2010). Individual-level physiological and behavioural responses may become co-opted at the group level if they increase overall defence for the individual, regardless of whether or not additional fitness benefits are also provided for surrounding conspecifics (Elliot & Hart, 2010; Brown & Taylor, 2010). Although social behaviours which have been suggested to contribute towards social immunisation, like social feeding (Rosengaus *et al.* 1999; Traniello *et al.* 2002) and allogrooming (Hamilton *et al.*, 2010; Konrad *et al.*, 2012), are uncommon outside the social insects (Cremer *et al.*, 2007), non-social species are known to undergo many physiological and behavioural changes during immune challenge. Whilst such changes may be an incidental byproduct of infection in non-social species as opposed to a purposeful communication signal, they could still be used as a cue to inform investment in prophylactic if individuals are able to 'eavesdrop' on their neighbours (Stowe *et al.*, 1995).

The mealworm beetle, *Tenebrio molitor*, is a non-social but gregarious stored product pest which undergoes frequent bursts of increased population density (Tschinkel & Willson, 1971) and exhibits several traits with density-dependent polyphenism (e.g. Barnes & Siva-Jothy, 2000). High population density, relatively low dispersal and high pathogen presence in a shared food source (Yezerski *et al.*, 2005; Otti *et al.*, 2014) are

ecological factors of this species which are thought increase vulnerability to pathogenesis (Schmid-Hempel 1998).

The chemosensory profile of *T. molitor* is known to vary with immune status (Nielsen & Holman, 2011), and females are able to discriminate against immune-stimulated males (Worden *et al.*, 2000; Sadd *et al.*, 2006; Krams *et al.*, 2011; Nielsen & Holman, 2011). Furthermore, *T. molitor* produce externalised secretions, such as volatile benzoquinones, which have antimicrobial action (Yezzerski *et al.*, 2000; Otti *et al.*, 2014). Secretion of these compounds is upregulated during tapeworm parasitism in *T. molitor* (Yan & Phillips, 1993), and the compounds have been shown to have a repellent effect on conspecifics and predators in closely-related *Tribolium spp.* (Suzuki, 1980), suggesting they may have some function as a group-level defence. The evolution of externalised immune defences may be favoured in such species with have large group sizes, use permanent and confined nests, and keep more permanent stores of food (Otti *et al.*, 2014).

Physiological and/or behavioural prophylaxes can reduce the costs of defence in environments where infection is more likely, and the potential for such preventative defences should thus be higher in gregarious species because: (i) the encounter rate with infectious agents may be expected to be higher due to density-dependent effects, (ii) there are likely to be stronger kin selection effects that reduce the threshold for the evolution of behavioural defences, and (iii) there should be a greater opportunity to detect cues of infection produced by conspecifics. I therefore investigated the potential for 'gregarious immunisation' in *T. molitor,* whereby uninfected individuals are predicted to reduce the costs associated with infection by investing in physiological or behavioural defences following cohabiting with an infected conspecific.

I used the Gram-positive bacteria *Staphylococcus aureus* as an infective agent, which has been shown to cause persistent infection and induce a long-lasting immune response (up to 28 days) in *T. molitor* (Haine *et al.*, 2008). The bacteria is an opportunistic pathogen which is ecologically relevant, as it is often present at high levels in grain stores (e.g. Yezerski *et al.*, 2005), and *T. molitor* endemically harbour high levels of the closely-related bacteria *S. epidermis* on their cuticle (e.g. Dobson, 2012). I predicted that cohabitation of an immunologically naïve individual with an infected conspecific would result in increased resistance and enhanced survival in the naïve individual during subsequent infection. Furthermore, to distinguish between the different potential routes of active and passive gregarious immunisation (Konrad *et al.*, 2012), I used either live or heat-killed bacteria to stimulate an immune response in 'sick' conspecifics. I predicted differences in immunity between naïve individuals cohabiting

with conspecifics with a live, transmissible bacterial infection and those cohabiting to conspecifics inoculated with heat-killed bacteria (i.e. a non-transmissible infection).

In this chapter, I examined:

- How immunity and survival respond to immune challenge in a naïve beetle that has cohabited with an infected conspecific
- The quantitative differences in induced immunity between naïve individuals housed with a conspecific with a live (transmissible) pathogen, and naïve individuals housed with a conspecific with a heat-killed (non-transmissible) pathogen

2.2. Methods

2.2.1. Insect culturing

Final-instar larvae of *Tenebrio molitor* were purchased from a commercial supplier (Live Foods UK) and maintained in an insectary at 26±2 °C under a 12/12hr light/dark cycle. Larvae were kept at a density of ~800 individuals per 30×15×10 cm box, and were provided with *ad libitum* access to Progrub (Livefoods Direct Ltd) and supplemented with freshly cut potato once per week. Pupae were collected between 2-3 days after pupation, and were sexed and weighed before being maintained in isolation in grid box containers. Only female pupae with a wet weight of 116–232mg were used (representing ± 2 S.D. around the population mean). Imagoes were provided with Progrub and a ~50mg potato supplement upon adult eclosion, and all treatments were performed 8-10 days after eclosion.

2.2.2. Bacterial culturing

Bacteria were derived from a single ancestral colony of erythromycin-resistant *Staphylococcus aureus (*JP015, SH1000 background; sourced from Simon Foster, University of Sheffield). The bacteria were revived from a frozen glycerin stock by plating onto Luria broth agar (2% LB, 1.5% agar) infused with 10µg/mL erythromycin, as well as 5.6µg/mL amphotericin-B to prevent fungal growth. Plates were incubated at 37°C for 24 h before a single colony was removed and suspended in 30mL Luria broth (2% LB) infused with 10µg/mL erythromycin, and cultured for 36-48 h in a shaking incubator (37°C, 110rpm), which is sufficient for the bacteria to reach stationary phase (Dobson, 2012).

Bacterial population density was estimated turbidometrically by measuring the optical density (OD650nm) of the bacterial suspension using a microplate reader (VersaMax), and referencing a previously calculated calibration curve. The bacterial suspension was then washed by two rounds of (i) pelleting by centrifugation (4500g for 20mins at 4°C), (ii) supernatant removal, (iii) pellet resuspension in 1mL sterile phosphate buffered saline (PBS), and (iv) recombination by pipetting and vortexing. The twice-washed solution was then used to prepare three different inoculates (see Table 2.1), which were adjusted to the appropriate concentrations by dilution with sterile PBS.

Table 2.1. Full treatments for infected individuals and subsequent assay challenges for paired naïve individuals.

Group	Infected treatment	Naïve treatment (antibacterial activity)	Naïve treatment (survival)
Live infection	$2.5x10^4$ CFUs live *S.aureus*	$2.5x10^6$ CFUs live *S.aureus*	$5x10^7$ CFUs live *S.aureus*
Heat-killed infection	$2.5x10^4$ CFUs heat-killed *S.aureus*	$2.5x10^6$ CFUs live *S.aureus*	$5x10^7$ CFUs live *S.aureus*
Procedural control	PBS	$2.5x10^6$ CFUs live *S.aureus*	$5x10^7$ CFUs live *S.aureus*
No-treatment control	- none -	$2.5x10^6$ CFUs live *S.aureus*	$5x10^7$ CFUs live *S.aureus*

2.2.3. Bacterial inoculate preparation

Three live inoculates were prepared (see Figure 2.1 for treatments): a $2.5x10^4$ CFUs/mL to be administered to live-infected cohabitants (Inf^{live}), and a $2.5x10^6$ CFUs/mL inoculate to be administered to naïve individuals following assigned to the antibacterial activity assay following cohabitation, and a $5x10^7$ CFUs/mL inoculate to be administered to naïve individuals assigned to the antibacterial activity assay following cohabitation. A $2.5x10^4$ CFUs/mL heat-killed inoculate, administered to heat-killed infected cohabitants (Inf^{heat}), was also prepared by heating the live bacterial solution in a waterbath at 95°C for 30 mins before leaving to cool. The effectiveness of heat-killing treatment upon bacterial mortality was confirmed by plating out 200µL of undiluted heat-treated inoculate on control agar (infused with only 5.6µg/mL amphotericin-B) and incubating for 48 h at 37°C, as no CFUs were detected on any plate (n=6).

2.2.4. *Staphylococcus aureus* LD_{50} calculation

In order to determine an appropriate infective dose of bacteria with which to gain a useful measure of host survival, the median lethal dose (LD_{50}) was calculated from a preliminary survival analysis which used a range of infective doses, from 10^2 to 10^8 CFUs (Appendix 1). The LD_{50} after 14 days was estimated to be 5.33×10^7 CFUs per 5µL inoculate.

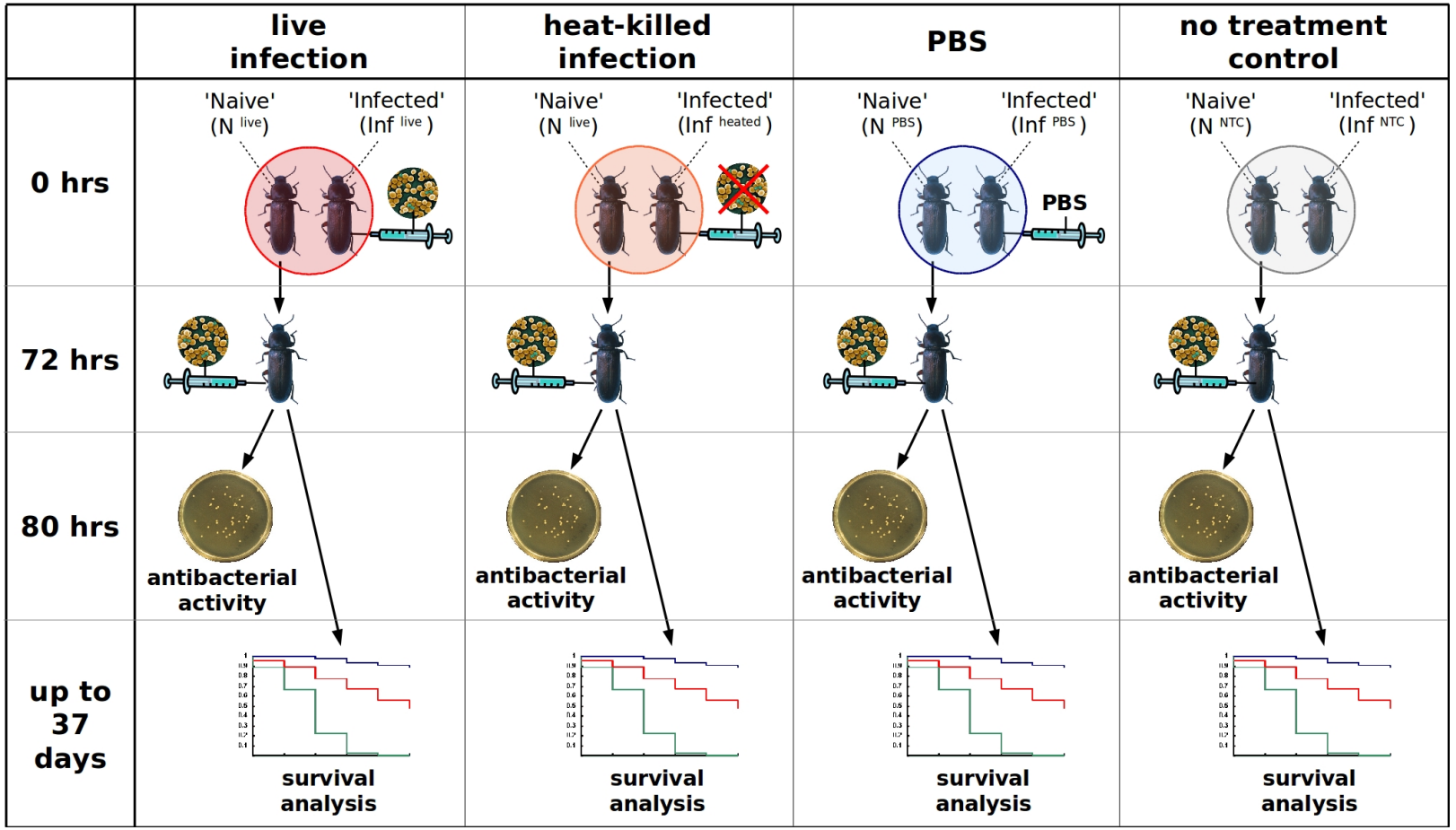


Figure 2.1. Summary of methods used in the experiment. Pairs of female beetles were housed together in Petri dishes (50mm diameter), with each pair consisting of one naïve individual ('N') and one infected individual ('Inf'). Groups differed according to the treatment administered to the infected (Inf) cohabitant: Inf^{live} received a live injection of *Staphylococcus aureus*, Inf^{heated} received an injection of heat-killed *S. aureus*, Inf^{PBS} received an injection of phosphate buffered saline (PBS) as a treatment control, and Inf^{NTC} remained unchallenged as a no treatment control. After 72h cohabitation, naive cohabitants (N) from each group were removed and received a subsequent immune challenge in the form of a live *S. aureus* infection. Half of these individuals had their haemolymph extracted 8h post-infection to measure their level of antibacterial activity, and half were assigned to a survival analysis for the following 37 days.

2.2.5. 'Infected' beetle treatments

'Infected' cohabitants were anaesthetised on ice before being inoculated using a sterile glass microcapillary which had been pulled to a fine point using an electrode puller (Narishige PC-10). The microcapillary was inserted through the pleural membrane between the seventh and eight abdominal sternites, and a syringe barrel was used to pneumatically introduce the inoculate to the haemocoel. 'Infected' cohabitants in the procedural control group (Inf^{PBS}) were injected with 5µL sterile, ice-cold PBS, whilst those in the no-treatment control group (Inf^{NTC}) remained unchallenged.

2.2.6. Cohabitation of infected-naïve pairs

Each treatment group consisting of female-female pairs, with each individual pair comprising one immune-challenged ('infected') individual and one unchallenged ('naïve') individual. Infected-naïve pairs were housed together for 72 h in a small Petri dish (50mm diameter) with *ad libitum* access to Progrub and a 100mg potato supplement provided. Cohabitants were individually labelled with a small spot of acrylic paint on the right elytron in order to differentiate them.

2.2.7. 'Naïve' beetle treatments

After 72 h of cohabitation, naïve individuals in each treatment group were removed from their pairs and randomly allocated to one of two sub-groups: one to quantify *in vivo* antibacterial activity, and one to measure long-term survival. Naïve individuals were anaesthetised on ice and given an immune challenge via the injection of 5µL of live bacterial inoculate, which contained of either $2.5x10^6$ CFUs (antibacterial activity assay) or $5x10^7$ CFUs (survival assay).

2.2.8. Measuring antibacterial activity in naïve beetles

At 8 h post-infection, naïve individuals assigned to the antibacterial activity assay were anaesthetised on ice before being perfusion bled with 500µL sterile PBS. Perfusion bleeds were performed by everting the genitalia and making a small incision, before inserting a syringe needle into the pleural membrane exposed between the head and thorax, and gently flushing 500µL sterile PBS through the haemocoel to be collected in a chilled, sterile centrifuge tube. Perfused haemolymph samples were then serially diluted up to 10^{-4} in PBS, and 200µL of each dilution was plated out onto erthyomycin-infused agar (10µg/mL erythromycin and 5.6µg/mL amphotericin-B). Plates were incubated for 48 h at 37°C before having their CFUs enumerated using an open-source colony counting

software, OpenCFU (Geissmann, 2013). The largest reliable colony count across plates was used as the final measurement of bacterial killing.

2.2.9. Measuring survival in naïve beetles

Naïve individuals assigned to the survival assay were housed individually in grid boxes with *ad libitum* access to food and a ~50mg potato supplement provided once per week. Survival was monitored over the next 37 days, and mortality was confirmed by the absence of movement in response to a physical stimulus. It was difficult to conclusively establish bacterial infection as being the primary cause of mortality, as very low residual loads of experimental *S. aureus* are left in *T. molitor* more than 24 h after infection (Haine *et al.*, 2008), making it difficult to a culture an enumerable number of colonies. The survival rates of naïve individuals were therefore compared to a baseline calculated from a control sample of untreated individuals of the same age whose survival was monitored over the same time period.

2.2.10. Statistical analysis

All statistical analyses were conducted using R statistical software (v3.1.2; R Development Core Team, 2014). As data on CFU counts were non-normally distributed, a negative binomial generalised linear model was fitted using the glm.nb function of the MASS package (Venables & Ripley, 2010). Naive individual body mass (calculated as pupal wet weight) was included as a covariate in the model (Table 2.2). To test for the effect of cohabitation treatment upon survival in naïve individuals, Kaplan–Meier survival models with a Weibull distribution were fitted using the survreg function of the survival package (Therneau, 2011). Individuals that were still alive at the end of the 37 day observation period were incorporated as right-censored data.

2.3. Results

2.3.1. Bacterial clearance in naïve beetles following infection

There was no significant effect of cohabitation type upon bacterial clearance rates in naïve individuals following subsequent infection (negative binomial GLM: $F = 1.339$; $df = 3, 72$; $p = 0.268$) (Figure 2.2). However, there was a non-significant positive correlation between between naïve individual body mass and antibacterial activity (Pearson's: $t = 1.82$, $df = 74$, $p = 0.074$). The inclusion of naïve weight as a covariate improved the predictive power of the negative binomial model (Table 2.2), and showed that weight was a significant predictor of antibacterial activity in naïve individuals (negative binomial GLM: $F = 5.40$; $df = 1, 68$; $p = 0.023$). There was a highly significant interaction effect between weight and cohabitation treatment upon the rate of antibacterial activity (negative binomial GLM: $F = 6.54; 5.397$, $df = 3, 68$; $p < 0.001$).

However, the effects of cohabitation treatment were opposite to that predicted *a priori*. Naïve individuals housed with a cohabitant suffering a heat-challenged bacterial challenge (N^{heated}) displayed the lowest rates of bacterial clearance (97.8 ± 0.84% [mean ± S.E.]). By contrast, naïve individuals housed with a live-infected cohabitant (N^{live}) exhibited the highest clearance rates (99.4 ± 0.36%). When body mass was accounted for, the difference in residual bacterial load between N^{heated} and N^{live} individuals was statistically significant (N^{heated} vs. N^{live} : $z = -2.76$, $p = 0.029$), as was the difference between N^{heated} and N^{NTC} individuals (N^{heated} vs. N^{NTC}: $z = 4.10$, $p < 0.001$) (Table 2.3). Another unexpected result was that antibacterial activity exhibited by naïve individuals in the no treatment control group (N^{NTC}) was significantly higher than that of naïve individuals in the procedural control group (N^{PBS}) (N^{NTC} vs. N^{PBS} : $z = 2.99$, $p = 0.015$), although N^{NTC} antibacterial activity did not differ significantly from N^{live} individuals (N^{live} vs. N^{NTC} : $z = 0.91$, $p = 0.798$) (Table 2.3).

Table 2.2. Likelihood of negative binomial regression models fitted to data on bacterial load (CFU counts) in previously naïve beetles. The log-likelihood score is significantly higher for the model which includes weight as a covariate, and provides a significantly better fit to the data .

Model	d.f.	logLik	Δ logLik	χ^2	P-value
. ~ 1 (null)	2	-333.16			
. ~ CFUs	5	-331.62	-1.54	3.0661	0.38154
. ~ . + weight	6	-330.77	-0.85	1.7009	0.19217
. ~ . + CFUs x weight	9	-326.12	-4.65	9.2992	0.02557

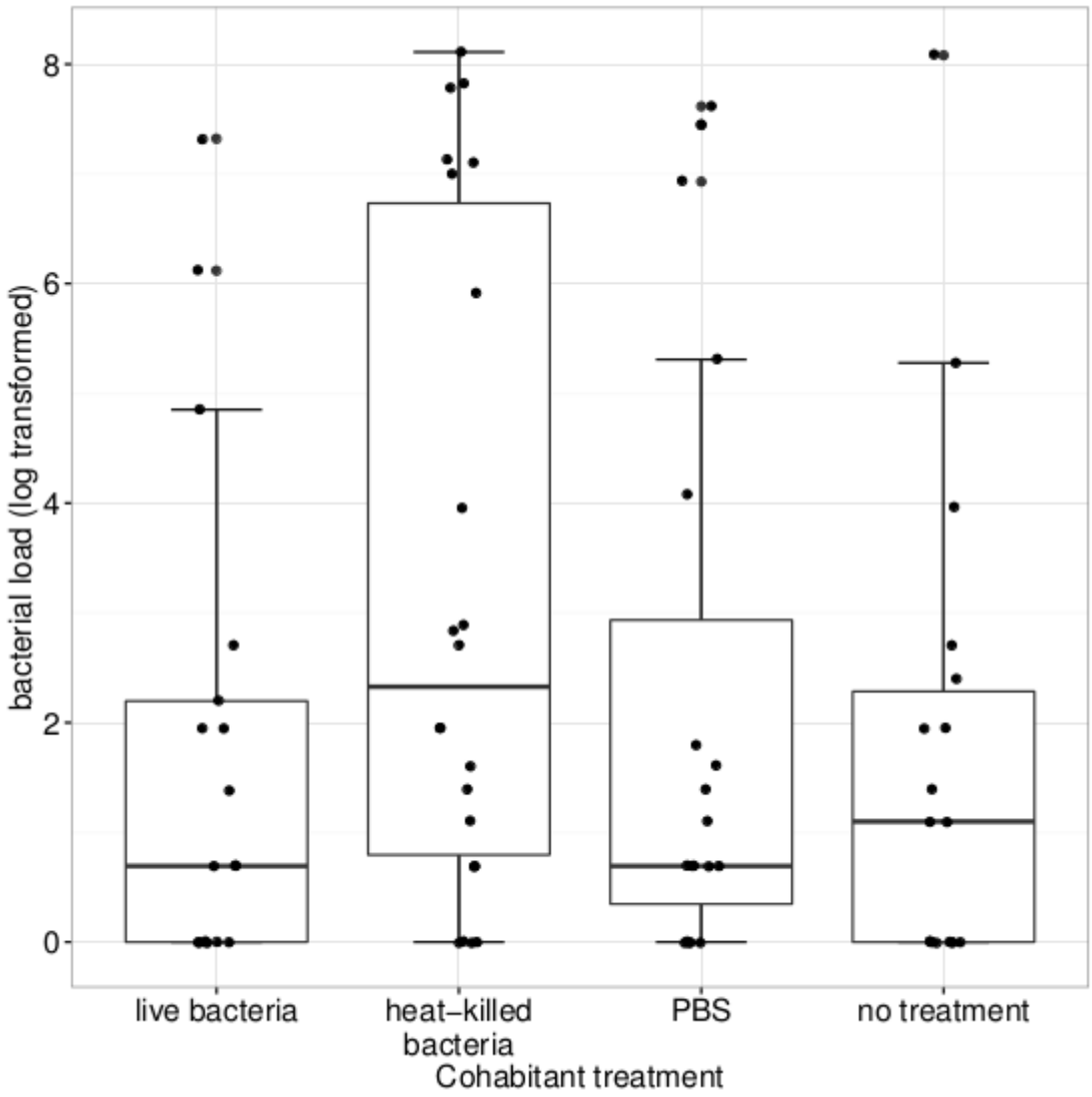


Figure 2.2. Effect of cohabitant treatment upon antibacterial activity in previously naïve beetles, as measured by the number of *Staphylococcus aureus* colony forming units (CFUs) recovered from their haemolymph 8 h after infection. Boxplots show the median and interquartile range (IQR), and whiskers extend to the minimum and maximum values within 1.5*IQR. Data are log-transformed to improve visibility.

Table 2.3. Multiple comparisons of mean bacterial load (CFUs) between naïve individuals in each cohabitation treatment. Tukey's contrasts were performed on fitted data from the negative binomial model which included naïve beetle weight as a covariate.

Comparison	Estimate	S.E.	z value	P-value
Heat-killed vs. Live	-23.13	8.39	-2.76	**0.029**
PBS vs. Live	-13.49	7.97	-1.69	0.326
PBS vs. Heat-killed	9.65	7.45	1.30	0.565
no treatment vs. Live	7.23	7.93	0.91	0.798
no treatment vs. Heat-killed	30.37	7.41	4.10	**<0.001**
No treatment vs. PBS	20.72	6.93	2.99	**0.015**

2.3.2. Survival in naïve beetles following infection

There was a significant effect of cohabitation type upon survival in naïve individuals following infection (Cox proportional hazards test: χ^2=10.48; df=3; p=0.015) (Figure 2.3). Survival was significantly lower in naïve individuals cohabiting with live-infected conspecifics (N^{live}) than naïve individuals in the PBS (N^{live} vs. N^{PBS} : z = 3.12; p = 0.002) or no treatment control (N^{live} vs. $N^{naïve}$: z=2.56, p=0.015) cohabitations. naïve individuals cohabiting with a conspecific with a heat-killed infection (N^{heated}) also showed some decrease in survival compared to controls, although this difference was not statistically significant (z=1.78; p=0.076).

The mortality of naïve individuals following infection was significantly greater for all cohabitation types than baseline levels (p<0.01); only 2/32 individuals from the uninfected baseline population died during the 37 day observation period. In contrast, mortality amongst infected naïve individuals was almost absolute; 95.2% (120/126) of individuals died within the 37 day observation period, with a mean survival time of 12.4 ± 1.1 days (mean ± S.E.) across all cohabitation treatments.

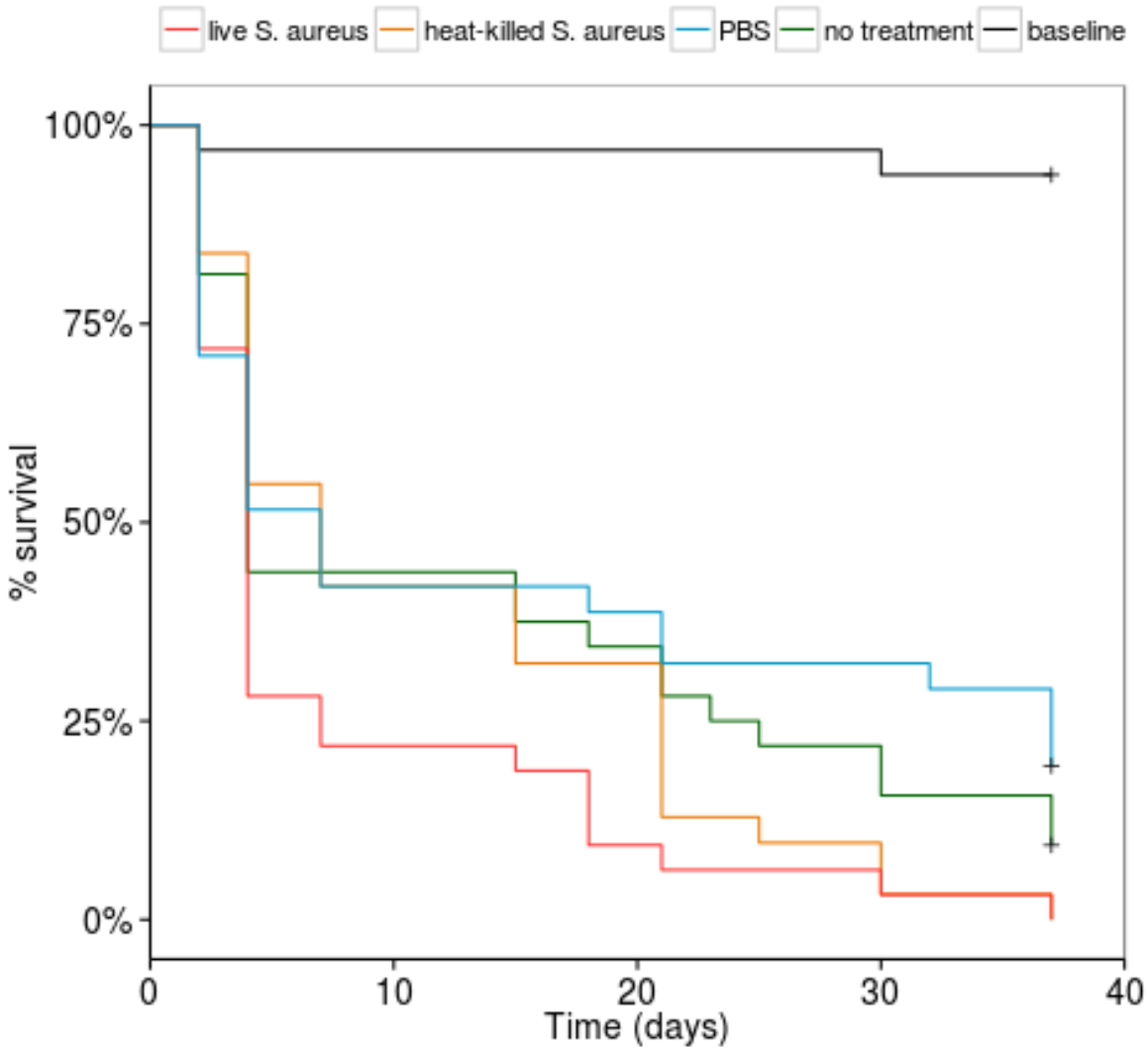


Figure 2.3. The effect of cohabitant treatment upon survival in previously naïve beetles following infection with *Staphylococcus aureus*. The black line depicts the baseline survival rate in a population of uninfected beetles of the same age and gender.

2.4. Discussion

This study found that the immune status of an infected conspecific had a significant effect on the response of a naïve individual to subsequent immune challenge. However, the directionality of this effect was not as predicted: naïve individuals housed with immune-challenged conspecifics were expected to exhibit enhanced resistance to subsequent infection. Instead, naïve beetles cohabiting with conspecifics with a live (and potentially transmissible) bacterial infection (N^{live}) showed reduced longevity in response to subsequent infection, whilst exhibiting no change in antibacterial activity (compared to naïve individuals exposed to a healthy conspecific [N^{NTC}]). Conversely, naïve beetles housed with conspecifics with a heat-killed (and non-transmissible) bacterial infection demonstrated significantly lower rates of bacterial clearance following subsequent infection, but did not show an associated decrease in longevity.

Although unexpected, these results raise some interesting points. Firstly, they suggest that the induction of resistance mechanisms against *S. aureus* infection by *T. molitor* may incur a significant survival cost for the host. Furthermore, the different responses of naïve individuals cohabiting with conspecifics with either a live (N^{live}) or heat-killed infection (N^{heated}) suggest that immune priming processes may act to augment tolerance, not resistance, against future infection. Finally, it is possible that artificial infections may not be sufficient to induce 'gregarious immunisation' if the route of immunisation relies on the direct transfer of low-level infection between hosts. Each of these points is discussed below.

2.4.1. Potential routes to social immunisation

A social transfer of immunity has been reported in eusocial insects, whereby immune-stimulated hosts provide a 'social immunisation' of healthy group members by augmenting their resistance against subsequent pathogenic infection (Rosengaus *et al.*, 1998; Traniello *et al.*, 2002; Hamilton *et al.*, 2010; Konrad *et al.*, 2012). Such social transfer of immunity may be acquired via two (non-exclusive) routes (Konrad *et al.*, 2012): through the active upregulation of an individual's own immune system following exposure to a parasitised neighbour (active social immunisation [e.g. Konrad *et al.*, 2012]), or via the social transfer of immune compounds between hosts, priming naïve hosts without requiring direct activation of their own immune systems (passive social immunisation [e.g. Rosengaus *et al.*, 1998; Traniello *et al.*, 2002; Hamilton *et al.*, 2010]).

2.4.1.1. Active immunisation

It is possible that naïve individuals housed with live-infected conspecifics show greater antibacterial activity (and suffered a concurrent reduction in longevity) in response to subsequent *S. aureus* infection due to previous contact with the pathogen via their neighbour during the cohabitation period. Physical interactions between hosts may result in the transfer of low-level infections (Yamada *et al.* 1992), causing naïve hosts to acquire a pathogenic load which is insufficient to cause systemic infection but large enough to provide long-lasting protection against the pathogen in question by priming the immune system (Rosengaus *et al.*, 1998; Konrad *et al.*, 2012). However, whilst physical contact is the most common transmission route for fungal pathogens and sexually-transmitted infections (STIs), most bacterial infections typically occur via oral uptake (Schmid-Hempel, 1998).

Ingestion of pathogenic material by naïve individuals is the most likely route of transmission in this study, as Inf^{live} individuals may have contaminated their surrounding food source through the excretion of live pathogens. For example, a large quantity of viable *Staphylococcus epidermidis* (the same amount as retained in the intestine) was found to be excreted from the nematode *Caenorhabditis elegans* after feeding on a bacterial lawn (Begun *et al.*, 2007). Although the transmission of live pathogens was only possible by live-infected cohabitants, individuals inoculated with heat-killed bacteria may still have excreted dead bacteria into their surrounding environment. Non-living bacterial material, such as lipolysaccharide (LPS), is known to elicit a potent and specific immune response in many insects, including *T. molitor* (Moret, 2006).

Active social immunisation may also be mediated by the dissemination of infection cues produced by infected conspecifics. As well as developing visual indicators of disease (e.g. spores on cuticle during fungal infection), hosts can exhibit manifold changes in behaviour ('sickness behaviours'; see Chapter 5) and chemosensory profile during an immune challenge. Chemosensory cues form a cornerstone of communication in insects (Singer, 1998; Wyatt, 2003), and represent the main route of communication in mealworm beetles, whose use of visual and auditory cues is relatively underdeveloped through adaptation to darkness (Wyatt, 2003; Carazo *et al.* 2004; Bryning *et al.*, 2005). Male *T. molitor* appear able to assess female reproductive status by chemosensory cues alone, demonstrating a preference for the odour of virgin over non-virgin females and sexually mature over immature females (Carazo *et al.* 2004). Furthermore, pheromone production in *T. molitor* varies with reproductive status (Seybold & Vanderwel, 2003) and condition (Rantala *et al.*, 2003), suggesting chemosensory cues are plastic with

regards to immune status.

Cuticular hydrocarbons (CHCs) are compounds present on the outer integument of insects which are used for intraspecific communication, and can transmit information about age, sex, reproductive status and colony membership (Singer, 1998; Wyatt, 2003). Fungal pathogens have been shown to degrade the levels CHCs in some insect hosts (Lecuona *et al.* 1991; Napolitano & Juarez, 1997), and quantitative changes in the hydrocarbon profile have been documented in honey bees and ants following parasitism with a virus (Baracchi *et al.*, 2012) and macroparasites (Salvy *et al.*, 2001; Trabalon *et al.* 1999). CHC modifications have also been reported in honey bees (*Apis mellifera*) and *T. molitor* following immunostimulation with lipopolysaccharide (LPS) (Richard *et al.*, 2008; Nielson & Holman, 2011), suggesting that at least some chemosensory cues of infection are produced by the host. However, it is unclear whether these are adaptive signals intended to communicate immune status with conspecifics or merely an incidental byproduct of the immune response (e.g. resource depletion, immunopathology).

Upon challenge, the fat body releases antimicrobial peptides (AMPs) and haemocytes into the haemolymph (Bulet & Stöcklin, 2004). These factors can be readily transported from the haemolymph to the epicuticular surface (Schal *et al.* 1998), and *Drosophila* have even been shown to directly express AMPs at their surface epithelium (Tzou *et al.*, 2002). Some endoparasites have also been observed to reduce haemolymph protein levels by up to 27% (Weinberg & Madel 1985). This suggests that internal changes in physiology during immune challenge can be translated into externalised cues of infection through their expression via cuticular pores or exocrine glands. The excretion of pathogenic or host-derived metabolic byproducts may also constitute signals of infection produced by parasitised hosts. In *T. molitor*, autoreactive damage incurred during an immune response can cause significant damage to the Malpighian tubules (Sadd & Siva-Jothy, 2006), which are essential regulators of osmoregulation. It is possible that activation of the immune response may lead to a build up of waste products within the haemocoel of parasitised hosts, which could in turn lead to the production of via the cuticle of exocrine glands. In mice, parasitised individuals have higher concentrations of plasma corticosterone in their urine, hormones which have been linked to the control of alarm odours (Dunn *et al.* 1987; Cocke *et al.*, 1993), and females can display avoidance of infected males using these cues (Kavaliers & Colwell, 1992; Penn & Potts, 1998; Arakawa *et al.*, 2011).

Live-infected cohabitants (Inf^{live}) in this study were likely to suffer greater levels of damage (immunopathogical *and* pathological) and resource depletion from defending

against a live and actively-evading pathogen; consequently, these individuals have a greater potential to undergo behavioural and chemosensory alterations during infection, and thus a greater capacity to produce detectable cues of infection with which to prime neighbouring conspecifics. However, whilst the results show that N^{live} cohabitants demonstrated higher levels of antibacterial activity than N^{heated}, activity levels were not greater than those displayed by naïve cohabitants exposed to healthy cohabitants (N^{NTC}, N^{PBS}) (Figure 2.2).

2.4.1.2. Passive immunisation

Passive social immunisation, whereby host immunity is activated indirectly by conspecifics as opposed to initiated by the host itself, may be mediated by the transfer of immune compounds between conspecifics (Rosengaus *et al.* 1999; Traniello *et al.* 2002; Feffermann *et al.* 2007). This may occur through an externalised route conducted over the body surface, such as contact with secreted substances during allogrooming (Traniello *et al.* 2002; Fernandez-Marin *et al.*,, 2006), or through an internalised route via the direct exchange of bodily fluids during social feeding behaviours such as trophallaxis (Rosengaus *et al.*, 1998; Hamilton *et al.*, 2010).

Whilst the transfer of immune factors has been suggested to be a ubiquitous immune defence across the social insects (Rosengaus, 2010), many social behaviours like trophallaxis and allogrooming are absent in non-social insects. However, externalised antimicrobial secretions are produced by many tenebrionid species, such as volatile benzoquinones in *T. molitor* and closely-related *Tribolium* species (Tschnikel, 1974). Whilst the primary purpose of these secretions may be to manage unwanted microbial growth in food stores (Yezzerski *et al.*, 2000; Otti *et al.*, 2014), benzoquinones also have a repellent effect on conspecifics and predators in *Tribolium* (Suzuki, 1980), and their secretion is also upregulated in *Tenebrio molitor* during parasitism by the rat tapeworm, *Hymenolepis diminuta* (Yan & Phillips, 1993). This suggests that these compounds may serve a secondary defensive function, perhaps as a group-level defence. For example, salicylaldehyde-based volatiles secreted by the brassy willow leaf beetle (*Phratora vitellinae*) have strong antimicrobial action, and when synergistically amplified in beetle aggregations, form a fumigant cloud which protects against fungal and bacterial pathogens in the microenvironment (Gross *et al.*, 1998, 2008).

Although externalised benzoquinone secretions could possibly act to passively immunise naïve individuals that ingest them, such volatile cues seem more likely to operate via the dissemination of olfactory cues through the environment. Furthermore,

this study finds differences between naïve individuals housed with live-infected conspecifics (N^{live}) and those housed with conspecifics receiving a heat-killed challenge (N^{heated}), suggesting that live pathogens may play an important role in the social transfer of immunity, either through direct pathogen transmission or through host detection of pathogen-derived cues (i.e. active routes of social immunisation).

2.4.2. Potential priming mechanisms

Immunity can be costly to use and maintain (Moret & Schmid-Hempel, 2000; Schmid-Hempel, 2003) and can inflict extensive immunopathological damage upon the host (e.g. Sadd & Siva-Jothy, 2006), meaning that maximised investment in physiological immune defence is not always the best way for the host to minimise the costs of parasitism (Hamilton *et al.*, 2007). Immune priming is therefore unlikely to be implemented by the constitutive upregulation of heavy-duty resistance mechanisms, and may rather involve a more a subtle form of immune activation.

2.4.2.1. Tolerance

Immune defence strategies can be broadly divided into groups: resistance, the ability to clear invading parasites, and tolerance, the ability to reduce detrimental effects of parasites (Schneider & Ayres, 2008; Medzhitov *et al.*, 2012). Insects possess an open haemocoel and an arsenal of cytotoxic and non-specific effector systems (Siva-Jothy *et al*., 2005); although these defences can be rapidly initiated, they can also incur fitness costs through immunopathological damage to self tissues (Sadd & Siva-Jothy; Long & Boots, 2011). Although quantitative resistance appeared to be lower in N^{heated} individuals, this was not associated with a notable decrease in survival, suggesting that higher parasite burdens during infection may not have been coupled with a significant fitness cost.

Tolerance of infection may also be accomplished through non-immunological responses which help to the host ameliorate the costs of parasitism without targeting the parasite at all. Terminal investment in reproduction (Clutton-Brock, 1984) is a common life-history 'escape attempt' (van Baalen, 1998; Minchella, 1985) which can help counter negative effects of infection upon personal condition through increasing host reproductive fitness. Although no measures of reproductive success were taken from naïve individuals in this study, it is possible that females, when threatened with an increased risk of pathogenesis, trade off physiological immune activity against reproductive investment. Future work may benefit from examining the effects of cohabitant immune status upon both immune and reproductive function in mixed-sex groups, in order to provide

threatened hosts with the ability to invest in reproduction as a life-history escape strategy. Furthermore, terminal investment in reproduction typically occurs in only males following infection (Polak & Starmer, 1998; Adamo, 1999; Abbot & Dill, 2001; Shoemaker *et al.*, 2006; Sadd *et al.*, 2006; Krams *et al.*, 2011; Nielson & Holman, 2011), as males are able to increase their reproductive success linearly with their number of matings, unlike females (Bateman, 1948). Investigating the effects of cohabitant immune status upon both naïve males and females may reveal if immune priming strategies vary between the sexes.

2.4.2.2. Sensitisation

A form of social immune priming is also known to exist in plants, whereby certain species are able to prime themselves against attack by responding to airborne volatile organic compounds (VOCs) produced by conspecifics experiencing herbivory or pathogenesis (Bate & Rothstein, 1998; Karban *et al.*, 2000; Arimura *et al.* 2002; Engelberth *et al.*, 2004; Karban *et al.*, 2006; Heil & Ton, 2008; Erb *et al.*, 2015). In primed plants, the physiological state is relatively modest compared to that during actual parasite attack (Bate & Rothstein, 1998; Arimura *et al.* 2002; Engelberth *et al.*, 2004); rather than being sensitive, the host is instead sensitised to a secondary danger signal produced only in the event of subsequent attack. This allows for a more rapid and potentiated immune response to be launched only if and when required (Conrath *et al.*, 2002; Heil & Ton, 2008), thus reducing the costly biochemical investment and immunopathology associated with full activation of the immune response (Heil, 2002; van Hulten *et al.*, 2006).

A similar form of sensitisation has been suggested to explain immune priming in invertebrates (Schulenburg *et al.*, 2007), although the exact underlying mechanisms are still unknown. It is possible that differences in sensitisation induced by cohabitant immune status could explain some of the variation in immunity and longevity measured between naïve individuals here, but this is purely speculative. Another experiment which measures levels of constitutive (before subsequent challenge) and inducible (after subsequent challenge) immune effectors following a similar cohabitation period may reveal a role for sensitisation in a gregarious immunisation process.

2.4.2.3. Secondary-mode prophylaxis vs. long-lasting immunity

If cohabitation with a conspecific with a heat-killed infection did help N^{heated} individuals to tolerate subsequent infection (as discussed above), then one may expect a similar

response from N^{live} individuals housed with live-infected conspecifics, yet this was not the case. The observed differences may stem from a distinction between the processes of social immunisation, secondary-mode prophylaxis and long-lasting immunity.

N^{live}, but not N^{heated}, individuals had the potential to acquire infection via pathogenic transmission from their cohabitant through the direct transfer of infection ('variolation'). Direct contact with the pathogen could have induced a long-lasting immune response in naïve hosts (e.g. Haine *et al.*, 2008; Johnston *et al.*, 2014) or otherwise augmented their responsive to secondary infection via a priming effect (e.g. Moret *et al.*, 2006). This would explain the observations of augmented levels of antibacterial activity in N^{live} individuals, as well as decreased longevity, as these individuals may have underwent two separate bouts of infection.

Long-lasting elevation of antimicrobial activity following infection has been reported in various insects, lasting from between 9–44 days (Faye *et al.* 1975; Azambuja *et al.* 1986; Bulet *et al.* 1992; Korner & Schmid-Hempel 2004; Haine *et al.*, 2008; Johnston *et al.*, 2014). A previous study which challenged *T. molitor* with a high dose of *S. aureus* found that antimicrobial activity was upregulated for up to 21 days, but also that the infective bacteria were able to recovered from host haemolymph for up to 21 days following infection (Haine *et al.*, 2008). Such long-lived immune responses have been suggested to prevent the evolution of pathogen resistance and manage persistent infections, with long-lasting expression of immune effectors acting to 'mop up' persistent pathogens which survive the first wave of host defences, and which can be more resistant to host defences as well as more virulent to the host (Haine *et al.,* 2008). Haine *et al.* (2008) suggest that such lasting immunity may have evolved to prevent the evolution of resistance in the infective bacterial population and/or to manage persistent infections, as opposed to being a prophylactic defence against reinfection from environmental bacteria, as in the case of secondary mode prophylaxis (Moret *et al.*, 2006). However, defending against a reemerging infection from persistent resident bacteria *in vivo* and defending against an entirely new infection from environmental bacteria are both essentially the same process (Dobson, 2012), and it is likely that a long-lasting immune response would provide protection against both potential routes of reinfection.

2.4.4. Non-adaptive explanations for effects of cohabitation

It is possible that the observed effects of treatment upon immunity were an artifact of the experimental design used, as the forced cohabitation of naïve individuals may have prevented the expression of behavioural defences against pathogenic threat, such as

physical avoidance of challenge conspecifics. Behavioural responses can provide hosts with a quick and low cost defence against potential pathogen transmission (Siva-Jothy *et al.*, 2005; Schulenberg & Ewbank 2007), and are likely to provide a greater fitness benefit when they allow the host to avoid pathogen exposure entirely, as opposed to just augmenting post-infection defences (Schaller & Park, 2011).

For example, *Drosophila melanogaster* and *D. simulans* both exhibit behavioural avoidance of a natural parasitoid wasp (*Leptopilina boulardi*) and prefer to oviposit in clean sites with an absence of wasps (Lefevre *et al.*, 2011). However, in the forced presence of wasps, only *D. melanogaster* females show a reduction in the number of eggs laid (Lefevre *et al.*, 2011), and *D. simulans* larvae infected by parasitoid wasps are better able to melanotically encapsulate and kill wasp eggs than *D. melanogaster* larvae (Schlenke *et al.*, 2007; Lefevre *et al.*, 2011). In other words, *D. simulans* continues to produce its normal complement of eggs as their offspring are more capable of resisting wasp infection via physiological immune defence, whilst *D. melanogaster,* whose offspring are less capable of mounting a successful physiological defence response, instead opt to limit their rate of oviposition. Whilst both host species exhibit similar avoidance behaviours in response to the costs of parasitism, differences in their resistance strategies emerge due to trade-offs between physiological and behavioural defences. Furthermore, forced cohabitation may have acted as a stressor upon naïve individuals due to their inability to escape from a potential threat; for example, mice housed with a tumour-bearing neighbour exhibit symptoms of both psychological and physical stress (Alves *et al.*, 2006).

The importance of sexual selection as a mediator of immune defence could not be accounted for in this experimental design as only female-female pairs were used. The absence of breeding partners may have prevented hosts from investing in reproduction as a non-immunological life-history escape response to counter the costs of infection. It is also possible that reliable signals of infection are only produced in the presence of members of the opposite sex. For example, in the burying beetle, *Nicrophorus vespilloides*, breeding females only release methyl geranate, a substance which indicates breeding status, in the presence of a male partner, i.e. a signal receiver (Steiger *et al.*, 2011). Such receiver-dependent chemical signalling is expected to evolve when costs are involved in the production or transmission of the signal (Steiger *et al.*, 2011).

Another, although less likely, possibility is that infected Inf^{live} cohabitants had their behaviour directly manipulated by the parasite. For instance, starved *Tenebrio molitor* and *Tribolium confusum* beetles have been shown to prefer to feed on the faeces

of rats infected with *Hymenolepis diminuta*, a tapeworm to which themselves and rats both play hosts (Evans *et al.* 1992; Pappas *et al.* 1995), and an attractive volatile attractant is thought to be present in infective faeces (Evans *et al.* 1992). It is possible that *S. aureus* is able to induce behavioural modifications in infected hosts which facilitate its transmission to neighbouring conspecifics.

2.4.5. Summary

This chapter did not find evidence for gregarious immunisation in *Tenebrio molitor*, as neither resistance nor survival were augmented in naïve individuals following exposure to a sick conspecific. However, it is possible that cohabitation with an immune-stimulated, but non-contagious, conspecific augments the tolerance of naïve hosts to future infection, since these individuals were found to withstand higher pathogen loads without suffering a reduction in longevity.

The key difference between individuals exposed to conspecifics with a live infection and those exposed to conspecifics with a heat-killed infection is proposed to rely on the active transfer of low-level infection between hosts ('variolation'). This may act in concert with passive route of immunisation (e.g. transfer of immune compounds between hosts) or an indirect active route (e.g. olfactory detection of pathogenic presence), but does not appear to have an additive effect upon the resultant induction of immunity in naïve hosts. Instead, it seems likely that direct contact between a live pathogen and the host immune system overrides other relatively subtle effects of gregarious immunisation, as infection often necessitates an immediate response in order to preserve host fitness.

In this chapter, I have shown that:

- Naïve beetles exhibit differences in their response to a subsequent infection following cohabitation with an immune-challenged conspecific
- Individuals exposed to conspecifics with a heat-killed bacterial infection (N^{heated}) exhibited relatively low rates of antibacterial activity in response to subsequent infection, but did not show a reduction in longevity
- Individuals exposed to conspecifics with a live bacterial infection (N^{live}) displayed higher levels of antibacterial activity (versus N^{heated}), yet suffered from increased mortality

CHAPTER THREE:

GENDER DIFFERENCES IN IMMUNE PRIMING OF NAÏVE *Tenebrio molitor* FOLLOWING COHABITATION WITH AN IMMUNE-CHALLENGED CONSPECIFIC

3.1. Introduction

Immune defences can be costly to maintain and use (Moret & Schmid-Hempel, 2000; Schmid-Hempel, 2003), and many immune effectors are induced only upon exposure to infection as opposed to being constitutively expressed (Hamilton *et al.*, 2007). Some invertebrates possess a form of adaptive immune memory, known as 'immune priming', whereby a primary pathogenic exposure can provide the host with enhanced protection against subsequent infections by the same pathogen (Kurtz & Franz, 2003; Moret *et al.*, 2006; Sadd & Schmid-Hempel, 2006; Pham *et al.*, 2007; Roth *et al.*, 2009b; Tidbury *et al.*, 2011).

Immune priming may also be induced before infection if the host is able to use environmental cues of pathogenic threat to invest in prophylactic protection against potential infection. For instance, insects can exhibit behavioural avoidance of infected conspecifics (Carver & Hurd, 1998; Worden *et al.*, 2000; Worden & Parker, 2005; Vainikka *et al.*, 2007) and free-living pathogens in the environment (Villani *et al.*, 1994; Myles, 2002; Zhang *et al.*, 2005; Meyling *et al.*, 2006; Ormond *et al.*, 2011; Yanagawa *et al.*, 2011), and may induce defences against indirect predictors of pathogenesis, such as high population density (Reeson *et al.*, 1998; Wilson & Reeson 1998; Barnes & Siva-Jothy, 2000).

Reliable indicators of disease produced by infected conspecifics should be beneficial to naïve hosts if they are able to use them to reduce their probability of infection, such as by stimulating investment in behavioural and/or physiological immune prophylaxes. Indeed, a social transfer of immunity, referred to as ‘social immunisation’, has been described in termites (Traniello *et al.*, 2002) and ants (Ugelvig & Cremer, 2007; Konrad *et al.*, 2012; Hamilton *et al.*, 2010), whereby healthy individuals housed with infected nestmates become better protected against subsequent infection by the same pathogen. naïve hosts have been suggested to become 'immunised' through one of three (non-exclusive) routes: (i) active induction of immunity following direct contact with the pathogen via the transmission of low-level infection from infected nestmates (Traniello *et al.*, 2002; Ugelvig & Cremer, 2007; Konrad *et al.*, 2012), (ii) active induction of

immunity following the detection of infection cues produced by infected nestmates (e.g. chemosensory, behavioural or visual changes), or (iii) passive induction of immunity following the social transfer of immune factors passed on from infected nestmates (Rosengaus & Traniello, 1999; Hamilton *et al.*, 2010).

The mealworm beetle, *Tenebrio molitor*, is a stored product pest which is non-social but gregarious, and has several features of its ecology in common with social insects, such as high population density, permanent nest sites with stored resources and high pathogen presence in their environment (Yezerski *et al.*, 2005; Otti *et al.*, 2014). These factors increase host vulnerability to pathogenesis (Schmid-Hempel 1998) and may predispose *T. molitor* to the development of group-level immune responses (Otti *et al.*, 2014). The chemosensory profile of *T. molitor* is known to vary according to reproductive status (Seybold & Vanderwel, 2003; Carazo *et al.*, 2004), condition (Rantala *et al.*, 2003) and during immune stimulation (Nielsen & Holman, 2011). Although female mealworm beetles are able to discriminate against the infection status of potential males (Worden, 2000; Sadd *et al.*, 2006; Krams *et al.*, 2011; Nielsen & Holman, 2011), little is known about whether naïve individuals (male or female) could utilise such cues of infection to inform a prophylactic investment in immunity to protect against a threat of parasitism.

The invertebrate immune system is broadly composed of constitutive defences, which are always ready to act but non-specific, and can cause immunopathological damage to the host (Sadd & Siva-Jothy, 2006), and inducible defences which are more specific but can take up to 48 h to reach peak activity (Haine *et al.*, 2008). In plants, which can also undergo immune priming in response to volatiles produced by infected or herbivorised conspecifics (Bate & Rothstein, 1998; Karban *et al.*, 2000; Arimura *et al.* 2002; Engelberth *et al.*, 2004; Karban *et al.*, 2006; Heil & Ton, 2008; Erb *et al.*, 2015), the physiological state of priming is relatively modest compared to that during actual parasite attack (Bate & Rothstein, 1998; Arimura *et al.* 2002; Engelberth *et al.*, 2004). In this way, primed hosts become sensitised to attack but not sensitive, able to launch a rapid and potentiated immuhne response in the event of subsequent attack whilst reducing the costs associated with an immediate full immune activation (Heil, 2002; van Hulten *et al.*, 2006). It is possible that priming in invertebrates is produced by a similar form of sensitisation, although the underlying mechanisms are mostly unknown (Schulenburg *et al.*, 2007).

Encapsulation is a fast-acting cellular response which defends the host by smothering invasive foreign bodies in haemocytes in order to starve parasites of oxygen

and nutrients. The cells in this capsule can undergo apoptosis and melanise through the action of cytotoxic quinones produced by the phenoloxidase cascade (Gillespie *et al.*, 1997). Phenoloxidase (PO) is an enzyme which catalyses the formation of melanin and the production of highly reactive and toxic quinones which form an essential part of constitutive defences in insects (Chapman, 1998), but which (alongside other defences such as reactive oxygen species) are known to be immunopathological (Nappi *et al.*, 1995; Sadd & Siva-Jothy, 2006). However, phenoloxidase is generally found in the host haemocoel in the form of an inactive precursor, prophenoloxidase (proPO), which becomes rapidly activated in the presence of microbial compounds (Cerenius *et al.*, 2008), thus limiting the immunopathological costs associated with its expression. PO activity and encapsulation ability are both positively correlated with pathogen resistance in insects (Braun *et al.*, 1998). The haemocoelic insertion of a nylon monofilament is an immune insult which incurs a significant fitness cost in *Tenebrio molitor* (Armitage, 2003; Sadd & Siva-Jothy, 2006), and also provides an effective way to measure cellular encapsulation against a novel antigen (e.g. König & Schmid-Hempel 1995; Schmid-Hempel & Schmid-Hempel 1998; Ryder & Siva-Jothy, 2001).

In Coleoptera, the cuticle forms a mechanical barrier which can provide a frontline defence to infection (Siva-Jothy *et al.*, 2005), and more melanised cuticles have been shown to provide increased resistance against the entomopathogenic fungi, *Metarizhium anisopliae*, which invades the host by penetrating the cuticle (Barnes & Siva-Jothy, 2000). Cuticular melanisation is also positively correlated with haemolymph levels of phenoloxidase and prophenoloxidase in *T. molitor*, as well as in crickets (Reeson *et al.*, 1998; Barnes & Siva-Jothy 2000), which in turn augments resistance against systemic infection, and the cuticle has been suggested to be a sink for harmful quinones recruited for but not used in sclerotisation (Chapman 1982). Sclerotised proteins are impermeable to many fungal secretions, resistant to enzyme degradation and can also mechanically prevent hyphal penetration (Hajek & St Leger 1994). However, cuticular melanisation is a density dependent polyphenism in *T. molitor* (i.e. dark cuticles are not a fixed trait in the population), suggesting that highly melanised cuticles may carry a hidden cost for the host. One potential explanation is that darker, thicker cuticles are less porous (Armitage, 2002), and may lower the attractiveness of the host by limiting the quantitative output of pheromones and cuticular hydrocarbons (CHCs) involved in sexual signalling.

In this chapter, I examine 'gregarious immunisation' in *Tenebrio molitor* by cohabiting immunologically naïve adults with an artificially immune-challenged conspecific in order

to determine whether socially-derived cues of infection alter immune investment. I use both male-male and female-female cohabiting pairs, although not mixed sex pairs in order to avoid the potentially confounding effects of mating upon immunity (Rolff & Siva-Jothy, 2002), to investigate the effects of gender upon gregarious immunisation. Gender is often neglected in studies on ecological immunity despite the often-striking differences between immune investment in males and females (Rolff, 2002; Zuk & Stoehr, 2002; Joop & Rolff, 2004).

The use of a artificial immune elicitor mimics a state of 'sickness' in the host whilst avoiding the potential for direct pathogen transmission between conspecifics, as well as avoiding potential parasitic manipulation of physiology or behaviour in the immune-stimulated host. The only remaining potential routes of gregarious immunisation in naïve conspecifics should therefore be: (i) via the transmission of an immune factor from the challenged conspecific to the naïve host, or (ii) via the detection of an infection cue produced by conspecifics during immune challenge, such as semiochemical cues or sickness behaviours (Konrad *et al.*, 2012).

Assuming a role for gregarious immunisation in this species, I predicted that exposure to an immune-challenged conspecific would upregulate immunity in naïve individuals. By measuring levels of constitutive immunity (before a subsequent challenge) and inducible immunity (post-challenge), I aimed to determine whether gregarious immunisation is achieved through full-scale activation of the immune system or by a more subtle priming response. Finally, positing the potential role of chemosensory signals in active gregarious immunisation and presuming a negative association between cuticle thickness and porosity, I predicted a negative correlation between the degree of cuticular melanisation in immune-challenged individuals and immune upregulation in the naïve individuals they were paired with.

In this chapter, I examine:

- The effects of cohabitation with an artificially immune-challenged conspecific upon investment in both cellular and humoral immunity in naïve beetles
- Gender differences in the immune responses of naïve cohabitants
- The role that cuticular melanisation (in both naïve and infected cohabitants) plays in these responses

3.2. Methods

3.2.1. Insect culture

Final-instar larvae of *Tenebrio molitor* were purchased from a commercial supplier (Live Foods UK) and maintained in an insectary at 26±2 °C under a 12/12hr light/dark cycle. Larvae were kept at densities of ~800 larvae per 30×15×10 cm box, and were provided with *ad libitum* access to Progrub (Livefoods Direct Ltd) and supplemented with freshly cut potato once per week.

Beetles were collected as pupae within 2-3 days of pupation, and were sexed, weighed and then maintained in isolation in grid box containers. (Only pupae with a wet weight of 78-112mg were used (representing ± 2 S.D. around the population mean). Upon imaginal eclosion, imagoes were provided with Progrub and a ~50mg potato supplement.

8-10 days after imaginal eclosion, adult males and females were randomly assigned to one of three treatments (Figure 3.1). Each treatment group consisted of pairs of beetles; one immune-challenged ('infected', hereafter referred to as 'Inf') individual and one untreated ('naïve', hereafter referred to as 'N') individual.

3.2.2. Infected beetle treatment

Infected individuals in the experimental group were immune challenged by the insertion of a ~2mm length of nylon monofilament (0.15mm diameter) into the haemocoel between the third and fourth abdominal sternites. Infected individuals in the experimental control group were given a sterile stab between the third and fourth abdominal sternites as a wounding control, and those in the no treatment control group were untreated.

3.2.3. Naïve beetle treatment

Infected-naïve pairs were housed together for 72 h in a small Petri dish (50mm diameter) with *ad libitum* access to Progrub and a 100mg potato supplement. After this cohabitation period, naïve individuals were removed and randomly assigned to either one of two sub-treatments: (i) a pre-challenge assay, in which haemolymph was extracted immediately to analyse constitutive levels of immunity following cohabitation, or (ii) a post-challenge assay, in which naïve individuals were given an immune challenge in the form of a nylon implant so as to quantify levels of inducible immunity, before having their haemolymph extracted for analysis and their nylon monofilament extracted 24 h later.

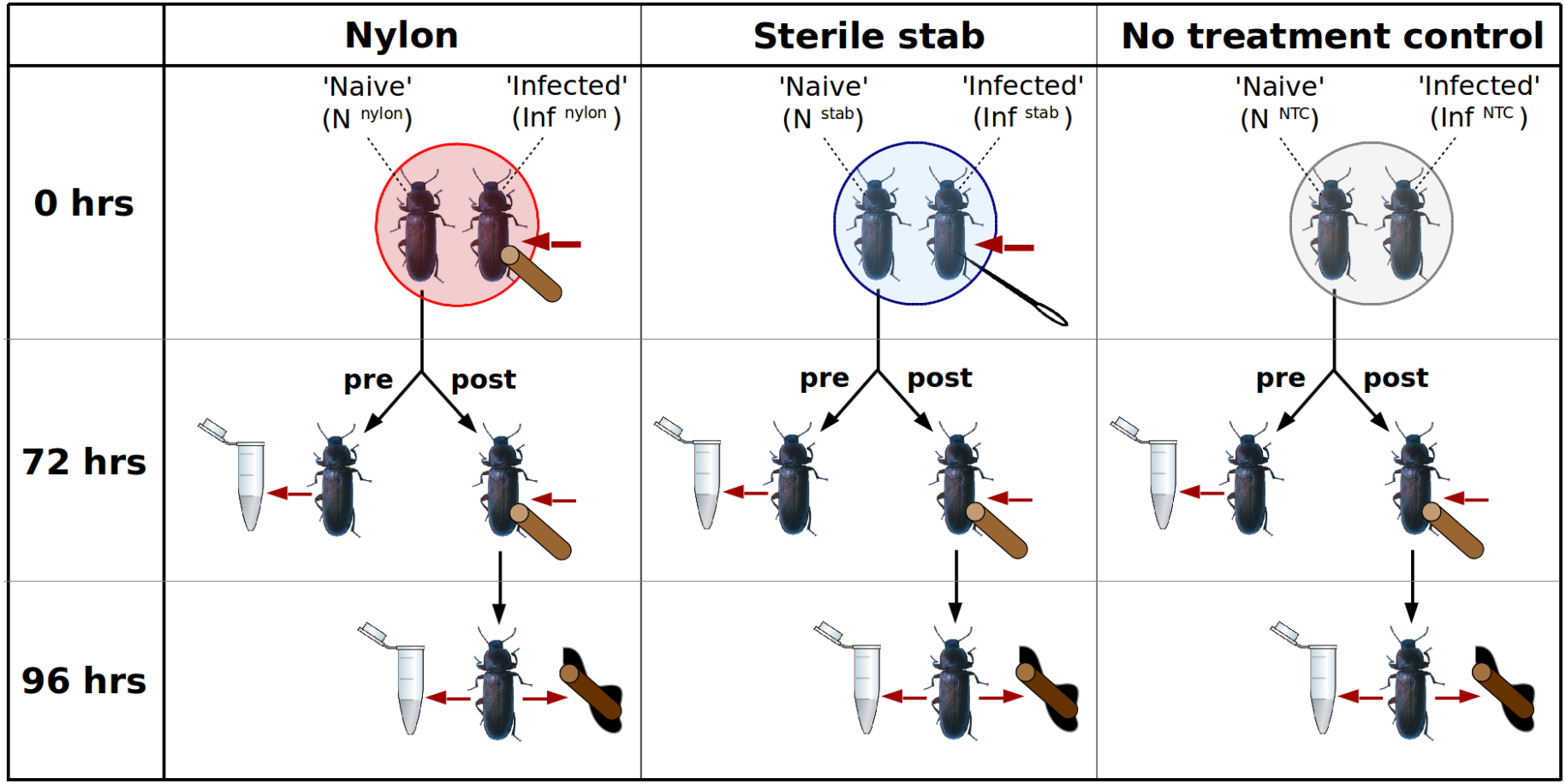


Figure 3.1. Summary of methods used in the experiment. Pairs of beetles (male-male and female-female) were housed together in Petri dishes (50mm diameter), with each pair consisting of one naïve individual ('N') and one infected individual ('Inf'). Groups differed according to the treatment administered to the infected (Inf) cohabitant: Inf^{nylon} received a nylon monofilament insertion, Inf^{stab} received a sterile stab as a treatment control, and Inf^{NTC} remained unchallenged as a non-treatment control. After 72h cohabitation, naïve cohabitants (N) from each group were removed and assigned to one of two assays. Those in the pre-infection ('pre') assay had their haemolymph extracted immediately after cohabitation to measure their level of constitutive immunity. Those in the post-challenge ('post') assay received a nylon monofilament insertion, and had their haemolymph and nylon filament extracted after 24 hours to measure their level of inducible immunity.

3.2.4. Haemolymph extraction

Naïve individuals were anaesthetised on ice and had their haemolymph extracted by perfusing the haemocoel with 500µL of sterile phosphate buffered saline (PBS). A 500µL perfusion avoids over-dilution of the haemolymph whilst ensuring a relatively comprehensive sampling of the haemocoel, as a 500µL perfusion has been shown to extract >90% of total haemocytes from *T. molitor* (Thompson, 2000). Perfused haemolymph was vortexed and split into two 200µL samples before being stored immediately at -80°C to disrupt haemocytes. Frozen samples were defrosted on ice for <15 mins and centrifuged for 15 mins at 4°C at 12000 rpm before the supernatant was extracted for analysis.

3.2.5. Nylon retrieval and elytra removal

After being perfusion bled, all naïve individuals and Inf^{nylon} infected individuals had their elytra removed and stored at -20°C for later analysis. All naïve and infected individuals that underwent nylon challenge were dissected to retrieve their nylon monofilaments, which were subsequently stored in 5% sodium azide solution and refrigerated for later analysis.

3.2.6. PO and proPO activity

Phenoloxidase (PO) activity and total PO activity (combined activity of phenoloxidase and prophenoloxidase [proPO]) were quantified using a modified version of the protocol reported by Stoks *et al.* (2006). I added 40µL haemolymph supernatant, 120µL distilled water and 20µL L-DOPA (4mg/mL in PBS) to each well of a 96-well microplate. ProPO was activated by the addition of 20µL alpha-chymotrypsin (2mg/mL in PBS), whilst for wells in which PO only was measured, 20µL PBS was added. Plates were incubated for 5 mins at room temperature before being placed in a plate reader (VersaMax) for 40 mins at 30°C, where optical density was measured at 495nm (OD_{495nm}) every 40 secs.

Enzyme activity, Vmax, was measured as the slope of the reaction curve during the linear phase under non-limiting substrate concentrations (Barnes & Siva-Jothy, 2000). This was quantified in R (v3.1.2; R Development Core Team, 2014) by applying a running median to smooth OD data before using a sliding window to determine the 10 minute period with the largest linear increase in OD. All values were standardised relative to a negative control run for each plate, which consisted of two wells with the same ingredients aside from 40µL PBS substituted for the haemolymph sample. A positive control was also run for each plate, with two wells each consisting of 40µL mushroom

tyrosinase (0.2mg/mL in PBS; Sigma-Aldrich T3824) in place of haemolymph. Each haemolymph sample was run in duplicate and a mean PO and total PO activity value calculated for each beetle.

3.2.7. Protein concentration

Total haemolymph protein concentration was estimated using the Bradford technique (Bradford, 1976). 5µL centrifuged haemolymph supernatant was added to 195µL Bradford reagent (Sigma: B6916) in microplate wells. Plates were incubated at room temperature for 5 mins before optical density was measured at 650nm (OD_{650nm}), and protein concentration estimated by referencing a calibration curve calculated using a serial dilution of a protein standard (bovine serum albumin; Sigma-Aldrich 82516). Haemolymph samples were run in duplicate and their mean value calculated.

3.2.8. Nylon analysis

Whilst nylon encapsulation and melanisation is a widely used immune assay in studies on insects (König & Schmid-Hempel 1995; Schmid-Hempel & Schmid-Hempel 1998; Ryder & Siva-Jothy, 2000), little detail has been published on the specifics of image acquisition and the quantification process, which can be highly subjective. A standardised and automated method of analysis was therefore developed. To achieve this, an automated image analysis script was developed in C++ language using the open-source image analysis library, OpenCV (http://opencv.willowgarage.com/). The image analysis script, calibration tool and sample images are documented online (https://github.com/JoGall/nylon-encapsulation/).

Nylon monofilaments were digitally photographed using a MicroPublisher 3.3 RTV camera (QImaging, Burnaby, BC, Canada) attached to a Leica dissecting stereoscope (Wetzlar, Hessen, Germany). In the image analysis script, each image was: (a) converted to greyscale, (b) adaptively thresholded in order to binarise the image and subtract the background, (c) eroded and dilated to smooth contour edges, (d) had the single largest contour found and extracted to select the desired foreground, (e) given an encapsulation score by enumerating the absolute number of pixels within the contour which have a lower brightness than a user-specified threshold, (f) given a melanisation score by calculating the mean pixel saturation of all pixels within the contour, (g) had the total length of the monofilament estimated by calculating the length of a minimum rotated rectangle fitted to the detected contour. Finally, measurements were scaled to true spatial units (mm^2) through calibration with a reference image of known size. Sample image

showing this procedure are provided in Figure A2.2.

The calibration stage is a vital subjective step in defining the level of encapsulation, as human judgment is required to distinguish between encapsulating cellular material (black) and non-cellular zones of darkening (more brown). The latter may be result of direct melanisation of the nylon monofilament or even melanisation of entangled (non-encapsulating) tissues, such as fat body (samples images provided in Figure A2.1). In order to calibrate the assay and find the most appropriate threshold, we manually estimated encapsulation for 10 nylon images using the thresholding function in ImageJ (http://imagej.nih.gov/ij). We then used the image analysis script to iterate over all possible thresholds (0-255) for the same images, and calculated the threshold whose result most closely matched the manual method (Figure A2.3).

3.2.9. Assessment of cuticular melanisation

Elytra were digitally photographed (setup as described above) under direct, intense illumination from a lightbox, as the elytra of lighter brown beetles is not readily distinguishable from black beetles under normal lighting conditions (Barnes & Siva-Jothy, 2000). In order to quantify cuticular luminescence, a similar image analysis script to the one described above was developed, again using the OpenCV library. In brief, each raw image was (a) converted to grayscale, (b) Gaussian blurred, (c) thresholded, and (d) had the single largest contour found and drawn. A darkness score was then given by the mean saturation of pixels in the detected contour. Elytron length was calculated by fitting a minimum rotated rectangle fitted to the detected contour and outputting its length, before scaling to true spatial units (mm^2) through calibration with a reference image. Image samples of this procedure are provided in Figure A2.4. Both elytra (left and right) were analysed for each beetle and mean values of luminescence and length calculated.

3.2.10. Statistical analysis

All statistical analyses were conducted using R (v3.1.2; R Development Core Team, 2014). Linear regressions were built for each response variable, with PO activity, total PO activity, nylon encapsulation and nylon melanisation data being log-transformed for normality. For each regression, statistical models were optimised using a stepwise procedure, working backwards from maximal models that included all main effects (sex [male or female], cohabitant treatment [nylon, sterile stab or no treatment control], and challenge status [pre-challenge or post-challenge]) and all possible interactions. Additional models were built to test for the effects of cohabitant treatment within each

gender independently by omitting sex and its interactions as fixed effects from the models. As body size is often positively correlated with the level of immune investment in many insect species, including *T. molitor* (e.g. Ryder & Siva-Jothy, 2001; Cotter *et al.*, 2004), pupal wet weight was initially included in the model, although proved to have no significant effect on the activity of any measured immune effector, nor did it have a significant interaction with any other fixed effect in the model. Furthermore, there were no significant correlations between beetle weight and the level of any measured immune effector (Spearman's rank correlation: $R^2 < 0.02$, df = 184; $p > 0.2$), and mean weight did not differ between males and females, so weight was excluded from the analysis.

3.3. Results

3.3.1 Gender differences in immune responses of naïve cohabitants

When combining data from both sexes (see Table 3.1), a significant gender by cohabitant treatment interactions was apparent for measured levels of PO activity ($F_{6,179} = 7.427$, $p < 0.001$) and total PO (combined PO and proPO) activity ($F_{8,177} = 5.569$, $p = 0.005$) in naïve individuals. There was also a significant interaction between naïve challenge status and cohabitant treatment upon total PO activity ($F_{8,177} = 3.362$, $p = 0.037$) and haemolymph protein concentration ($F_{4,181} = 4.880$, $p=0.028$) in naïve individuals, and a marginally non-significant main effect of naïve challenge status upon PO activity ($F_{6,179} = 3.829$, $p < 0.052$). There were no significant predictors of nylon encapsulation or melanisation rates in naïve individuals, although there was a non-significant interaction between gender and cohabitant treatment upon nylon encapsulation ($F_{5,89} = 2.920$, $p = 0.059$).

3.3.1.1 Immunity in naïve females

Additional models fitted only the female data show no significant effect of cohabitant treatment upon any measure of immunity in naïve individuals (Table 3.2), despite a non-significant trend for higher PO activity in naïve females paired with immune-stimulated conspecifics ($F_{3,92} = 2.929$, $p = 0.058$). There was a significant effect of challenge status upon PO ($F_{3,92} = 6.241$, $p = 0.014$) and total PO activity ($F_{3,92} = 5.040$, $p = 0.027$) in naïve females, although activated titres recorded after subsequent immune challenge were unexpectedly lower than constitutive levels recorded before challenge (Figure 3.2), as were recorded concentrations of haemolymph protein (Figure 3.4).

3.3.1.2 Immunity in naïve males

Individual models fitted to male data show a significant interaction between cohabitant treatment and naïve immune status upon all immune measures except nylon melanisation (Table 3.2), although the interaction effect upon nylon melanisation was marginally non-significant ($F_{5,83} = 2.99$, $p = 0.061$). However, the effect of cohabitant treatment upon immunity in males is contrary to that predicted: instead of demonstrating augmented defence following cohabitation with an immune-stimulated cohabitant, N^{nylon} males exhibited lower levels of PO and total PO activity (Figure 3.2), nylon encapsulation (Figure 3.2) and haemolymph protein concentration (Figure 3.4) compared to control males housed with non-challenged cohabitants. For all immune measures but nylon melanisation, the effects of cohabitant treatment were only apparent in constitutive levels

of immunity, measured in naïve individuals before immune challenge.

3.3.2. Effects of cuticular melanisation

In N^{nylon} / Inf^{nylon} cohabiting pairs, there was a significant positive correlation between the cuticle darkness of challenged individuals and nylon melanisation in their naïve cohabitants (t_{28}=2.11, p=0.044; Table 3.4, Figure 3.5A), and a significant negative correlation between Inf^{nylon} cuticle darkness and N^{nylon} protein concentration (t_{58}=2.12, p=0.038; Table 3.4, Figure 3.5B). There were no such correlates between Inf^{nylon} cuticle darkness and either PO or total PO activity in N^{nylon} individuals, although there was a non-significant trend for an association between Inf^{nylon} cuticle darkness and N^{nylon} encapsulation (t_{28}=1.87, p=0.073; Table 3.4).

In contrast with previous studies (e.g. Reeson *et al.*, 1998; Barnes & Siva-Jothy, 2000; Armitage & Siva-Jothy, 2005), there were no striking associations between cuticle darkness and any measure of immunity measured within the same individuals (i.e. darker naïve individuals did not produce stronger immune responses) (summarised in Table 3.3). Although there was some evidence for a positive correlation between cuticular melanisation and haemolymph protein concentration in naïve individuals (p = 0.021), this difference was non-significant after correcting for multiple comparisons. As expected, PO activity and total PO activity were highly correlated (p<0.001), and both were also positively correlated with haemolymph protein (p<0.001). Nylon encapsulation and melanisation also showed a significant positive correlation with one another (p<0.001).

Table 3.1. Parameter estimates for linear models fitted to each measure of immunity in naïve beetles, for both sexes combined. Nylon encapsulation and melanisation were only measured in challenged naïve individuals ('post-challenge'), so 'treatment' and its interactions are omitted as an effect from these models. Significant effects ($p<0.05$) are highlighted in bold. Parameters for main effects are excluded in models where their interactions are significant.

	PO activity			Total PO activity			Haemolymph protein		
Trait	d.f.	F	P-value	d.f.	F	P-value	d.f.	F	P-value
sex	-	-	-	-	-	-	1	0.004	0.948
treatment	-	-	-	-	-	-	2	0.614	0.542
challenge	1	3.829	0.052	-	-	-	**1**	**4.880**	**0.028**
sex x treatment	**2**	**7.427**	**<0.001**	**2**	**5.569**	**0.005**	2	2.352	0.098
sex x challenge	1	2.829	0.094	1	1.843	0.176	1	1.369	0.257
treatment x challenge	2	1.919	0.150	**2**	**3.362**	**0.037**	2	0.028	0.867
sex x treatment x challenge	2	1.112	0.331	2	1.929	0.148	2	2.206	0.113

	Nylon encapsulation			Nylon melanisation		
Trait	d.f.	F	P-value	d.f.	F	P-value
sex	-	-	-	1	0.495	0.484
treatment	-	-	-	2	0.757	0.472
sex x treatment	2	2.920	0.059	2	1.832	0.166

Table 3.2. Parameter estimates for linear models fitted to each measure of immunity in naïve beetles, for both sexes combined. Nylon encapsulation and melanisation could only be measured in challenged naïve individuals ('post-challenge'), so 'treatment' and its interactions are omitted as an effect from these models. Significant effects ($p<0.05$) are highlighted in bold. Parameters for main effects are excluded in models where their interactions are significant.

MALES

	PO activity			Total PO activity			Haemolymph protein		
Trait	d.f.	F	P-value	d.f.	F	P-value	d.f.	F	P-value
treatment	-	-	-	-	-	-	-	-	-
challenge	-	-	-	-	-	-	-	-	-
treatment x challenge	**2**	**3.311**	**0.041**	**2**	**4.682**	**0.012**	**2**	**3.642**	**0.030**

	Nylon encapsulation			Nylon melanisation		
Trait	d.f.	F	P-value	d.f.	F	P-value
treatment	**2**	**3.514**	**0.039**	2	2.990	0.061

FEMALES

	PO activity			Total PO activity			Haemolymph protein		
Trait	d.f.	F	P-value	d.f.	F	P-value	d.f.	F	P-value
treatment	2	2.484	0.089	2	2.929	0.058	2	0.585	0.559
challenge	**1**	**6.241**	**0.014**	**1**	**5.040**	**0.027**	1	2.954	0.089
treatment x challenge	2	0.067	0.935	2	0.190	0.827	2	0.060	0.942

	Nylon encapsulation			Nylon melanisation		
Trait	d.f.	F	P-value	d.f.	F	P-value
treatment	2	0.640	0.532	2	0.105	0.901

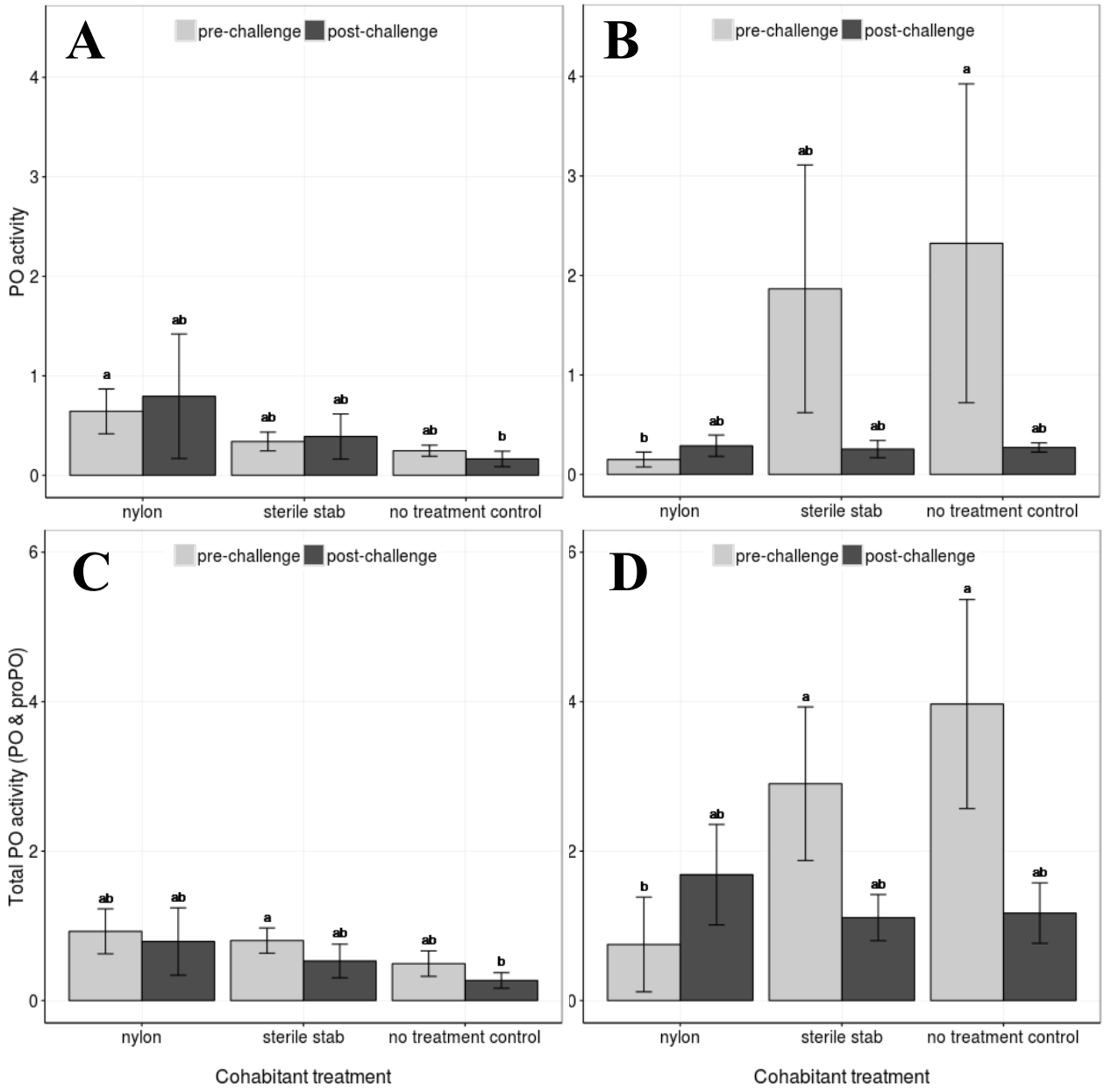


Figure 3.2. Haemolymph phenoloxidase (PO) activity (A, B) and total phenoloxidase (PO and proPO) activity (C, D) in naïve females (left column) and males (right column), measured either before (pre-challenge; light grey bars) or after a subsequent immune challenge (post-challenge; dark grey bars). Bars indicate mean ± S.E.. Bars that do not share a letter differ significantly ($p<0.05$; Tukey's HSD tests on linear models fitted independently to data from each figure).

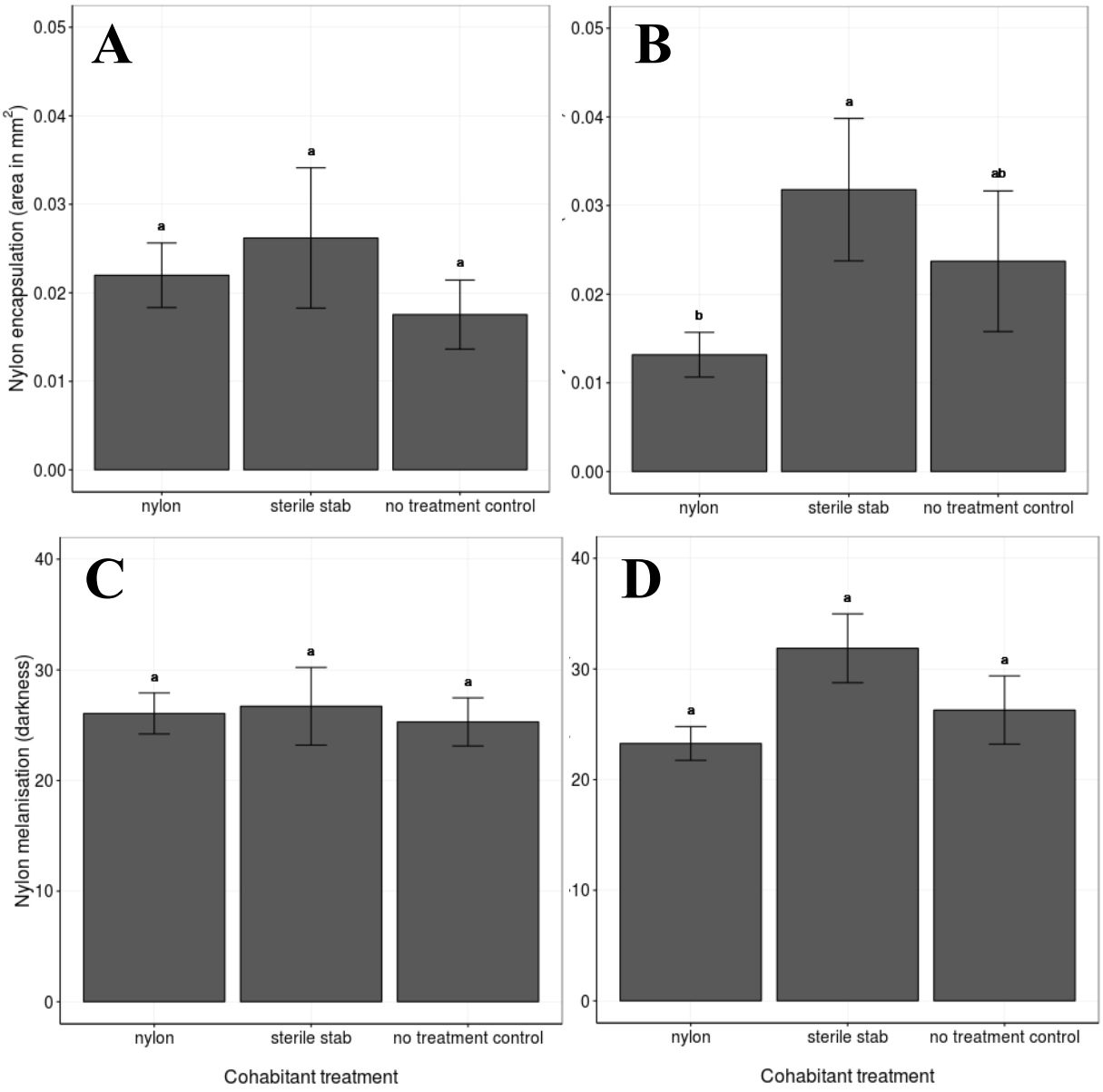


Figure 3.3. Levels of nylon encapsulation (A, B) and nylon melanisation (C, D) in naïve females (left column) and males (right column). Bars indicate mean ± S.E.. Bars that do not share a letter differ significantly ($p<0.05$; Tukey's HSD tests on linear models fitted independently to data from each figure).).

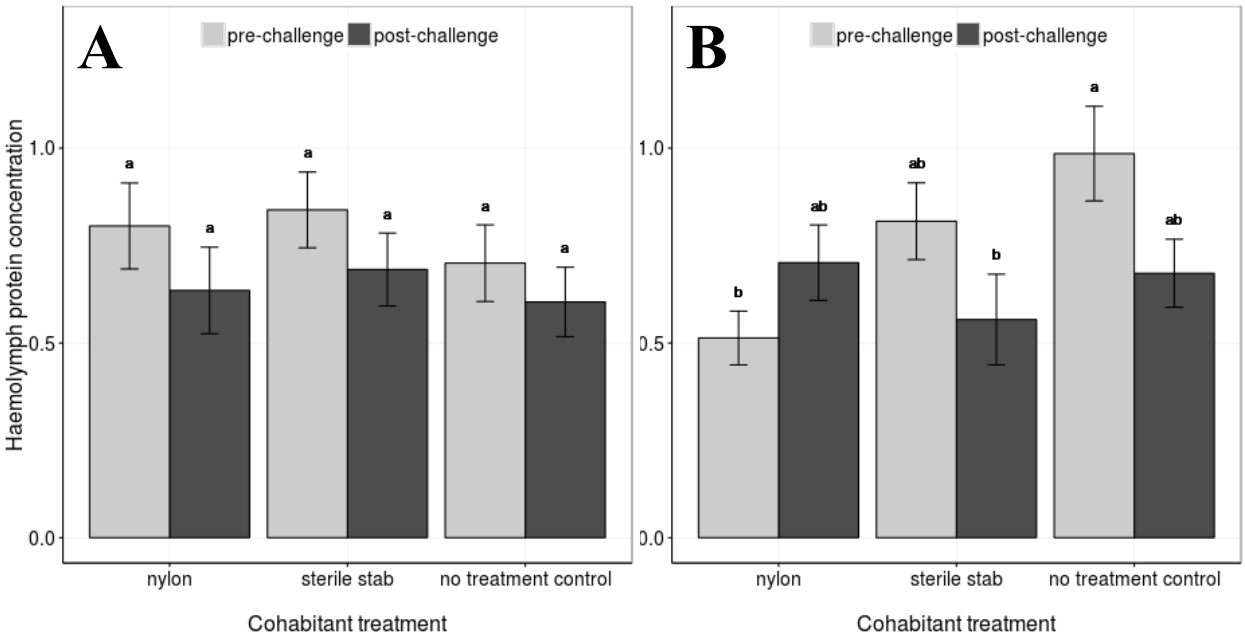


Figure 3.4. Haemolymph protein concentrations in naive females (A) and males (B), measured either before (pre-challenge; light grey bars) or after a subsequent immune challenge (post-challenge; dark grey bars). Bars indicate mean ± S.E.. Bars that do not share a letter differ significantly ($p<0.05$; Tukey's HSD tests on linear models fitted independently to data from each figure).

Table 3.3. Correlations between immune measures taken from naïve beetles (males and females combined). Spearman's rank correlation coefficients are displayed below the diagonal and raw p-values above it. Significant correlations ($p<0.05$) are highlighted in bold; asterisks indicate correlations that were no longer significant after Holm-Bonferroni correction for multiple comparisons.

Trait	Haemolymph protein	PO activity	Total PO activity	Nylon melanisation	Nylon encapsulation	Cuticle darkness
Protein	-	**<0.001**	**<0.001**	0.555	0.525	**0.021***
PO activity	**0.667**	-	**<0.001**	0.235	0.598	0.121
Total PO activity	**0.735**	**0.904**	-	0.122	0.553	0.180
Nylon melanisation	-0.108	0.216	0.279	-	**<0.001**	0.959
Nylon encapsulation	-0.117	0.097	0.109	**0.801**	-	0.906
Cuticle darkness	**0.296***	0.202	0.176	0.010	0.022	-

Table 3.4. Parameters of linear models fitted between cuticle darkess in immune-challenged beetles and each measure of immunity taken from their paired naïve cohabitants. Significant correlations ($p<0.05$) are highlighted in bold, and plots of their correlation shown below (Figure 3.5).

Trait	estimate	S.E.	d.f.	t	P-value
. ~ haemolymph protein	**16.49**	**7.78**	**58**	**2.12**	**0.038**
. ~ PO activity	238.56	194.85	58	1.22	0.226
. ~ total PO activity	117.85	139.07	58	0.85	0.400
. ~ nylon melanisation	**-0.83**	**0.39**	**28**	**2.11**	**0.044**
. ~ nylon encapsulation	-502.84	267.65	28	1.88	0.071

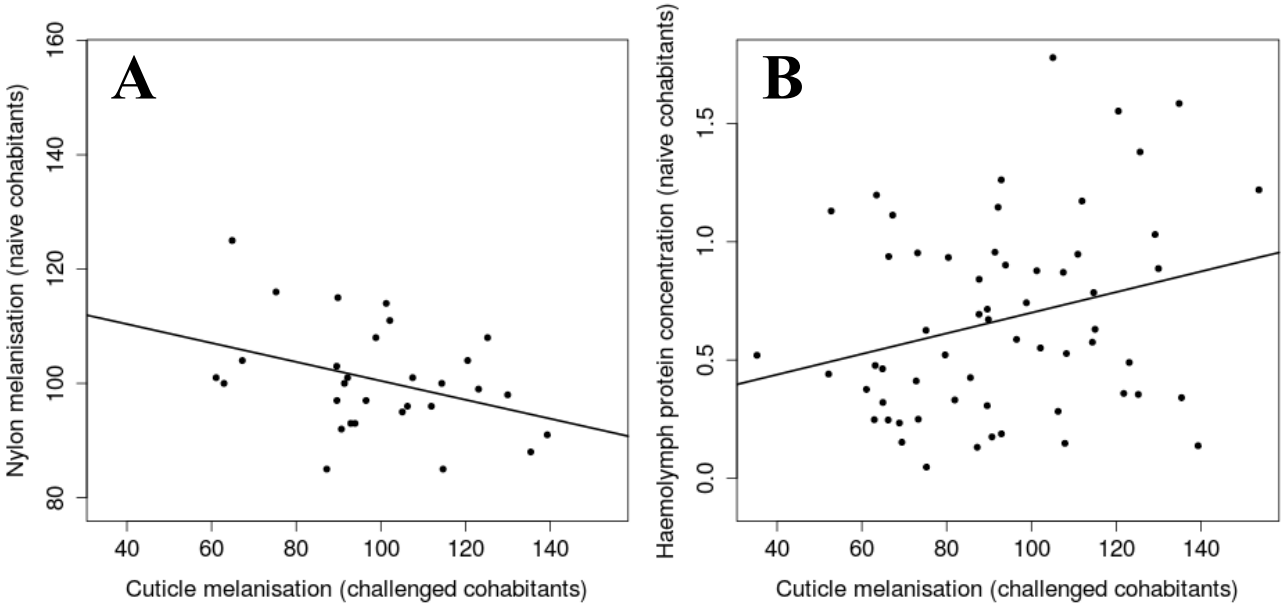


Figure 3.5. Correlations between cuticle darkness in immune-challenged beetles and nylon melanisation (A) and haemolymph protein concentration (B) in paired naïve cohabitants. Lines show a linear regression fitted to the data (nylon melanisation: adjusted $R^2 = 0.056$, df = 1,58, p-value: 0.038 ; haemolymph protein concentration: adjusted $R^2 = 0.107$, df = 1,28, p-value: 0.044).

3.4. Discussion

In this chapter, I found that cohabitation with an immune-stimulated conspecific of the same sex alters immune investment in previously naïve *Tenebrio molitor* males, though not in females. However, the directionality of this effect was not as predicted *a priori*, as naïve males housed with challenged neighbours did not exhibit augmented immunity, but rather showed significantly lower levels of constitutive haemolymph PO and total PO (PO plus proPO) activity, and a significantly reduced ability to encapsulate a subsequent nylon challenge. Although these data suggest that *T. molitor* males at least may be able to discriminate against conspecific immune status, the measured responses seem to be inconsistent with a process of gregarious immunisation. The data also suggest there are gender differences in how naïve individuals respond to environmental threats of pathogenesis.

3.4.1. Gender differences and the role of sexual selection

An important factor which may have influenced interactions between cohabitants and explained the observed gender differences is the differential potential for the adaptive modification of attractiveness traits by parasitised males and females. Immunity is traded-off against other physiologies and life-history traits (Zahavi 1975; Stearns 1992; Sheldon & Verhulst 1996), and hosts must distribute their finite pool of resources so as to maximise lifetime reproductive success. The terminal investment hypothesis suggests that hosts should invest more in current reproductive output if the chance of surviving to next reproduction is low (Clutton-Brock, 1984), thus ameliorating the costs of parasitism. However, males typically benefit more from terminal reproductive investment as their reproductive success increases linearly with their number of matings, unlike females (Bateman, 1948). Females should instead be selected to invest more in immunity and other strategies which improve survival in order to preserve their capacity for future reproduction (Rolff, 2002).

There is support for terminal investment in males of several insect species, with increased investment in reproductive traits, such as pheromone production, copulatory activity and sperm production, shown following pathogenesis (Polak & Starmer, 1998; Adamo, 1999; Abbot & Dill, 2001; Shoemaker *et al.*, 2006; Sadd *et al.*, 2006; Krams *et al.*, 2011). In *T. molitor*, males and females both undergo quantitative modifications of their cuticular hydrocarbon (CHC) profile during parasitism (Nielson & Holman, 2011), as documented in several eusocial insect species (Trabalon *et al.*, 2000; Salvy *et al.*, 2001; Richard *et al.*, 2008; Evans & Spivak 2010). Furthermore, *T. molitor* females are

preferentially attracted to the CHCs of immune-challenged males over unchallenged males (Nielson & Holman, 2011; c.f. Worden *et al.*, 2000), perhaps because males increase the quantity of sex pheromones produced during infection (Sadd *et al.*, 2006).

Sexually transmitted infections (STIs) are widespread in insects and are commonly detrimental to fertility (e.g. Knell & Webberley, 2004), meaning that insects, particularly females, should be under selection to avoid mating with infected conspecifics (Loehle, 1997). However, in some species, males infected with STIs are able to attract even more mating partners than uninfected males (Knell & Webberley, 2004; Goodacre & Martin, 2012; Adamo *et al.*, 2014). A host that behaves as if it were sick will not attract mates (Knell & Webberley, 2004), meaning that suppression of sickness behaviours should be advantageous for preserving individual fitness, particularly for males. A common sickness behaviour is reduced mating effort, such as decreased calling effort in infected male *Gryllus campestris* crickets (Adamo *et al.*, 2014), which is linked to a reduction in male mating success (Gerhardt & Huber, 2002; Adamo *et al.*, 2014). However, parasites, particularly those that are transmitted through sexual contact (STIs), gain an advantage from the suppression of such host sickness behaviours as they are more likely to be transmitted if infected hosts continue normal rates of sexual contact. Indeed, STIs are more likely to be asymptomatic than other diseases (Lockhart *et al.*, 1996; Mackey & Immerman, 2003; Antonovics *et al.*, 2011).

In this context, signalling by males during infection can be seen as a dishonest indicator of immune status (Sadd *et al.*, 2006), and one which may lead to sexual conflict as females stand to suffer greater fitness costs than males by mating with an infected partner. Females may incur direct fitness costs through reduced longevity and/or reproductive success if the pathogen is transmitted. Immediate reproductive success may also be reduced as parasitised males often undergo changes in their reproductive physiology; for example, *Tribolium castaneum* males suffer decreases in sperm production, sperm competitive ability and seminal fluid quality following parasitism with the rat tapeworm, *Hymenolepis diminuta* (Carver & Hurd, 1998; Pai & Yan, 2003). Furthermore, there may also be additional costs to indirect (kin) fitness if parental pathogens are transmitted vertically to offspring, or if parental parasite susceptibility is inherited genetically in the offspring (Carazo *et al.*, 2004). *Tenebrio molitor* females have been found to be preferentially attracted by filter paper discs of healthy males than those infected with the rat tapeworm, (Worden *et al.* 2000; Worden & Parker, 2005), and females mating with more attractive males have been shown to produce more offspring (Worden *et al.*, 2000) and have increased longevity (Vainikka *et al.*, 2007). Finally, in

species with sex-biased dispersal or parental care, one may expect greater investment in immunity by the sex which has the closest contact with kin, which is most commonly the female (Wilson & Cotter, 2013).

It has been suggested that the cost to females of assessing male signal honesty may outweigh the costs of mating with an infected male (Nielson & Holman, 2011). Female *T. molitor* are known to mate with multiple males (Drnevich *et al.*, 2000), and can increase their lifetime reproductive success through polyandry (Worden & Parker, 2005). Nevertheless, one may still expect mate choice to be plastic with regards to mate infection status, as some pathogens (e.g. castrating parasites or obligate killers) can inflict severe costs upon their host. Arguably, it is more favourable for hosts to employ an 'always avoid' strategy in response to infected conspecifics, avoiding these individuals regardless of the possible type or intensity of infection; false negatives in the identification of parasitism stand to incur greater fitness costs than false positives (i.e. accidental exposure to contagious conspecifics versus accidental avoidance of healthy conspecifics). This is more likely to be true for gregarious species with a balanced sex ratio, such as *T. molitor*, as there are no shortage of prospective mates available.

On the other hand, the observed differences between males and females may be partially explained by differences in conspecific exposure, as naïve individuals were only housed with conspecifics of the same sex. If, for example, females were to exhibit a stronger immune response than males in response to immune challenge, one may expect females to produce a greater amount of immune-related cues. As discussed, females often invest more heavily in immunity than males due to life-history differences between the sexes (Rolff, 2002), and this may affect the production of externalised chemosensory cues of infection during immune challenge given mechanistic links between internal physiology and externalised cues of immunity (e.g. Schal *et al.*, 1998; Tzou *et al.*, 2002). Here however, nylon-challenged males and females (Inf^{nylon}) did not significantly differ in their levels of immunity (data not shown), although it is possible that differences in other unmeasured immune effectors exist which lead to the production of different immune-related cues by each sex. Alternatively, as male *T. molitor* are known to increase their expression of sex pheromones following immune challenge (Sadd *et al.*, 2006; Nielson & Holman, 2011), and the apparently immunosuppressive response exhibited by naïve male cohabitants could be representative of a more general stress response, perhaps brought on by competition with an attractive rival male.

Same-sex pairs were used in this experiment in order to avoid the potentially confounding effects of mating upon immunity (e.g. Rolff & Siva-Jothy, 2002), but it is

likely that different processes would occur between mixed-sex pairs of immune-challenged and naïve individuals. For example, female burying beetles advertise their breeding status via the emission of methyl geranate, a compound structurally related to juvenile hormone, and increase emission when in the presence of a male partner (Steiger *et al.*, 2011). This suggests the cue is not a byproduct of breeding status but rather an intentional signal intended for communication; the authors argue that such receiver-dependent signal transmission may have evolved to reduce the costs of maintaining or transmitting a signal (Steiger *et al.*, 2011). It is possible that similar selection pressures favour the production of signals which indicate immune status to potential mates and/or non-mate conspecifics (particularly if one considers a social framework in which individuals stand to gain benefits to inclusive fitness by reducing the risk of pathogen transmission to nearby kin in the population).

In the case of reproductive signalling, *T. molitor* males prefer mature over immature females, and virgin over mated females (Carazo *et al.*, 2004). Given that both previously mated and unmated females benefit from mating with multiple males (Drnevich *et al.* 2001; Worden & Parker 2005), signalling of reproductive status may not be in the best interests of females (Svensson 1996). Carazo et al (2004) suggest that reproductive signalling in *T. molitor* females and males is not an example of 'true communication' (i.e. where information benefits both sender and receiver), but of specialisation restricted to the receiver, who is able to 'spy' or 'eavesdrop' on a conspecific to gauge their suitability as a mate (Bradbury & Vehrencamp 1998; Sorensen & Stacey 1999; Wyatt 2003). In non-social species, where the direct costs to personal fitness during infection are likely to be higher than the indirect fitness costs to kin through transmission, it seems probable that externalised cues of infection are similarly only produced as a byproduct, as opposed to being intended as a communication signal. Hosts that are noticeably sick are typically less likely to attract mates (Knell & Webberley, 2004), and one may even expect infected individuals to attempt to mask the symptoms of their infection, such as through increased sexual signalling in immune-challenged *T. molitor* males (Sadd *et al.*, 2006; Nielson & Holman, 2011).

3.4.2. Haemolymph protein and illness-induced anorexia

Naïve males which cohabited with nylon-challenged conspecifics demonstrated lower concentrations of protein in their haemolymph, and this reduction coincided with reductions in encapsulation ability and in PO and total PO activity (Figure 3.4b). Haemolymph protein concentrations can be used as a measure of physiological condition

(Cotter *et al.*, 2004, 2008), and previous studies have found a positive correlation between immunocompetence and haemolymph protein concentration in insects (Adamo, 2004; Lee *et al.,* 2004; Povey *et al.*, 2009). Lowered protein levels may be a consequence of reduced dietary intake of protein (Lee *et al.,* 2004; Povey *et al.*, 2009), and it is possible that N^{nylon} naïve individuals engaged in dietary restriction as an adaptive response to avoiding infection. Illness-induced anorexia is a common sickness behaviour which may serve to starve pathogens of key micronutrients such as iron (Hart, 1988), prevent the spread of pathogens from the gut to the blood during a systemic infection (Dunn *et al.*, 1994), and/or to prevent the diversion of resources away from immunity and into digestion (Weers & Ryan, 2006; Adamo *et al.*, 2008; Adamo *et al.*, 2010). However, anorexia may also be used as a preventative strategy initiated in response to environmental cues of pathogenic threat, and may prevent the host from consuming contaminated food (e.g. Zhang *et al.*, 2005). It is possible that anorexia was the primary response to cohabitation elicited by N^{nylon} naïve males, and that this resulted in lower immunocompetence as a secondary effect of nutrient deprivation (e.g. Siva-Jothy & Thompson, 2002; Lee *et al.*, 2008). Future work may benefit from investigating the impact of cohabitation with an infected conspecific upon feeding rates to determine the role that anorexia may play in prophylactic defence.

3.4.3. Role of cuticular melanisation

There was a positive association between the degree of cuticular melanisation in infected (Inf) cohabitants and the level of nylon melanisation and encapsulation observed in their paired naïve (N) neighbours, as well as a negative correlation between Inf cuticular melanisation and N haemolymph protein concentration. In Coleoptera, the highly melanised cuticle forms a mechanical barrier which provides a frontline defence to infection, and more melanised cuticles have been shown to provide increased resistance against entomopathogenic fungi, such as *Metarizhium anisopliae*, which invade the host by penetrating the cuticle (Gershenzon 1994; Verhoog *et al.* 1996; Reeson *et al.*, 1998; Barnes & Siva-Jothy 2000). Cuticular melanisation is also positively correlated with haemolymph levels of PO and proPO (Reeson *et al.*, 1998; Barnes & Siva-Jothy 2000), which in turn augments resistance against systemic infection. However, a large amount of variation in cuticular darkness exists within natural *T. molitor* populations (Barnes & Siva-Jothy, 2000) and dark cuticles are not a fixed trait, suggesting that a highly sclerotised cuticle may carry some fitness cost.

One explanation is that thicker cuticles contain less pores, making darker

individuals appear less attractive to mates due to the constrained production of sex pheromones and other volatiles (Armitage, 2002). However, the data in this study suggest that naïve individuals respond more strongly to immune-challenged neighbours with darker, thicker cuticles, suggesting that volatile signals may play an important role in hosts determining the immune status of conspecifics. However, infected individuals with darker cuticles are also more likely to induce a stronger immune response (Reeson *et al.*, 1998; Barnes & Siva-Jothy 2000), which may induce a stronger response in naïve individuals if: (i) internal physiology is able to modify the externalised CHC profile during immune challenge (e.g. Tzou et al., 2002; Schal, 2003), (ii) immune-related cues are produced in the excreta and able to be detected by neighbouring hosts, as infected individuals exhibiting a stronger immune response may be expected to suffer a greater degree of immunopathological damage to tissues, particularly those involved in osmoregulation and excretion (Sadd & Siva-Jothy, 2006), or (iii) visual cues are involved in discrimination, as immune activity may also be positively correlated with the extremity of sickness behaviours displayed by infected individuals (Adamo, 2006).

Correlation between internalised upregulation of the phenoloxidase cascade and externalised upregulation of cuticular melanisation may be pleiotropic, with one unable to come without the other (Armitage & Siva-Jothy, 2005). The cuticle acts as a sink for harmful quinones recruited for but not used in sclerotisation (Chapman, 1998), and plays a major role in defence by resisting enzymatic degradation and hyphal penetration by fungal pathogens (Söderhäll & Ajaxon, 1982; Hajek & St Leger, 1994). Thus, melanin may enhance disease resistance in insects by augmenting the chemical defences of the cuticle as well as improving its physical impermeability. It is possible that such chemical defences alter interactions with cuticular micriobiota in heavily melanised individuals, producing different chemosensory signals which can be detected by nearby conspecifics. If social (or gregarious) immunisation relies on chemosensory cues of infection, as has been suggested (Konrad *et al.*, 2012), the data in this chapter do not support the idea, since naïve individuals paired with challenged conspecifics possessing thicker cuticles upregulated their immune system to a greater degree than naïve individuals paired with conspecifics with more tan cuticules.

3.4.4. Non-adaptive explanations

Data from Chapter 2 showed that naïve females housed with a conspecific with a live bacterial infection exhibited reduced longevity in response to subsequent infection, despite showing an increase in antibacterial activity. Survival may be impacted by a

trade-off between immunity and other physiologies or life-history traits (Zuk & Stoehr, 2002; Schmid-Hempel, 2003), as hosts may not always minimise their fitness losses during infection simply by increasing their investment in immunity, and may choose to invest in life-history 'escape attempts', such as increasing their reproductive effort (van Baalen, 1998; Minchella, 1985). Another possibility is that trade-offs existed between our measures of immunity and other immune effectors that were not measured, such as lysozyme-like activity, which has been found to be negative correlated with phenoloxidase activity in previous studies (Cotter *et al*., 2004b; Adamo, 2004).

Alternatively, mortality may have been a non-adaptive consequence of the type of exposure; for example, naïve individuals were not able to physically escape from their immune-challenged cohabitants, rendering potential behavioural immune responses, such as physical avoidance of infected neighbours, redundant. Although there is evidence for social immunisation in termites (Traniello *et al.*, 2002), another study found no difference between isolated and grouped termites in their ability to encapsulate a nylon implant (Calleri *et al.*, 2006). The authors suggest that social processes may not affect the cellular aspect of innate immunity, and suggest that group-level immune strategies like social immunisation provide a prophylactic 'frontline defence' against pathogenesis which provides no benefit to hosts once a full-scale infection occurs (Calleri *et al.*, 2006). Once the pathogen invades the host, the induction of a physiological immune response is likely to lead to a lesser reduction in host fitness defence than continued expression of behavioural defences. However, increased mortality and increased antibacterial activity were only observed in female *T. molitor* exposed to conspecifics infected with live bacteria (Chapter 2), and not those housed with neighbours treated with heat-killed bacteria (a non-transmissible infection), suggesting a potential importance of the transmission of low-level infection ('variolation') between hosts (Yamada *et al.*, 1992; Rosengaus *et al.*, 1998; Konrad *et al.*, 2012).

As an inert and non-transmissible elicitor, the nylon implant used to stimulate immunity in this study resembles the heat-killed bacterial challenge used in the previous chapter. It is interesting, therefore, that the reduction of PO and total PO activity observed in N^{nylon} males in this study mirrors the downregulation of antibacterial activity exhibited in $N^{heat\text{-}killed}$ females in the previous study. However, gender dimorphism in life-history caution comparisons of immunity between the sexes, and there is little similarity between N^{nylon} females in this experiment and $N^{heat\text{-}killed}$ females in the previous chapter. Furthermore, the two immune insults are likely have very different effects upon the host, both mechanistically (e.g. cellular versus humoral effectors, specific versus general

recognition) and spatially (nylon insertion likely results in a more localised immune response [e.g. Sadd & Siva-Jothy, 2006] than the haemocoelic injection of bacteria, which likely results in systemic infection).

Immune priming in invertebrates appears to rely on the upregulated expression of specific cellular recognition receptors following contact with pathogen-associated molecular patterns (PAMPs; Schulenburg *et al.*, 2007), and augmented defence against secondary pathogenesis is likely mediated by upregulated and/or more rapid expression of highly specific immune effectors, such as antimicrobial peptides (AMPs; Schulenburg *et al.*, 2007; Cerenius *et al.*, 2010). It is possible that the type of immune challenge used in this study was not appropriate to stimulate a specific form of gregarious immunisation in naïve cohabitants, nor the production of specific immune cues by infected cohabitants, as nylon monofilaments provoke a non-specific immune response due to their lack of specific PAMPs.

It seems probable that there are differences between the cues produced by nylon-challenged individuals and cues produced by individuals during a natural (virulent) pathogenic infection. Potential damage resulting from the pathogen or from greater levels of host-induced immunopathology (e.g. Sadd & Siva-Jothy, 2006) during a live infection may influence the production of chemosensory cues. In the case of nylon implantation, the encapsulation response is a localised response to a spatially discrete foreign body, and the production of immunological effectors is therefore more concentrated in the vicinity of the immune insult, as opposed to be systemic throughout the haemocoel (e.g. Sadd & Siva-Jothy, 2006). If alterations in the chemosensory profile of an individual are mediated by the transport of immunological compounds from within the haemocoel through the cuticle (Schal, 2003), then one may expect the intensity of the (internal) immune response to be correlated with the level of changes in (external) CHCs (and other chemosensory compounds). Therefore, it is possible that nylon implantation, whilst sufficient to stimulate a costly physiological immune response, is insufficient to stimulate the production of externalised cues of infection, which may explain the apparent lack of gregarious immunisation in this study.

3.4.5. Summary

This chapter did not find evidence for a gregarious immunisation process in *Tenebrio molitor*, as naïve individuals did not exhibit enhanced immunity following cohabitation with an immune-challenged conspecific. However, males paired with a 'sick' conspecific showed a downregulation of several immune effectors, suggesting that *T. molitor* are able

to detect and respond to socially-derived cues of infection produced by conspecifics. Furthermore, there appeared to be an association between the level of cuticular melanisation in immune-challenged individuals and the resultant levels of immunity in naïve cohabitants they were housed with, although the reason for this is unknown. It is possibly that darker beetles, which often exhibit greater immunocompetence, produce different cues of infection that are detected by naïve conspecifics and used to inform immune priming.

In this chapter, I have shown:

- Cohabitation with an artificially immune-challenged conspecific can alter immune investment in naïve males, but not females
- In males, this effect was opposite to that predicted: naïve individuals housed with nylon-challenged conspecifics displayed significantly lower constitutive levels of haemolymph PO and total PO (PO plus proPO) activity, and significantly lower rates of nylon encapsulation following challenge
- Gender differences in immune responses, as males exhibited an unexpectedly higher level of PO and total PO activity than females, as well as interaction effects between gender and cohabitation type
- A potential role of cuticular melanisation in this process: there was a positive correlation between cuticle darkness in immune-challenged individuals and resultant immune activity in their paired naïve cohabitants

CHAPTER FOUR:

DEVELOPMENT OF AN AUTOMATED BEHAVIOURAL TRACKING SYSTEM

4.1. Introduction

Locomotor activity impacts almost all aspects of a mobile animal's ecology. Movement underpins key fitness-driving traits, such as foraging, mating, courtship, learning processes, and immunity (Martin, 2004). Many of the most commonly measured behaviours in animals, such as ambulation, freezing (resting immobile), jumping, vectorial information (speed, acceleration), positional information (e.g. site preference, orientation angle) – as well as psychological measures typically considered in only vertebrate studies, such as anxiety, obsession and aggression – are emergent from tracking the movement vectors of an individual, i.e. the organism's spatial coordinates over time.

The quantification of the complex movement patterns of mobile organisms has become an integral subject in biological research, and has been facilitated by recent advances in automated tracking methods. Automated systems provide a much higher throughput than manual methods, and tend to be more reliable due to the consistency of a processing algorithm, which does not suffer observer fatigue or drift (Noldus *et al.*, 2001). Digital methods can also yield behavioural metrics that would be difficult or impossible to quantify manually, such as velocity, acceleration and turning angle, as well as calculating time and spatial location with a high degree of accuracy.

As computing capabilities have increased and costs decreased over the last decade, automated tracking systems have progressed from rudimentary and often unreliable analogue systems which described mostly discrete behaviours, to digitised methods which can detail an array of continuous kinematic variables. Several digitiser-based video tracking systems are commercially available, but there are drawbacks with many of these. Firstly, many trackers are developed only for the most well-studied model organisms (e.g. *Drosophila* [Gomez-Marin *et al.*, 2012, Dankert *et al.*, 2009], *C. elegans* [Swierczek, *et al.*, 2011], zebrafish [Beyan & Fisher, 2013], mice [de Chaumont *et al.*, 2012]), being highly tailored to the particular morphology and movement patterns of their target species. Secondly, many of these programs are designed to address specific behavioural paradigms, and thus offer little flexibility, often requiring specialised apparatus or the use of highly specific experimental designs. Thirdly, much available tracking software is proprietary and requires a substantial fee (up to $10,000), as well as additional annual

license fees to use (e.g. EthoVision [Noldus *et al.*, 2001], ANY-maze [http://www.anymaze.com]). Of the free and open-source options available, several tend to parse output files (containing the tracked X,Y-coordinates) with toolboxes reliant on proprietary software, such as MATLAB (e.g. Ctrax, Flytrax [Branson *et al.*, 2012]). Finally, of the published open-source tracking software, many are largely inflexible and onerous to modify, necessitating in-depth knowledge of the relevant programming language as well as a substantial investment of time. Several have not been kept updated sufficiently, and are unstable to use or are entirely non-functional (e.g. SwisTrack [Lochmatter *et al.*, 2008], MotMot [Straw & Dickinson, 2009]).

Advancements in computer-vision capabilities and the increasing availability and support of open-source libraries, such as OpenCV (Willowgarage, http://opencv.willowgarage.com), are creating a new and accessible ecosystem of highly customisable and affordable tools for biologists to study the behaviour of almost any organism.

This chapter details the development of an automated behavioural tracking platform for insects, 'UbiTrail'. The software was designed to be (i) versatile, working with a range of morphologically and behaviourally distinct insect species and within a range of non-specialised experimental designs, (ii) affordable, using exclusively open-source software and inexpensive hardware, and (iii) robust, working under imperfect lighting conditions, handling insect occlusion and other experimental variables that lead to lower repeatability. A statistical package was also developed to define and analyse key behavioural metrics from the tracking data.

The system was developed primarily to collect quantitative data on immune-induced behavioural modifications ('sickness behaviours'). Such data are generally lacking in insects (Adamo, 2006). The system was also used in experiments to investigate whether behavioural cues of infection are produced by immune-challenged *Tenebrio molitor* (Chapter 5), and whether naïve beetles display a behavioural avoidance of infected conspecifics as a form of immune defence (Appendix 4).

In this chapter, I:

- Develop a computer vision system to track the movement of insects
- Develop a statistical package to define and extract key behavioural metrics from tracked coordinates and produce biologically meaningful quantitative data

4.2. Methods

4.2.1. Description of the system

The tracking software, UbiTrail, was written in C++ using the OpenCV library (Willowgarage, http://opencv.willowgarage.com), and under the CodeBlocks design environment (http://codeblocks.org). The software source code and compilers for Unix and Microsoft Windows operating systems are freely available online (http://sourceforge.net/projects/ubitrail), as is the associated package for statistical analysis, Rubitrail, as well as a user manual, sample videos and sample data.

The details of the image analysis process are described in detail in Figure 4.1. In brief, the software uses a dynamic learning algorithm to learn to identify moving foreground objects during an initial training period (default value of 500 frames, ~25 seconds). In order to solve ambiguities in foreground detection, a likelihood model is built on the fly, based upon several key features of known foreground, including contour shape, pixel colour and distance between current contour and last detected contour, with the most likely single contour being taken as foreground.

4.2.2. Using the software

UbiTrail currently works with digital video files as input, although an option for real-time analysis is under development. Videos can be recorded using an inexpensive USB video camera (any webcam with a resolution of 640x480 pixels or better is suitable) and are easily captured using the open-source multimedia player, VLC (VideoLAN, http://videolan.org/vlc).

After recording a video, the user is able to define a mask to denote the position of areas within the arena, as well as sub-territories within individual areas, if desired. The user is then able to adjust several processing parameters in order to optimise tracking accuracy, such as sensitivity (which determines how likely noise is to be detected as motion) and the number of frames used to train the motion detector.

The software can be implemented either via the command line or using a graphical user interface (GUI). The GUI is a simple assistant which allows the user to interactively define the inputs and output options, preview the defined mask over the video, and visualise the actual tracking process on-the-fly (Figure 4.2). Command line usage can increase efficiency by allowing the user to iteratively analyse multiple videos without the need for continual input.

The software outputs a CSV file containing an X,Y coordinate, timestamp, area ID and territory ID (if applicable) for each detected object in each frame of a video. Also

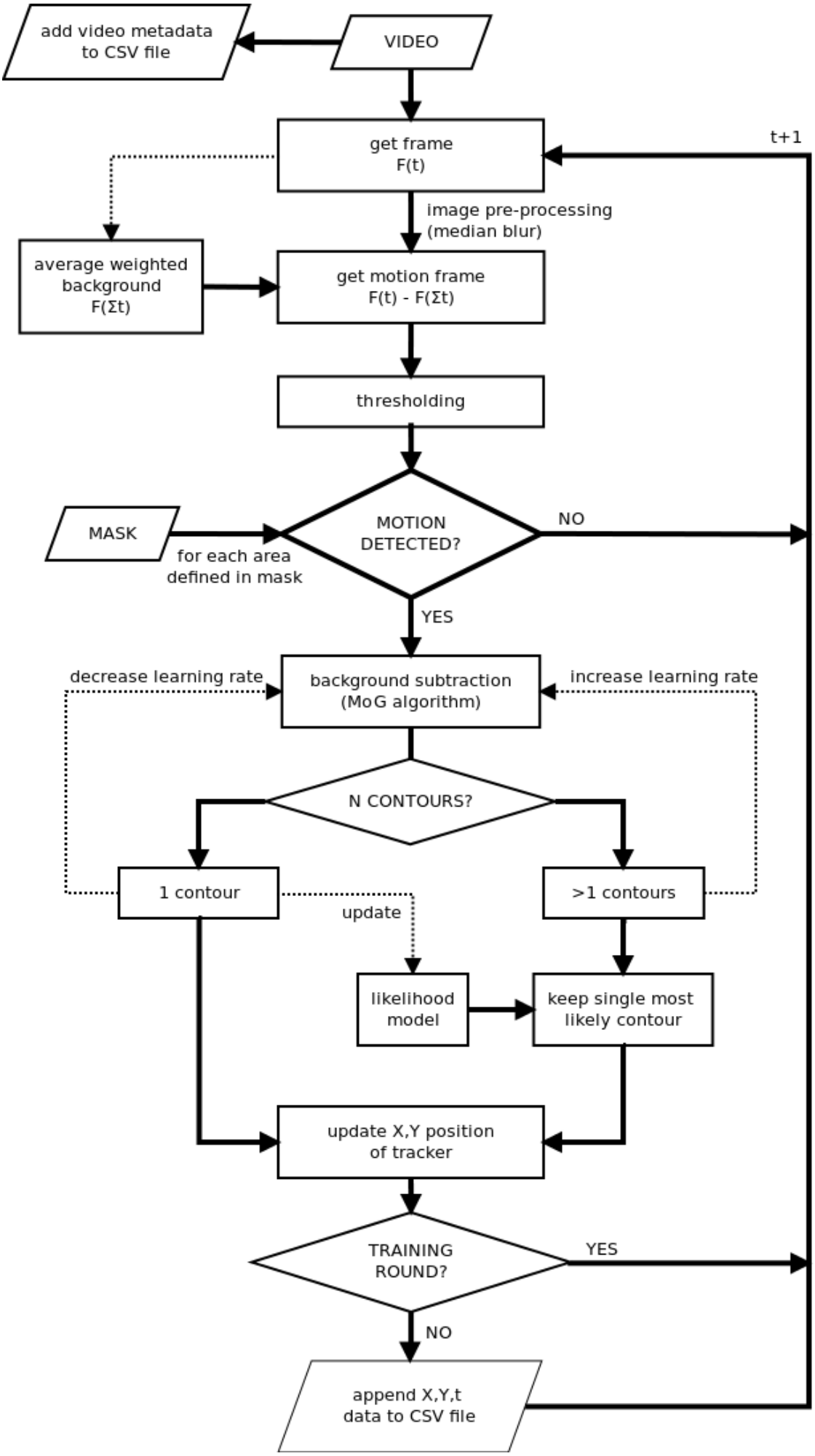

add video metadata to CSV file
VIDEO
get frame F(t)
t+1
image pre-processing (median blur)
average weighted background F(Σt)
get motion frame F(t) - F(Σt)
thresholding
MASK
for each area defined in mask
MOTION DETECTED?
NO
YES
decrease learning rate
background subtraction (MoG algorithm)
increase learning rate
N CONTOURS?
1 contour
update
>1 contours
likelihood model
keep single most likely contour
update X,Y position of tracker
TRAINING ROUND?
YES
NO
append X,Y,t data to CSV file

Figure 4.1. (left) Flow diagram of tracking software process. UbiTrail takes a digital video file as input and returns a CSV file containing tracked X,Y-coordinates for each frame in each defined area, as well as a header containing metainformation extracted from the video. Each frame, F(t), is extracted individually from the video and de-noised using a (9x9) median blur filter. A motion frame is then produced by subtracting the current frame from a running weighted average of previous frames, F(t-1), which is used to model the background. At this point, the mask is applied to split the frame up into individual areas. In areas where motion is detected, a dynamic learning algorithm based upon a mixture of Gaussian (MoG) background subtraction method is used to identify moving foreground objects. The MoG algorithm is trained separately for each individual area, with the rate of learning being increased following ambiguous frames in which the movement of more than one foreground object is detected, and decreased following unambiguous frames in which movement of exactly one foreground object is detected. In order to solve ambiguities in foreground detection, an on-the-fly likelihood model is built based upon several key features of known foreground objects, including contour shape, mean and standard deviation of pixel colour in the red, green and blue channels, and distance between centre of the current contour and centre of the last detected contour. A log-likelihood, L, is then calculated under the assumption of normal distribution, where:

$$\mathrm{L} = \sum_{i=0}^{n} l_i$$

$$\mathrm{l}_i = \ln\left(\frac{1}{s_i\sqrt{2\pi}} e^{-\frac{(x_i - m_i)^2}{2s_i^2}}\right)$$

and where **n** is the total number of features, **i** is a given feature, $\mathbf{x_i}$ = value of feature **i**, $\mathbf{m_i}$ is the psuedo-mean of feature **i**, and $\mathbf{s_i}$ is the pseudo-standard-deviation of feature **i**. When more than one contour is detected, the contour with the maximum log-likelihood is taken as the foreground object. An initial training round (default = 500 frames) is used to train the background subtraction algorithm and build a suitable likelihood model for foreground detection, ensuring valid foreground detection throughout the video analysis.

included is a header containing metainformation, such as name, duration, and number of frames per second, as well as the X,Y-coordinates of each detected area. Video files of the tracking process can also be optionally returned, either as a single video of the global arena or as separate videos for each individual area.

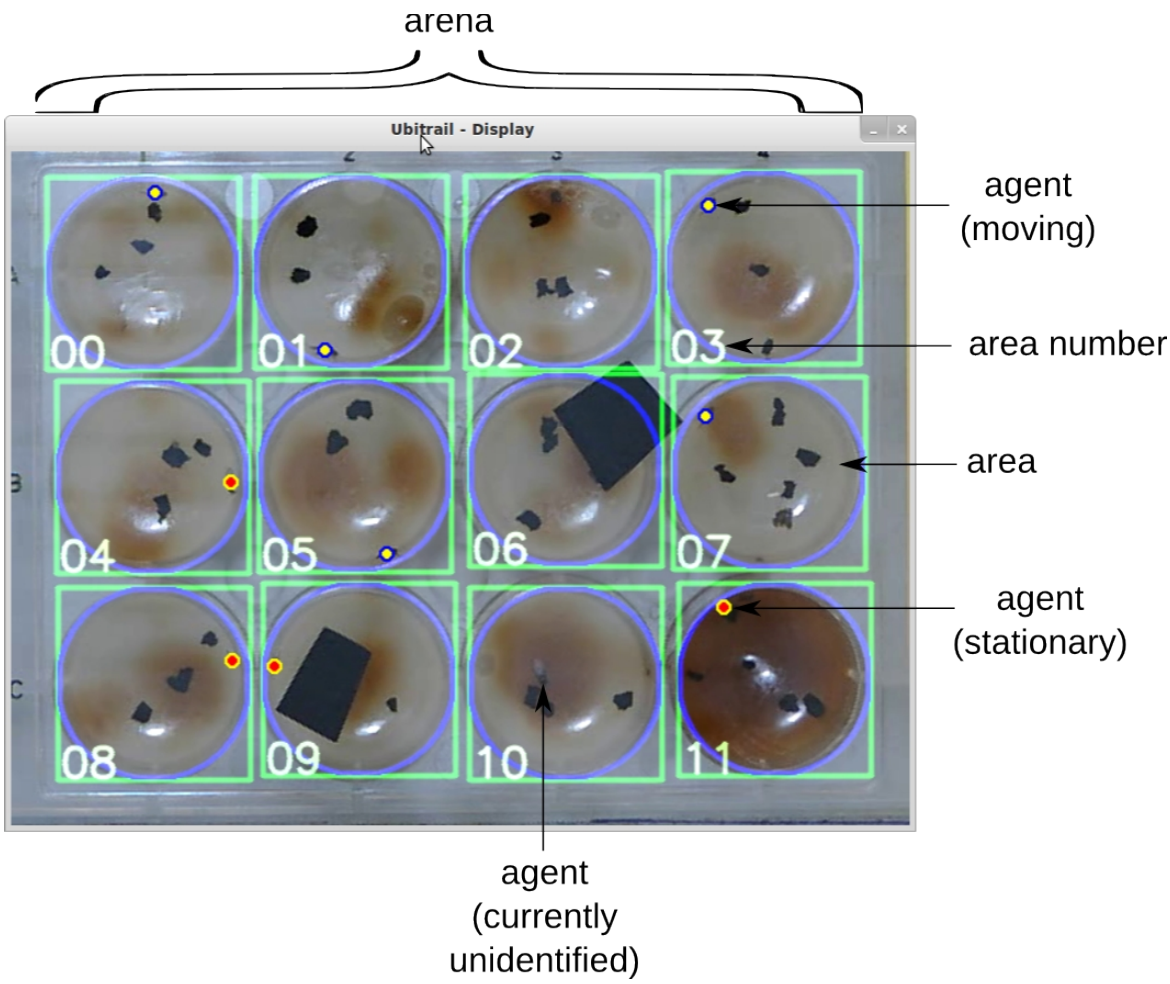


Figure 4.2. The graphical user interface (GUI) of the UbiTrail software, with key elements labelled. The outline of the mask which defines each individual arena is shown in blue, with its assigned area number depicted inside the green square. The above sample shows a test on *Drosophila melanogaster* adults, where variation in background luminosity was created to test for the robustness of background subtraction, and covering objects (unlabelled black objects) were introduced to test for the effects of occlusion upon insect tracking.

4.2.3. Rubitrail analysis package

The analysis software, Rubitrail, is a package written for the open-source statistics software R (R Core Development Team, http://r-project.org). The package extracts multiple features from the raw data outputted by the tracking software, including velocity, turning angles, activity levels and positional information, as well as allowing the user to define their own additional variables for analysis. Whilst all scripts within the package are fully customisable, a single master function is included to aid user accessibility, requiring as input only a list of CSV files for analysis and a scale calibration (pixels/mm).

4.2.3.1. Pre-processing data

4.2.3.1.1. Undistortion

Fisheye lenses and low-cost wide-angle lenses can produce a significant degree of barrel distortion in the images they capture, having the potential to impact the validity of detected movements in a tracking software (Figure 4.3). This can be corrected using a simple algorithmic transformation:

$$R = r(a.r^3 + b.r^2 + c.r + d)$$

where **r** is the distance of a given pixel to the centre of the uncorrected image and **R** is the distance of the pixel in the corrected image. This transformation can either be applied before tracking analysis by transforming each image frame of the raw video, or after tracking by transforming the detected X,Y-coordinates; Rubitrail utilises the latter method. Ready-made parameter sets for particular cameras can be found online (e.g. http://sourceforge.net/projects/hugin/files/PTLens%20Database), or can be calculated manually by taking a calibration image (see Figure 4.3) and using the undistortion feature in a number of image manipulation programs (e.g. ImageMagick, http://imagemagick.org).

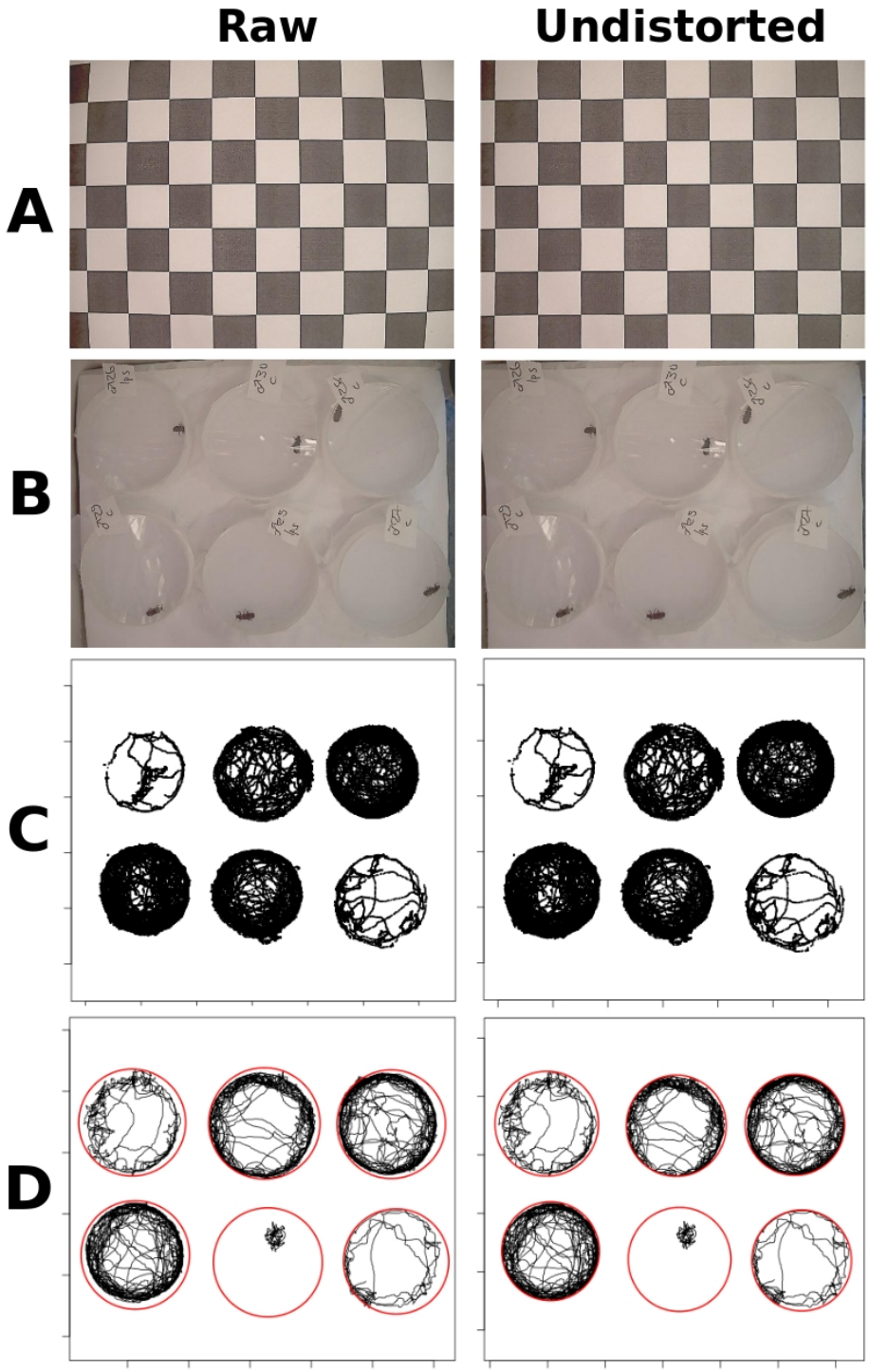


Figure 4.3. The effects of lens undistortion upon images and tracked coordinates. (A) An image of a chessboard pattern is captured during the recording stage and used to calibrate parameters for an undistortion matrix. The effects of undistortion (raw [left] vs. processed [right]) are shown for (B) raw video frames, (C) tracked movement vectors, and (D) fitting a minimal enclosing circle to arenas.

4.2.3.1.2. Linear interpolation

In frames where insects are occluded by obstacles or glare, or a contour is otherwise not found, X,Y position is inferred using linear interpolation. X,Y-coordinates are not inferred for training frames, where the insect has yet to be detected. In instances where no movement is detected throughout the entire video, zero velocity is inferred for all frames, but all other metrics regarding positional information are defined as NA. In cases where movement is not detected in the latter frames of a video (e.g. the insect does not move in the final two minutes of analysis), the last confirmed object position is used as the X,Y-coordinates for the remaining frames.

4.2.3.1.3. Trajectory smoothing

Camera noise, lighting abnormalities, non-locomotory insect movements (e.g. grooming, antennation) and imperfections in foreground segmentation can cause false movements to be identified, increasing the noise in detected X,Y trajectories. Furthermore, lateral oscillation in the detection of moving objects is common (Hen *et al.*, 2004); this may be due to alternated movement between the insect head and posterior ('tail') between frames. Both of these factors are manifest in the tracked coordinates as a relatively small jitter, with perturbations no larger than the maximum length of the tracked insect. Two methods were used to correct this noise.

Firstly, trajectories were smoothed by using a simple moving median with a window size of 20 data points (1s) with a 1 point step size (0.05s). These values were found to preserve overall trajectory information and provide greater accuracy in determining velocity, turning angles and overall activity level (Figure 4.4). Secondly, due to the size of the smoothing window, insects were often falsely determined to have negligible, but non-zero, velocity (>0 mm/s). A movement threshold was implemented to filter smoothed velocity data, with near-zero velocities of <1mm/s (~2 pixels/s; a value used in similar tracking software [Valente *et al.*, 2007; Robie *et al.*, 2010; Colomb *et al.*, 2012]) redefined as zero velocity (=0mm/s) (Figure 4.5).

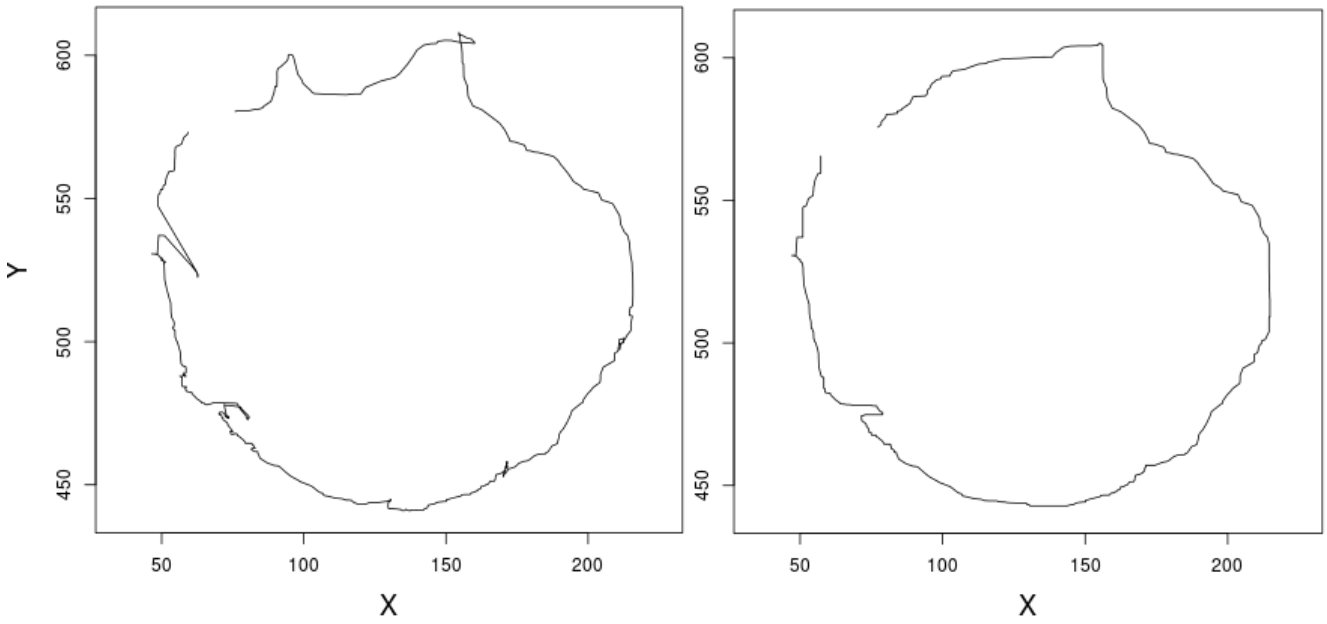


Figure 4.4. Smoothing of tracked x,y-coordinates. (A) shows a 60s sample of raw trajectories outputted by the tracking software, whilst (B) shows the same trajectory after application of a rolling median with window size of 3s (60 frames).

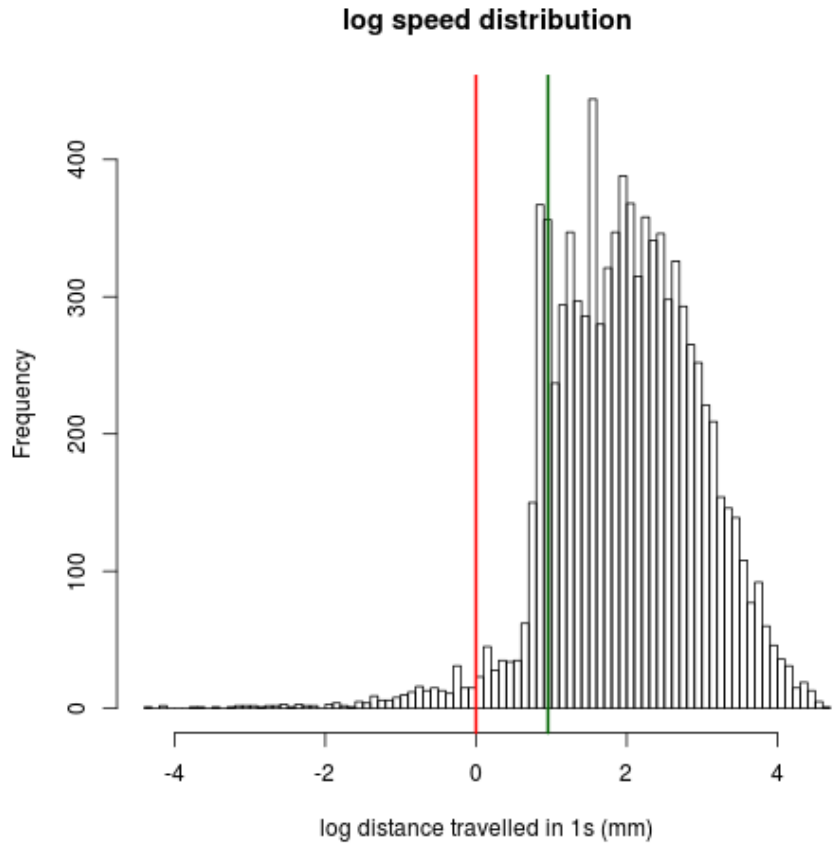


Figure 4.5. Histogram of smoothed velocity frequency (logarithmic scale), with lower threshold (red line: 0 mm/s) and upper thresholds defined (green line: 1 mm/s)

4.2.3.2. Extracting metrics

Several key metrics regarding movement were calculated from the smoothed trajectory data.

4.2.3.2.1. Velocity metrics

Distance moved was calculated as the Pythagorean distance between smoothed X,Y-coordinates in successive frames. Summing each movement length over the entire analysis yielded the total distance travelled (mm). Dividing distance travelled by time gave instant velocity (mm/s), and the first derivative of instant velocity was used to define acceleration (mm/s^2).

4.2.3.2.2. Angular metrics

Turning angle was calculated as the angle between successive velocity vectors (Figure 4.6). Considering the movement from P_0 to P_1, α_0 is the absolute movement angle, the turning angle, γ, can be calculated as $\alpha_0 - \alpha_1$. Movement paths of walking insects are generally continuous, and do not have discrete break points that make it easy to define moves; a common solution is to resample movement at regular time intervals and connect successive positions with linear interpolation. Here, smoothed data were down-sampled to a rate of 1 frame per second (Figure 4.6). Meander is a measure of movement tortuosity which combines turning angle with distance travelled, and increased meander is generally associated with navigational uncertainty (Collins *et al.*, 1994). Meander is calculated by dividing the turning angle by the instantaneous velocity (θ * mm/s) (Martin *et al.*, 2004). Turnaround events were defined as turns of 180° ± 25° which were completed within the space of one second (example highlighted in Figure 4.6e).

Many animals show a tendency to turn around an arena (Yaski *et al.*, 2011), a behaviour which is often interpreted as an escape response. Escape responses are well-studied in cockroaches, which rapidly turn directly away (180°) from threatening stimuli, such as a puff of wind, and accelerate away (Domenici *et al.*, 2008). A similar response is observed in *Tenebrio molitor* (pers. obs.), although this behaviour may equally be representative of roving behaviour or foraging activity, as opposed to an anti-predation or stress response.

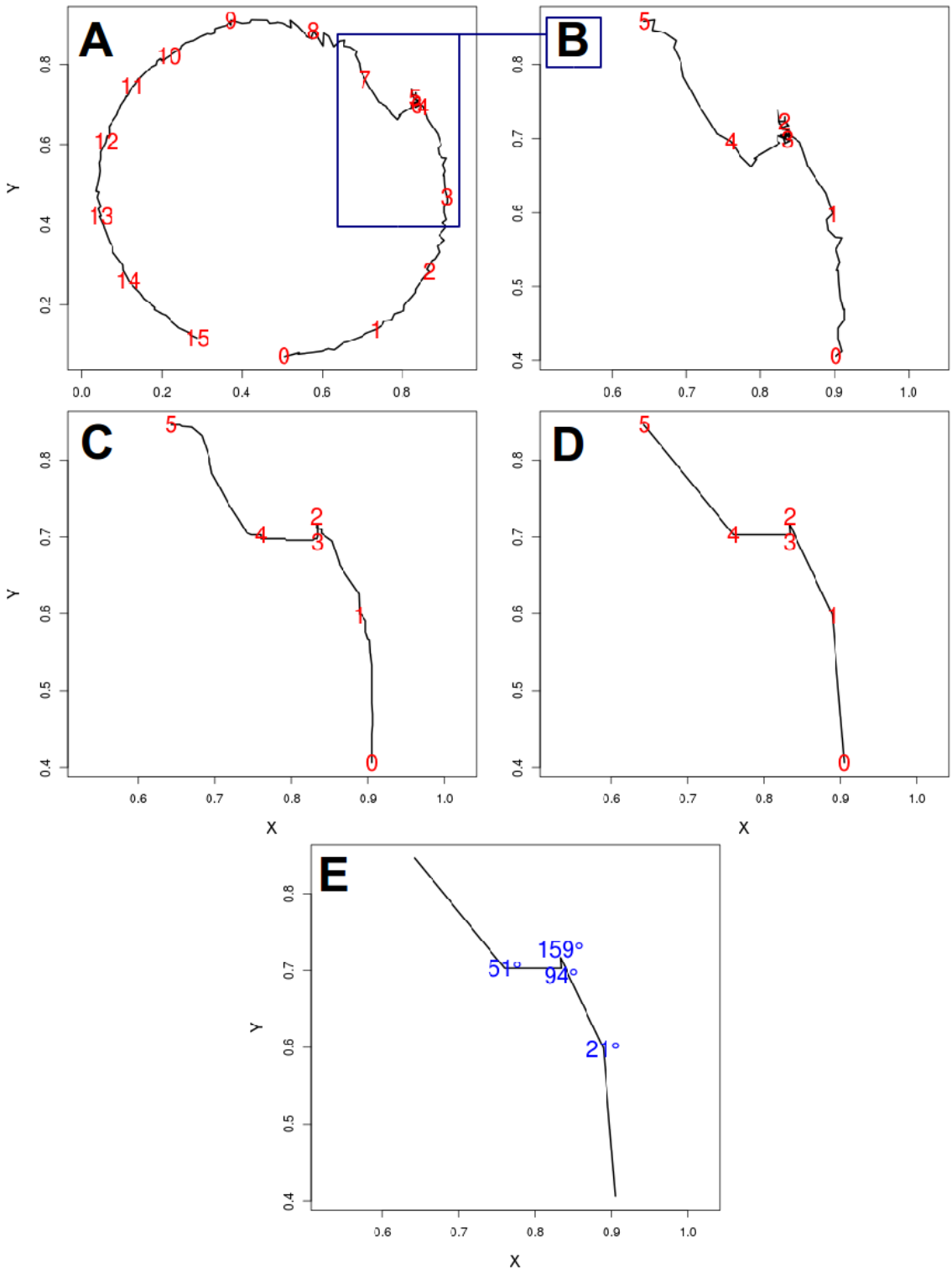


Figure 4.6. Calculation of turning angles. (A) shows a 15s sample (300 frames) of raw tracked X,Y-coordinates, with corresponding number of seconds overlaid (red text). The area inside the blue box is a 5s subsample which is zoomed on in (B-E), where (B) shows the same raw X,Y-coordinates with number of seconds (red text), (C) shows coordinates that have been smoothed using a rolling median with a window of 20 frames [1s]), (D) shows coordinates that have been smoothed (window=20) and then resampled at 20Hz (1 frame per s), and (E) shows the final relative turning angles (in degrees; blue text) calculated from smoothed and resampled coordinates in (D).

4.2.3.2.3. Activity metrics

Run length encoding (RLE) was used to temporally smooth velocity in order to derive activity metrics, allowing identification of stationary and mobile phases. RLE is a form of data compression which identifies patterns in consecutive sequences (runs) of data. For example, a binary sequence of characters, "AAAAABBABBB", may be run length encoded as, "5A, 2B, 1A, 3B". Here, information on mobility was calculated by run length encoding smoothed and thresholded velocity data to determine whether movement speeds were above or below a subjectively-defined threshold velocity (1mm/s) (Figure 4.7). Owing to noise between frames in detected velocities, a sliding window of 3s was used to classify movement transitions (see Figure 4.8); i.e. when velocity was above the defined threshold (>=1mm/s) threshold for a period of >=3s, the insect entered a movement phase, and when its speed fell below 1mm/s for a period of >3s, the insect entered a stationary phase. The absolute number of phases transitions and mean duration of mobile and stationary phases was calculated for each insect.

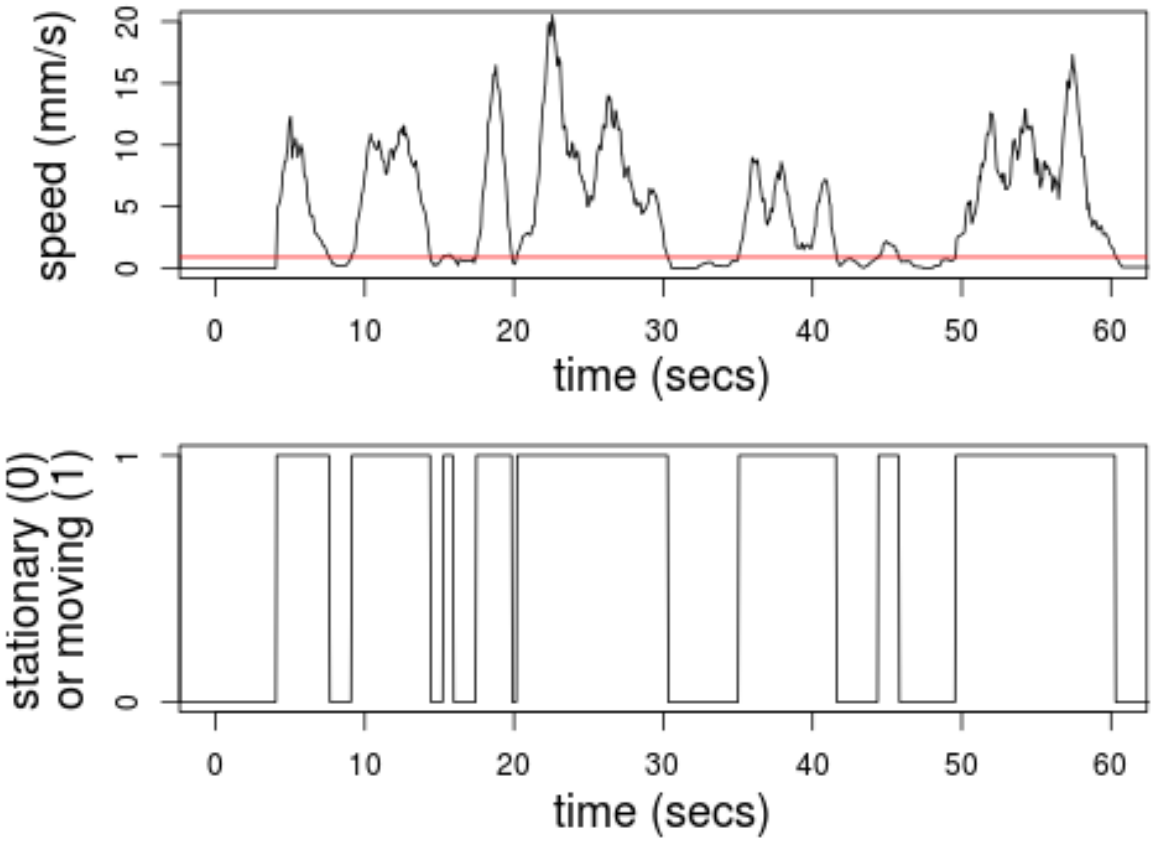


Figure 4.7. Determination of insect movement (mobile vs. stationary) using run length encoding. The top plot shows a 60 s sample of smoothed insect movement speed (mm/s), where the red line represents the user-defined speed threshold; here, 1mm/s. The bottom plot shows the same 60 s sample but speed has been run length encoded into a binary format, whereby the insect is classified as mobile (1) when moving faster than the speed threshold and stationary (0) when moving slower.

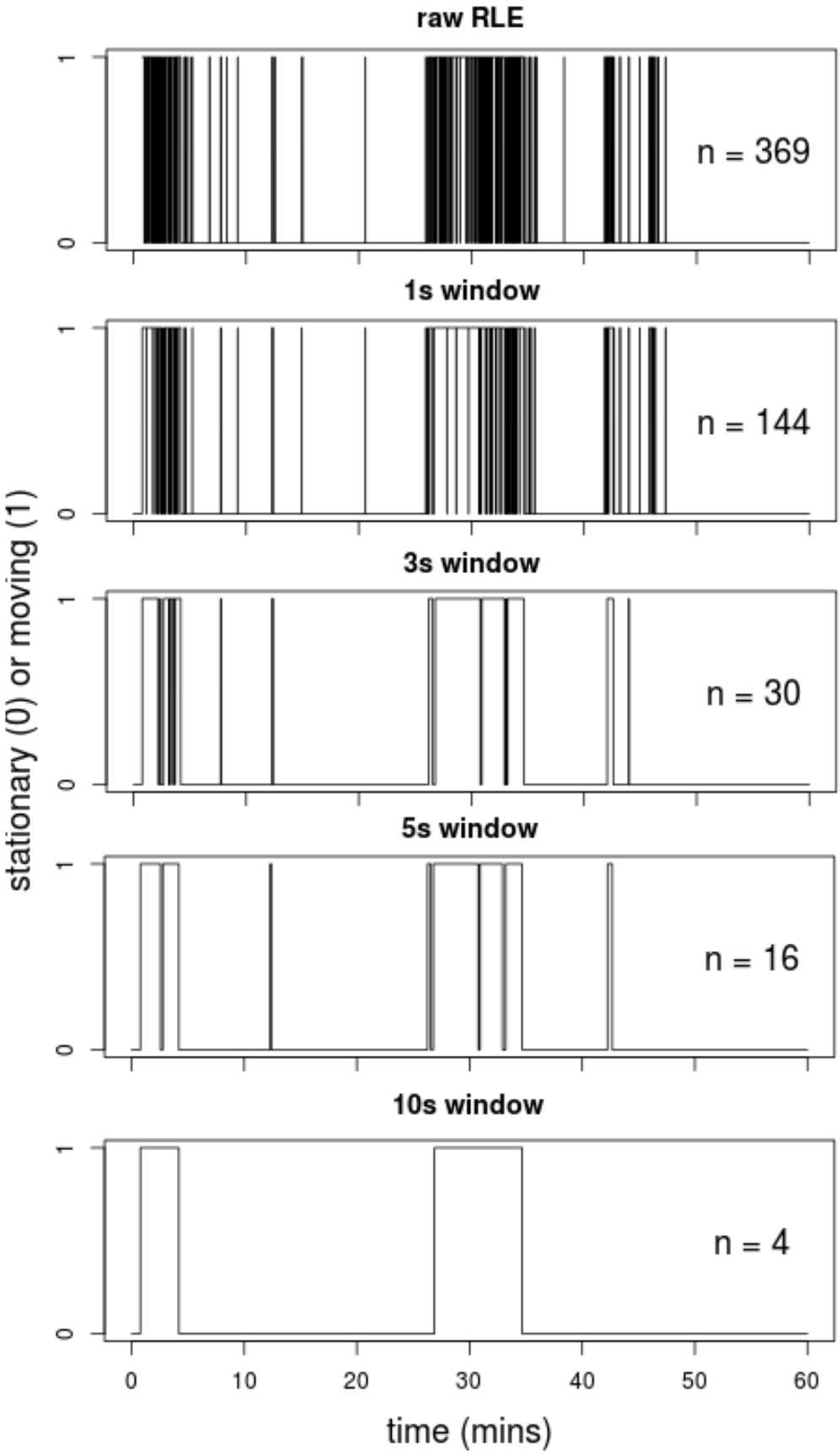


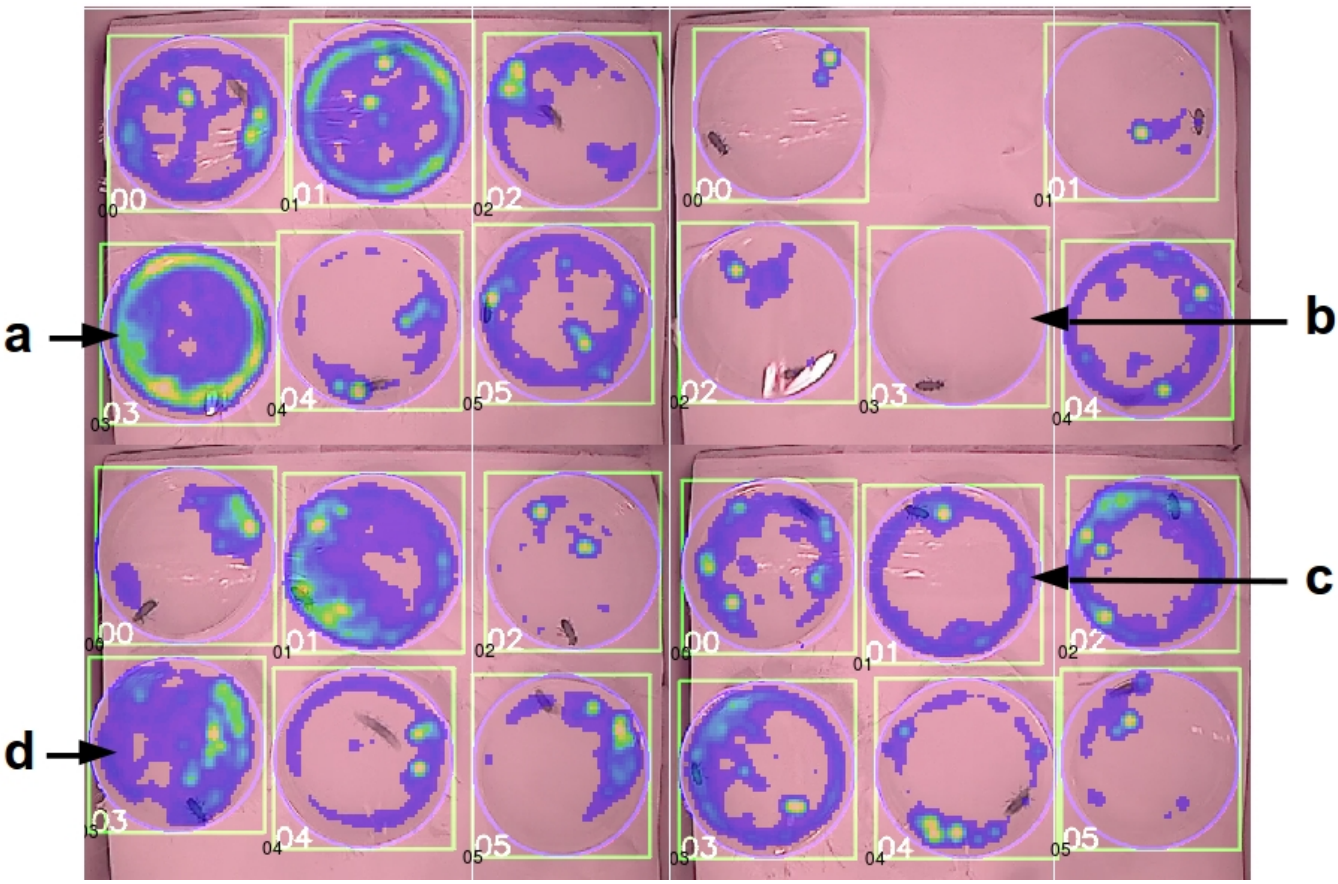
Figure 4.8. A sample of different sliding window sizes for smoothing run length encoded movement data, from raw data (1 frame interval) to 10s (200 frame interval). n indicates the number of defined transitions between mobile and stationary phases, which can be seen become eroded as the size of the sliding window increases. A sliding window of 3s was used in the final analysis as this was found to preserve biologically meaningful pausing events whilst still reducing noise from oversampling.

4.2.3.2.4. Spatial analysis

'Heat maps' can be outputted to provide a fast and intuitive overview of an insect's location during the course of the experiment (Figure 4.9). Two metrics, thigmotaxis and exploration, were also developed in order to quantify the amount of time spent in certain zones of the arena. Thigmotaxis (or centrophobism) is the tendency of many animals to display a central zone avoidance in open-field experiments, with individuals moving in the peripheral areas where they can physically touch the walls of the arena, and avoiding central areas, which are presumed more threatening as they leave the insect more vulnerable to predation (e.g. Gotz & Biesinger, 1985; Colomb *et al.*, 2012). Exploratory (or roving) behaviour is often defined in vertebrates alongside such metrics as shyness/boldness, aggression and neophobia (Dingemanse *et al.*, 2002), but can be simply defined as the propensity of an individual to move around their environment.

In order to normalise the spatial locations for each arena, a minimum enclosing circle was fitted to each arena to determine its exact boundaries, before tracked Cartesian coordinates (x,y) for each arena were converted to polar coordinates (r,θ). To quantify thigmotaxis, each defined minimum enclosing circle was divided into two zones of equal area: an inner disc and an outer ring (Figure 4.10), and each r,θ-coordinate was defined as belonging in the inner or outer zone based upon its distance from the centre of the arena.

To quantify exploration, each circular arena was divided into a network of 96 cells of equal area by a series of concentric circles and line segments (Figure 4.11). The grid cell location of each r,θ-coordinate in a trajectory path is determined, and a measurement of proportion of territories visited (number of unique cells visited / total number of cells) is calculated for each insect over the course of observation.


a
b
c
d

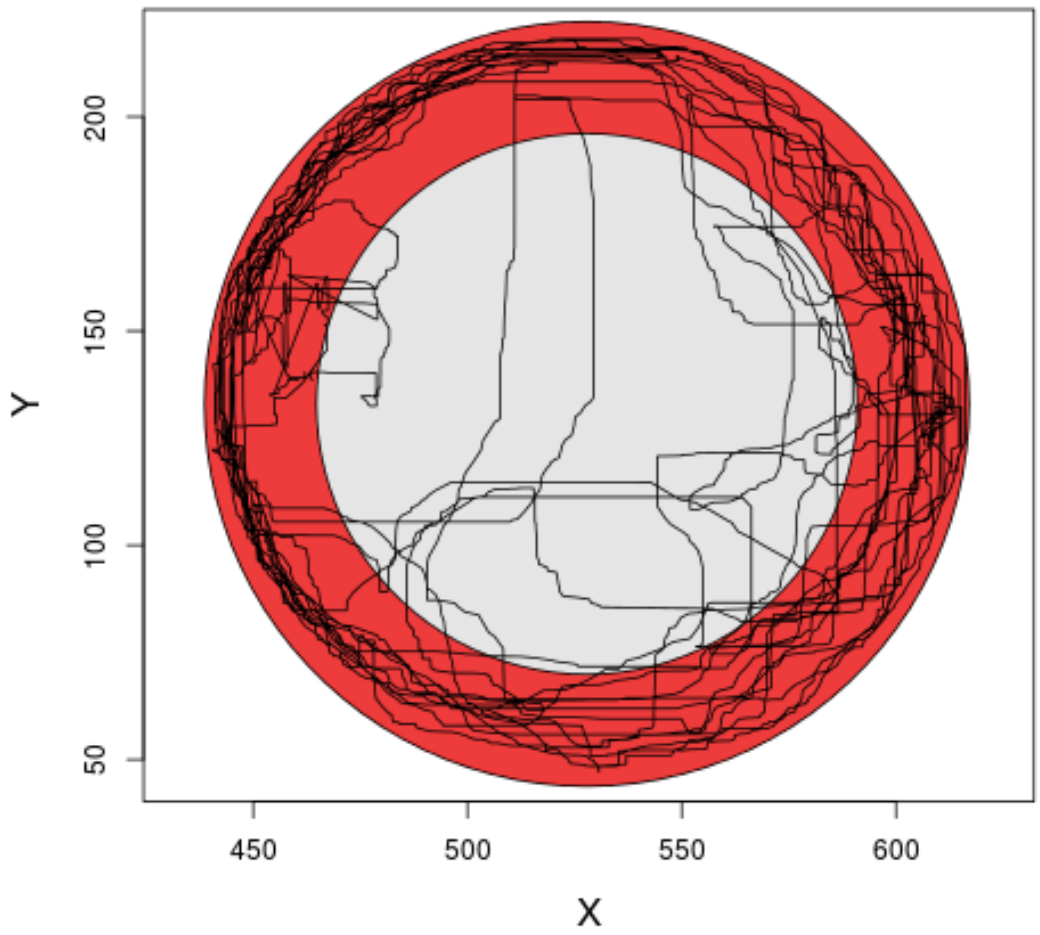


Figure 4.9. Sample 'heatmaps' showing the frequency insect locations over the course of a 60 minute recording, with yellow areas being visited frequently and blue areas infrequently. Each background image is a frame taken from the raw video analysed by the tracker. (a) shows an insect which displayed a high level of exploration as well as a relatively high degree of thigmotaxis. (b) shows an insect which did not display sufficient movement during the recording to be tracked (<500 frames [25s] in which motion was detected). (c) shows an insect with a high degree of thigmotaxis but a relatively low level of exploration. (d) shows an insect with a high level of exploration, although with movement being concentrated primarily on the right hand side of the arena.**Figure 4.10.** Visualisation of the thigmotaxis metric. A minimum enclosing circle (outer boundary of red ring) is fitted to each circular arena, which is then divided into two zones of equal area, an inner zone (shown in red) and an outer zone (shown in grey). The radius of the inner circle, r_{inner}, is $\sqrt{2}$ times smaller than the radius of the outer enclosing circle, r_{outer}. Each X,Y-coordinate in a trajectory path (black line) is designated as being in the inner or outer zone based upon its Pythagorean distance from the midpoint of the arena. I.e. a coordinate (x_t,y_t) is classified as being in the outer zone if:

$$\sqrt{(x_{mid} - x_t)^2 + (y_{mid} - y_t)^2} > \frac{r_{outer}}{\sqrt{2}}$$

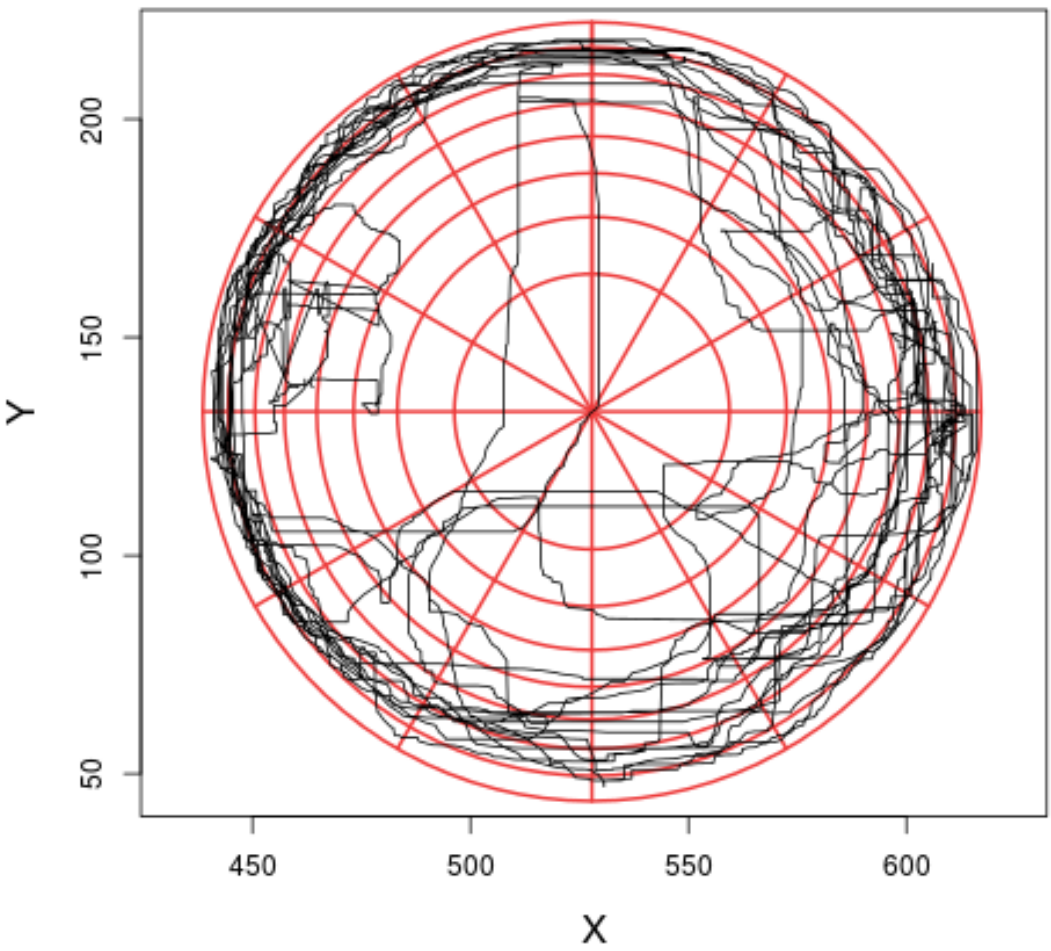


Figure 4.11. Visualisation of the exploration metric. A minimum enclosing circle is fitted to each circular arena, which is then divided into a network of grid cells of equal area by concentric circles and line segments. Here, 8 concentric circles and 12 line segments (shown above in red) compose a grid of 96 cells. Given a number of circles i:n, the radius of circle i, r_i, is given by the formula:

$$r_i = r_n \cdot \sqrt{\frac{i}{n}}$$

where r_n is the radius of the outermost circle enclosing the arena. Given a number of line segments, j:n, the angle of segment j, θ_j, is given by the formula:

$$\theta_j = \frac{2\pi j}{n}$$

The cell location of each coordinate in a smoothed trajectory path (shown above in black) is then determined, and the total number of unique cells visited by the insect used as a measure of exploration.

4.2.4. Testing tracking accuracy

Implemented smoothing and thresholding procedures acted to eliminate the majority of false artifacts from raw trajectories. To quantify the remaining level of unreliability in the system and measure its accuracy, the tracker was compared to human users. Videos were manually authenticated by producing a series of images at random points during the analysis, and asking human users to estimate the x,y position of objects in the image using a simple interactive C++ application (Figure 4.13). For the same frames, human-estimated object coordinates were compared with raw object coordinates detected by the software, and with processed object coordinates returned after movement thresholding and smoothing in order to gain a correlative measure of accuracy (Figures 4.14 & 4.15).

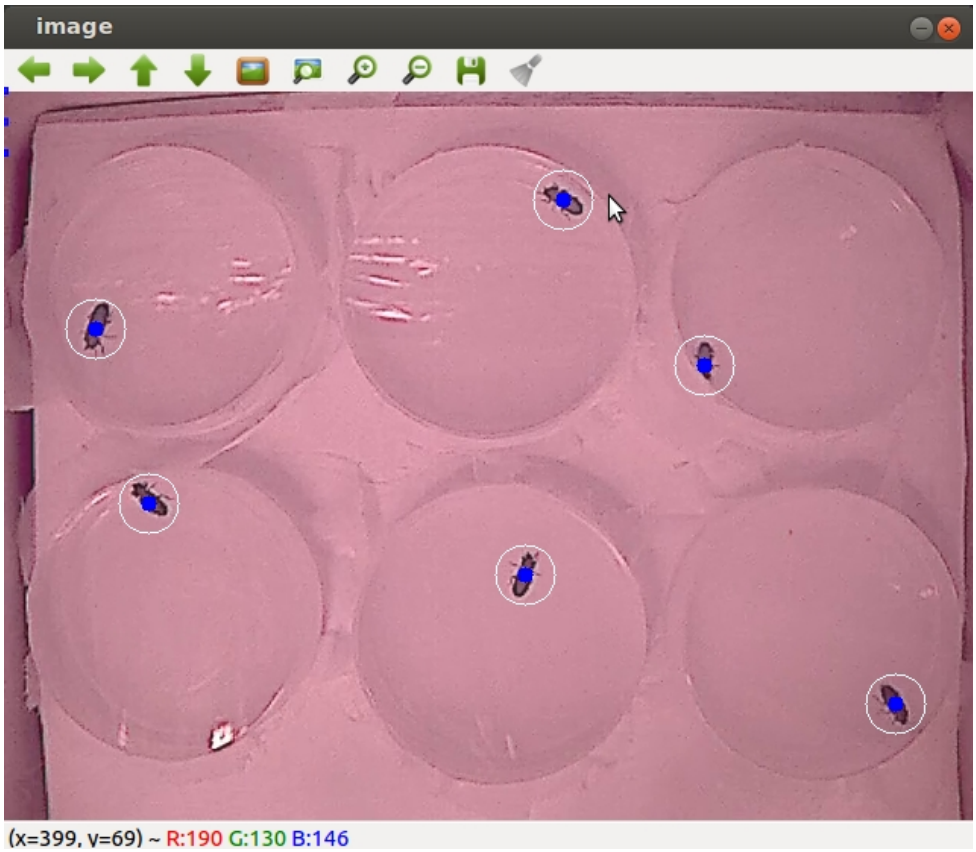


Figure 4.13. A sample of the human scoring test application used to examine automated tracking accuracy. Raw frames were randomly extracted from a video and opened in a simple C++ application. Users then clicked the point at which they deemed the centre of mass of insect to be (shown as blue dots with white circle). User-defined x,y-coordinates were then compared with coordinates defined by the tracking software for the same frames (see Figure 4.14 & 4.15).

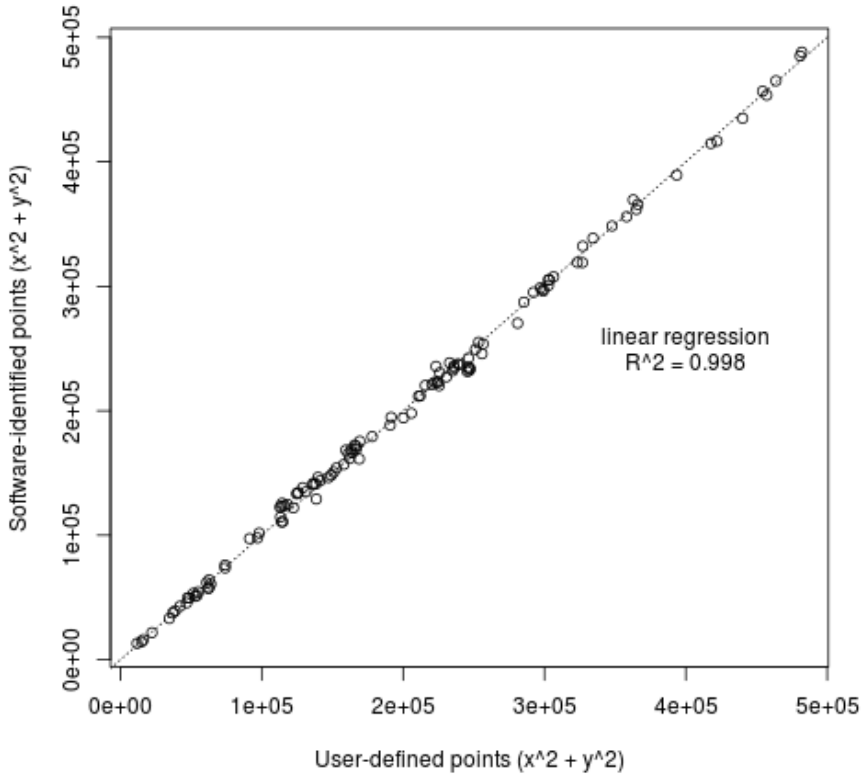


Figure 4.14. Correlation between user-defined X,Y-coordinates of insect location and coordinates outputted by the automated tracker.

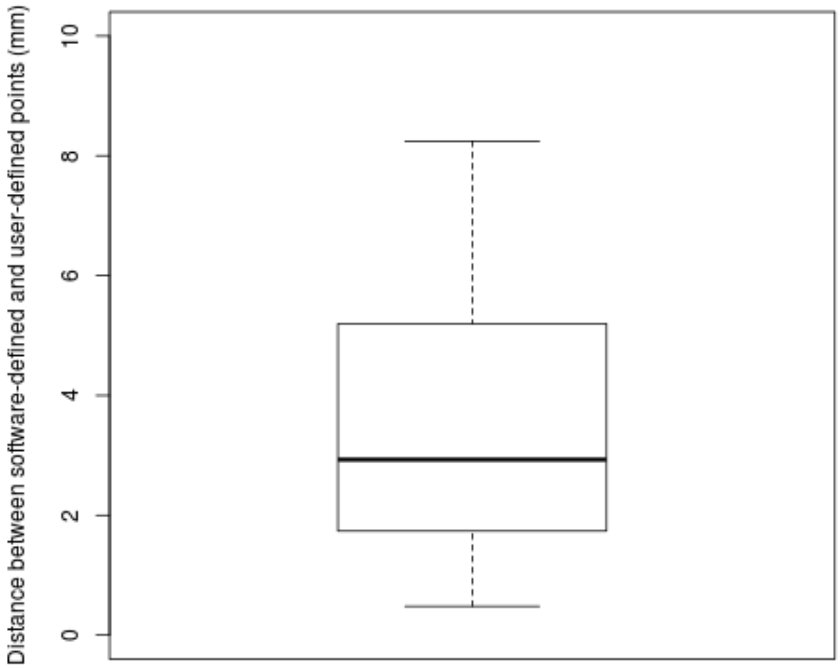


Figure 4.15. Boxplot showing Pythagorian distance (in mm) between tracked X,Y-coordinates of insects outputted by the software and coordinates defined by users. Boxplots show the median and interquartile range (IQR), and whiskers represent 1.5*IQR. For comparison, the mean body length of adult *T. molitor* is ~18mm.

4.3. Summary

The developed tracker, UbiTrail, is capable of recording the trajectory of up to 24 insects simultaneously with relatively high spatial (up to 0.5mm / pixel) and temporal resolution (up to 30Hz). A range of biologically meaningful behavioural metrics have been defined in order to produce quantitative data on insect locomotion, including information on velocity, turning angles and location, as well as several more specific behaviours such as turnarounds, thigmotaxis and exploration.

The tracker is: (i) versatile, having been tested on a range of insect species, including *Tenebrio molitor, Drosophila* spp. (both adults and larvae), ants (*Lasius niger*), aphids (*Acyrthosiphon pisum*) and bean weevils (*Acanthoscelides obtectus*), (ii) robust, working with relatively low resolution video images (640 x 480 px), imperfect and variable lighting conditions and moderate levels of visual occlusion and background variation, and (iii) accurate, as tracked coordinates of *T. molitor* were found to closely match (<10% of the body length of the insect away from) coordinates determined by human users. Furthermore, the system is inexpensive as the software makes use of only open-source tools and does not require specialised apparatus for experimental set ups or recording. Finally, the inclusion of a graphical user interface (GUI) for video analysis and R package, Rubitrail, for statistical analysis, aim to maximise accessibility to the user and allow for tracking analysis 'straight out of the box'.

The developed system is used in Chapter 5 to investigate locomotory sickness behaviours in *T. molitor*, and help determine whether behavioural cues of infection produced by conspecifics play a role in immune priming in naïve conspecifics.

In this chapter, I have:

- Developed a computer vision system to automatically track the movement of insects
- Developed a statistical package to define and extract key behavioural metrics from tracked coordinates and gain biologically meaningful quantitative data

CHAPTER FIVE:
SICKNESS BEHAVIOUR IN *Tenebrio molitor*

5.1. Introduction

The physiological immune system is an important defence against parasites, but can be considered as the final line of defence in a series of barriers which include behaviour, morphology and life-history (Combes 2001; Rigby *et al.*, 2002). Physiological responses are often costly (Barnes & Siva-Jothy 2000; Siva-Jothy *et al.*, 2005; Wilson & Cotter 2008) and can be thought of as an 'emergency service' (Siva-Jothy *et al.*, 2005). Behavioural defences, on the other hand, are generally less costly to implement (Siva-Jothy *et al.*, 2005; Schulenburg & Ewbank 2007), are faster to enact and have a greater degree of plasticity (West-Eberhard, 1989), and can be as effective as physiological immune responses at preserving host fitness in the face of parasitism (Moore, 2002; Schmid-Hempel *et al.*, 2003).

Hosts can use a range of behavioural responses to reduce their risk of contracting an infection in order to confer qualitative resistance against pathogenesis, i.e. complete avoidance of infection (de Roode & Lefevre, 2012). However, hosts will not always manage to avoid contact with parasites. Once a pathogen successfully invades a host, the host can employ a further suite of distinct behavioural responses which limit the negative fitness consequences of infection and provide quantitative resistance, which reduces parasite load (de Roode & Lefevre, 2012). Behavioural changes induced during infection are collectively termed 'sickness behaviours' (Hart, 1988; Moore, 2002), and complement or even replace the action of costly physiological immune effectors. Generally, sickness behaviours induce a state of energy conservation or otherwise allow quantitative resistance of infection through resistance mechanisms which either limit pathogen growth, or through tolerance mechanisms which limit damage caused during infection (pathological damage, immunopathological damage, and costs of resource expenditure) (Schneider & Ayres, 2008; de Roode & Lefevre, 2012). Sickness behaviours have been documented in a range of vertebrate (Penn & Potts, 1995; Owen-Ashley & Wingfield, 2012) and invertebrate hosts (Adamo, 2006), and the most common responses include reduced activity, lower levels of exploration, reduced food and water intake, decreased social contacts, fever and decreased reproductive behaviour (Johnson, 2002; Adamo, 2006; Dantzer *et al.*, 2009).

Some sickness behaviours may be a maladaptive consequence of parasitism caused by pathogenic damage, self-inflicted immunopathological damage or resource depletion (Johnson, 2002), and some may even be the direct result of parasitic manipulation which aims to increase the fitness of the parasite by prolonging infection or facilitating transmission to other susceptible hosts (Poulin, 1995; Adamo, 2012). However, many sickness behaviours have been suggested to constitute adaptive, host-induced changes which help augment host resistance, facilitate recovery or hinder transmission between hosts (Moore, 2002). Behavioural sickness has been described as a 'motivational state' which allows sickened hosts to re-prioritise their resource investment (Hart, 1988; Johnson, 2002; Dantzer, 2009).

However, not all responses will be necessarily based upon immune defence, as hosts may also minimise the fitness costs associated with infection and immune activation by investing in other beneficial life-history traits, particularly reproduction. In some cases, if the chance of recovery is sufficiently low or the the risk of reinfection sufficiently high, it may be more beneficial for the host to counter fitness losses by refocussing energy expenditure solely into reproduction through terminal investment (Clutton-Brock, 1984). These non-immunological responses have been referred to as life-history 'escape attempts', which form a tolerance strategy that allows the host to ameliorate the costs of infection (van Baalen, 1998; Minchella, 1985). There are examples of both increased and decreased locomotory activity following infection in different insect species (reviewed in de Roode & Lefevre, 2012). One study found that *Drosophila* species infected with *Wolbachia* exhibited either decreased or increased locomotor activity depending on the host species and the bacterial strain (Peng *et al.* 2008), suggesting that sickness behaviours may have a high degree of specificity and plasticity.

Sensing pathogens in the environment has most commonly been attributed to chemosensory cues detected via the olfactory and gustatory systems, and the detection of immune status in conspecifics has been suggested to utilise similar mechanisms (Konrad *et al.*, 2012). However, but little is known about the potential role of visual and/or behavioural cues in this process, although behavioural cues are thought to mediate avoidance of infected potential mates in rats (Penn & Potts, 1995; Kavaliers & Colwell, 1995) and attraction to infected same-sex conspecifics in house finches (Hawley *et al.*, 2007), and visual cues of infection (e.g. cuticular spore attachment) may be used to stimulate hygienic grooming behaviour in ants (Marikovsky, 1962). Whilst immune-induced chemosensory changes are partially understood in *Tenebrio molitor* (Nielsen & Holman, 2011), knowledge of locomotory and behavioural changes during immune

challenge is lacking. Sickness behaviours exhibited by infected individuals may interact with behavioural resistance mechanisms induced by non-infected individuals; for example, if hosts greatly reduce their range of movement, social contacts and/or sexual activity during infection, we may not expect naïve hosts to actively invest in physiological or behavioural prophylaxes towards a sick neighbour, as protection would already be passively afforded.

This study investigates the potential for sickness behaviours during artificial immune stimulation in the mealworm beetle, *Tenebrio molitor*. The use of lipolysaccharide (LPS), a non-pathogenic immunogen, to insult the host immune system eliminates the possibility of parasitic manipulation of host behaviour. By using the tracking software developed in Chapter 4 to generate quantitative behavioural data, I hope to identify sickness behaviours in immune-stimulated beetles. I examine behaviour in both males and females to search for potential gender differences, and compare host responses to both primary and secondary immune challenge in order to investigate the role of immune priming upon sickness behaviour. This approach can establish whether behavioural cues of infection play a role in socially-informed prophylactic immune strategies, such as gregarious immunisation (see Chapters 2 & 3) or behavioural avoidance (see Appendix 4).

In this chapter, I examined:

- The effect of a non-pathogenic immune challenge upon the expression of locomotory sickness behaviours
- The effect of gender upon behavioural responses to immune challenge
- Differences in locomotion expressed immediately (1 h) after immune challenge and those expressed after a recovery period (7 days after challenge)

5.2. Methods

5.2.1. Insect culturing

Final-instar larvae of *Tenebrio molitor* were purchased from a commercial supplier (Live Foods UK) and maintained in an insectary at 26±2 °C under a 12/12hr light/dark cycle. Larvae were kept at densities of ~800 larvae per 30×15×10 cm box, and were provided with *ad libitum* access to Progrub (Livefoods Direct Ltd) and supplemented with freshly cut potato once per week. Pupae were collected between 1–3 days after pupation, and were sexed and weighed before being maintained in isolation in grid box containers. Both male and female imagoes were provided with Progrub and a ~50mg potato supplement upon adult eclosion, and treatments being performed 8-10 days after eclosion.

5.2.2. Insect treatments

Preliminary trials showed the level of individual variation in all measured behaviours to be high, with levels of variation within sexes and treatment (i.e. naïve vs. immune-challenged) greater than the levels of variation between them (data not shown). A paired sample approach was therefore adopted to compare behaviour during immune challenge with baseline levels of unchallenged behaviour in the same individuals. Beetles were observed for a 1 h period on two occasions, one week apart (i.e. day 0 & day 7). They were randomly assigned to one of three treatment groups: 'infection', 'recovery' or 'control' (**see Table 5.1**). Individuals in the 'infection' group were initially immunologically naive and challenged with LPS one week later, whilst those in the 'recovery' group were initially immune challenged and observed in the absence of any further immune challenge the next week. Those in the 'control' group remained unchallenged in both observation periods in order to control for any potential effects of habituation (e.g. Sokolowski et al., 2012).

Before each observation, individuals assigned to be immune-challenged were anaesthetised on ice for 5 minutes before injection with 5µL of 0.5mg/mL lipopolysaccharide (LPS; Sigma L2630), suspended in sterile, ice-cold phosphate buffered saline (PBS). Injections were performed by inserting a sterile glass microcapillary, which had been pulled to a fine point using an electrode puller (Narishige PC-10), through the pleural membrane between the seventh and eight abdominal sternites, and using a syringe barrel to pneumatically introduce the inoculate to the haemocoel. Individuals with no immune-challenge scheduled were also anaesthetised on ice for 5 minutes but received no immune insult. Immediately after treatment, beetles were placed into Petri dishes (90mm diameter) with filter paper on the bottom surface. Petri dishes were then sealed with masking tape to prevent individuals from escaping and limit

potential communication of olfactory or visual cues between arenas. Petri dishes were then placed in the observation chamber (Figure 5.1), and beetles were given 5 minutes to acclimatise before their movement was video recorded for 60 minutes. After recording, beetles were removed and housed individually in plastic grid boxes with *ad libitum* access to Progrub and a potato supplement provided once per week.

5.2.3. Experimental apparatus

Video capture took place in an upright, temperature-controlled incubator (SLS Qualicool) which was maintained at 26°C. Petri dishes were placed atop a sheet of white paper in order to provide good background contrast for tracking. A USB webcam was held in place above the observation area by a standard retort clamp stand, and connected to an external laptop (Intel Core 2 Duo 2.00 Ghz processor, 1 GB RAM, 120 GB HDD, Ubuntu 12.04). Light was provided by an anglepoise lamp fitted with a red fluorescent light bulb; Tenebrionids are highly photosensitive, but, like most insects, lack the photoreceptors to detect red light (Crozier, 1924). Foil was also affixed to the internal walls of the incubator to help diffuse light more evenly and reduce glare on the Petri dish lids. The experimental setup is shown in Figure 5.1. Videos were recorded using the free and open-source media player VLC (https://www.videolan.org/vlc). A calibration image of a chessboard was taken before each new trial in order to later correct for lens distortion introduced by the webcam (see Chapter 4; Figure 4.3).

Table 5.1. List of treatments used in each observation period. A paired sample design was adopted using the same individuals over two observation periods to test for the presence of sickness behaviour ('infection') or recovery behaviour ('recovery'). A treatment was also included in which individuals remained unchallenged in both observation periods in order to control for any potential effects of habituation ('control').

Group	Day 0	Day 7
Infection	- no treatment -	**LPS**
Recovery	**LPS**	- no treatment -
Control	- no treatment -	- no treatment -

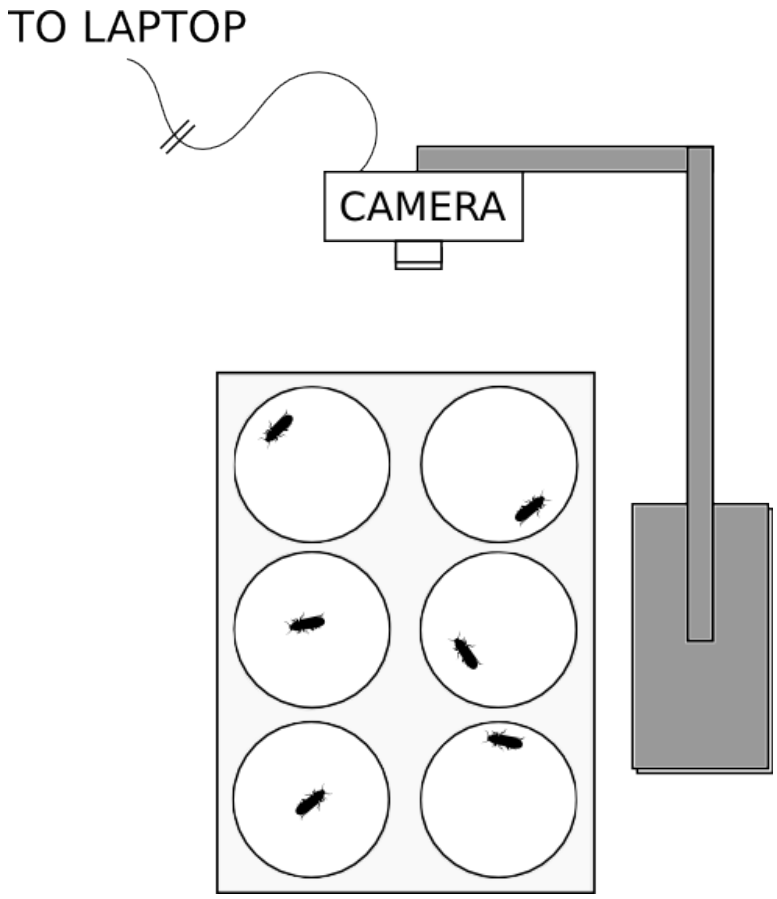


Figure 5.1. Experimental apparatus used to record beetle behaviour. Beetles were observed in a homogeneously illuminated incubator inside Petri dishes lined with filter paper discs, and filmed from above with a USB camera connected to a nearby laptop.

5.2.4. Video tracking and calculation of behavioural metrics

Videos were analysed with UbiTrail (see Chapter 4), and the raw x,y-coordinates calculated for each beetle were processed in R (v3.1.2; R Development Core Team, 2014) using the co-developed package, RUbiTrail (see Chapter 4). A number of behavioural metrics were extracted from the trajectory data of each insect, based on movement (speed, acceleration, total distance travelled, number of pauses, pause duration, walk duration), orientation (turning angle, meander, turnarounds [~180° turns]) and positioning (thigmotaxis [~centrophobism], roving behaviour [~exploration]). The definitions and biological significance of these metrics are discussed in detail in Chapter 4.

5.2.5. Statistical analysis

For each individual, differences in medians between the two observation periods (i.e. Day 7 median – Day 0 median) were calculated for each behavioural metric. Medians were

chosen to describe behavioural metrics as means exhibited clearly non-normal distributions. A multivariate analysis of variance (MANOVA) was built to test for the main effects of sex (male, female) and treatment (infection, recovery, control), as well as a sex by treatment interaction, upon all behavioural metrics combined. Post-hoc one-sample t-tests were then conducted on each behavioural metric independently to determine the directionality and significance of individual behavioural changes. Finally, a principle components analysis (PCA) was performed on the raw differences data using the princomp() function in R.

5.3. Results

5.3.1. MANOVA

Analysis of data for both sexes combined showed no significant effects of gender or immune treatment upon overall behaviour (all metrics combined; Table 5.2). However, individual ANOVAs (on each metric individually) revealed a significant effect of gender upon turning angle and total time spent stationary, a significant effect of treatment upon pause duration, and a significant gender by treatment interaction effect upon acceleration (Table 5.3).

5.3.2. Post-hoc t-tests

Post-hoc t-tests found no significant behavioural changes in either males or females in the 'infection' group, which remained immunologically naïve at day 0 and were challenged with LPS on day 7 (Table 5.4; Figure 5.2). However, behavioural differences were apparent in the 'recovery' group, in which individuals were challenged with LPS on day 0 but were unchallenged on day 7 (Table 5.4; Figure 5.2). Recovering males showed significant increases in metrics of movement (larger distance travelled, increased acceleration) and activity (increased walk duration, reduced time spent stationary). These males also exhibited significant changes in trajectory (increased turning angle, more turnarounds), although these differences may have been an artifact of cohabitation as control males who remained unchallenged in both observation periods showed a similar increase in turning angle and number of turnarounds, as well as a decrease in their overall speed (Table 5.4). Recovering females, on the other hand, exhibited few changes in behaviour. Only an increase in the number of pauses was apparent in the second observation period, although this difference was mirrored in the control group, suggesting it may also be a consequence of habituation rather than immune status (Table 5.4). Immune status appeared to have no effect upon positioning in the arena for either gender, as no effect of treatment was found upon thigmotaxis or exploration.

5.3.3. Behavioural correlations

Before conducting the principal components analysis (PCA), behavioural metrics that were highly correlated with one another were discarded; median values for speed, meander, thigmotaxis, exploration, number of pauses, duration of pauses and duration of walks were thus retained. The first three principal components (PCs) explained 31.5%, 28.5% and 13.0% of the total variance between individuals, respectively. PC loadings showed that speed and walk duration (PC1), number of pauses (PC2), and exploration

(PC3) are the most influential factors in these components (Table 5.5). However, the PCA failed to demonstrate clear differences between gender or between treatments (Figure 5.3). Combined with the MANOVA (Tables 5.2 & 5.3), these results suggest that immune stimulation with LPS does not greatly affect overall patterns of locomotion in either males or females, but may nevertheless cause differences in more subtle behavioural features, such as speed, turning or activity levels, and that these responses may also vary between males and females.

Table 5.2. Results of MANOVA conducted on differences combined for both genders and all treatment groups. Raw differences in medians were calculated for each individual between the first replicate (Day 0) and the second (Day 7).

Terms	d.f.	Pillai	F	num d.f.	den d.f.	P-value
sex	1	0.195	1.153	12	57	0.338
treat	2	0.379	1.129	24	116	0.324
sex x treatment	2	0.314	0.900	24	116	0.601
Residuals	68					

Table 5.3. Individual ANOVA results for each behavioural metric included in MANOVA. Significant effects ($p<0.05$) are highlighted in bold.

	Acceleration					Distance travelled					Exploration				
Term	df	Sum Sq	Mean Sq	F	P value	df	Sum Sq	Mean Sq	F	P value	df	Sum Sq	Mean Sq	F	P value
sex	1	8.57E-002	8.57E-002	0.030	0.862	1	3.32E+008	3.32E+008	1.919	0.170	1	2.76E-002	2.76E-002	1.418	0.238
treat	2	5.18E+000	2.59E+000	0.917	0.404	2	2.18E+008	1.09E+008	0.630	0.536	2	4.92E-002	2.46E-002	1.262	0.290
sex x treatment	**2**	**2.45E+001**	**1.22E+001**	**4.332**	**0.017**	2	4.74E+008	2.37E+008	1.370	0.261	2	3.38E-002	1.69E-002	0.866	0.425
Residuals	68	1.92E+002	2.83E+000			68	1.18E+010	1.73E+008			68	1.33E+000	1.95E-002		

	Meander					Number of pauses					Speed				
Term	df	Sum Sq	Mean Sq	F	P value	df	Sum Sq	Mean Sq	F	P value	df	Sum Sq	Mean Sq	F	P value
sex	1	5.53E+000	5.53E+000	0.858	0.358	1	2.33E+000	2.33E+000	0.019	0.890	1	1.60E+000	1.60E+000	0.089	0.766
treat	2	1.21E+001	6.05E+000	0.939	0.396	2	2.63E+002	1.31E+002	1.086	0.343	2	2.11E+001	1.05E+001	0.591	0.557
sex x treatment	2	1.51E+000	7.57E-001	0.117	0.889	2	1.61E+002	8.07E+001	0.667	0.516	2	2.02E+001	1.01E+001	0.567	0.570
Residuals	68	4.38E+002	6.44E+000			68	8.23E+003	1.21E+002			68	1.21E+003	1.79E+001		

	Pause duration					Thigmotaxis					Total time stationary				
Term	df	Sum Sq	Mean Sq	F	P value	df	Sum Sq	Mean Sq	F	P value	df	Sum Sq	Mean Sq	F	P value
sex	1	1.37E+011	1.37E+011	1.610	0.209	1	1.38E-001	1.38E-001	0.764	0.385	**1**	**4.11E-001**	**4.11E-001**	**4.099**	**0.047**
treat	**2**	**7.40E+011**	**3.70E+011**	**4.339**	**0.017**	2	1.03E-003	5.13E-004	0.003	0.997	2	2.45E-001	1.22E-001	1.221	0.301
sex x treatment	2	1.52E+010	7.59E+009	0.089	0.915	2	4.24E-002	2.12E-002	0.118	0.889	2	4.20E-001	2.10E-001	2.093	0.131
Residuals	68	5.80E+012	8.53E+010			68	1.22E+001	1.80E-001	NA	NA	68	6.82E+000	1.00E-001		

	Turnarounds					Turning angle					Walk duration				
Term	df	Sum Sq	Mean Sq	F	P value	df	Sum Sq	Mean Sq	F	P value	df	Sum Sq	Mean Sq	F	P value
sex	1	7.06E+002	7.06E+002	1.916	0.171	**1**	**3.66E+002**	**3.66E+002**	**5.148**	**0.026**	1	4.26E+010	4.26E+010	2.125	0.150
treat	2	1.59E+003	7.97E+002	2.162	0.123	2	3.85E+002	1.92E+002	2.704	0.074	2	7.33E+010	3.66E+010	1.826	0.169
sex x treatment	2	7.67E+002	3.83E+002	1.041	0.359	2	2.35E+002	1.17E+002	1.648	0.200	2	3.43E+010	1.71E+010	0.855	0.430
Residuals	68	2.51E+004	3.68E+002			68	4.84E+003	7.12E+001			68	1.36E+012	2.01E+010		

Table 5.4. Summary of post-hoc t-tests conducted for each behavioural metric within each sex and within each treatment.

MALES

	Infection					Recovery					Control				
Metric	% change	S.E.	t	df	p	% change	S.E.	t	df	p	% change	S.E.	t	df	p
Acceleration	-50.96	55.62	0.785	12	0.447	**+526.69**	**181.22**	**-2.748**	**12**	**0.018**	-45.24	26.69	1.666	12	0.122
Distance travelled	-3.80	34.09	0.042	12	0.968	**+114.50**	**34.57**	**-3.022**	**12**	**0.011**	+12.07	18.28	-0.730	12	0.479
Exploration	-5.67	11.09	0.406	12	0.692	+19.85	10.91	-1.615	12	0.132	+1.39	3.30	-0.918	12	0.377
Meander	+3.49	17.45	-0.121	12	0.905	+52.88	27.19	-1.557	12	0.145	+48.39	23.74	-2.148	12	0.053
Number of pauses	+15.24	20.61	-0.867	12	0.403	+12.79	20.29	-0.426	12	0.678	+47.85	25.22	-1.723	12	0.111
Pause duration	+267.98	275.56	-0.925	12	0.373	-86.70	60.59	1.409	12	0.184	-62.39	27.69	1.947	12	0.075
Speed	-16.40	19.02	0.801	12	0.439	+8.49	16.46	-0.201	12	0.844	**-26.48**	**11.84**	**2.245**	**12**	**0.044**
Thigmotaxis	+9.55	19.54	-0.226	12	0.825	-0.71	16.76	0.244	12	0.811	+19.07	17.88	-1.060	12	0.310
Total time stationary	-17.41	12.84	1.441	12	0.175	**-48.74**	**9.70**	**4.568**	**12**	**<0.001**	-17.21	9.62	1.792	12	0.098
Turnarounds	+13.98	22.08	-0.583	12	0.571	**+139.55**	**34.81**	**-3.602**	**12**	**0.004**	**+71.43**	**33.13**	**-2.391**	**12**	**0.034**
Turning angle	+59.15	109.34	-0.540	12	0.599	**+386.35**	**19.83**	**-4.663**	**12**	**<0.001**	**+376.78**	**147.03**	**-2.606**	**12**	**0.023**
Walk duration	+158.74	136.33	-1.188	12	0.258	**+191.65**	**74.99**	**-2.537**	**12**	**0.026**	-2.58	41.72	-0.219	12	0.830

FEMALES

	Infection					Recovery					Control				
Metric	% change	S.E.	t	df	p	% change	S.E.	t	df	p	% change	S.E.	t	df	p
Acceleration	+88.63	55.82	-1.588	13	0.136	+1.76	52.13	-0.034	16	0.974	-17.50	33.38	1.168	10	0.270
Distance travelled	+10.32	21.19	-0.487	13	0.634	+20.27	46.10	-0.440	16	0.666	-14.13	24.09	0.079	10	0.938
Exploration	-3.68	9.85	0.374	13	0.715	-0.43	6.01	0.071	16	0.944	-0.94	8.53	0.049	10	0.962
Meander	+2.29	26.44	-0.087	13	0.932	+21.45	26.88	-0.798	16	0.436	+42.55	26.99	-1.452	10	0.177
Number of pauses	+11.25	26.86	-0.419	13	0.682	**+50.00**	**17.52**	**-2.855**	**16**	**0.011**	**+39.57**	**17.60**	**-2.563**	**10**	**0.028**
Pause duration	+187.76	138.64	-1.354	13	0.199	-92.13	50.91	1.810	16	0.089	-16.58	25.69	0.499	10	0.629
Speed	-5.68	19.80	0.287	13	0.779	-5.27	26.16	0.201	16	0.843	-29.26	26.48	0.555	10	0.591
Thigmotaxis	+27.62	23.19	-1.191	13	0.255	+24.19	15.92	-1.520	16	0.148	+12.32	16.18	-0.724	10	0.486
Total time stationary	-17.94	12.97	1.383	13	0.190	+7.66	19.54	-0.392	16	0.700	-7.21	13.92	0.618	10	0.550
Turnarounds	+24.01	39.20	-0.612	13	0.551	+51.56	25.23	-2.044	16	0.058	+58.30	26.43	-2.032	10	0.070
Turning angle	-8.69	98.19	0.089	13	0.931	+18.48	57.81	-0.320	16	0.753	+118.74	91.59	-1.300	10	0.223
Walk duration	+118.03	115.34	-1.023	13	0.325	-64.37	31.14	2.067	16	0.055	-35.81	26.86	1.683	10	0.123

Differences in medians were calculated for each individual between the first replicate (Day 0) and the second (Day 7), and are expressed as a percentage of the population mean of the first replicate. One sample t-tests were conducted on each set of raw differences to determine significantly if they differed significantly from zero. Raw p-values are presented, with significant effects ($p<0.05$) highlighted in bold.

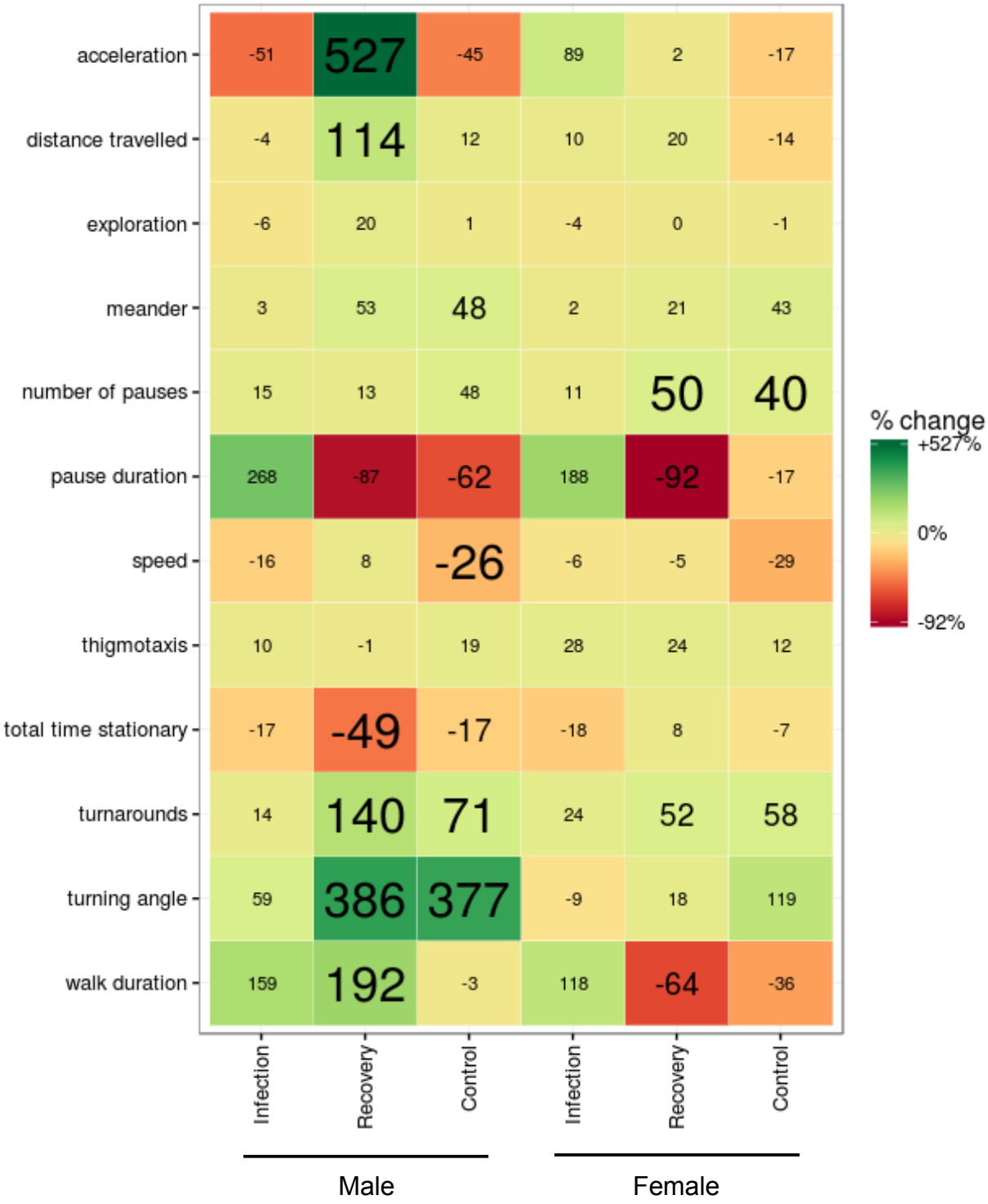


Figure 5.2. Heatmap demonstrating effects of immune status upon differences in measured behavioural metrics, split by gender. Red colours indicate a decrease in median values between observation periods and green colours indicate an increase. Values are the mean of raw differences in medians for each individual between replicates, expressed as a percentage of the first replicate median. For example, for males in the 'infection' group, median acceleration was 0.817 mm.s-2 on Day 0, and the mean of median differences between individuals on Day 7 and Day 0 was -0.416 mm.s-2, resulting in a mean difference of -51% (-0.416 / 0.817 = -0.509). Text size is inversely proportional to the p-value derived from each t-test.

Table 5.5. Principal component (PC) loadings for the first three components extracted from analysis of behavioural differences. PC1 explains 31.5% of variation between individuals, PC2 explains 28.5% and PC3 explains 13%. Loadings in bold are >70% of the largest loading and indicate the most significant factors in the component (Mardia et al. 1979).

	PC1	PC2	PC3
exploration	0.30	0.40	**0.59**
meander	-0.49	0.28	0.01
number of pauses	-0.18	**0.59**	0.05
pause duration	-0.16	-0.57	0.08
speed	**0.57**	0.03	0.00
thigmotaxis	0.30	0.25	-0.78
walk duration	**0.44**	-0.15	0.19

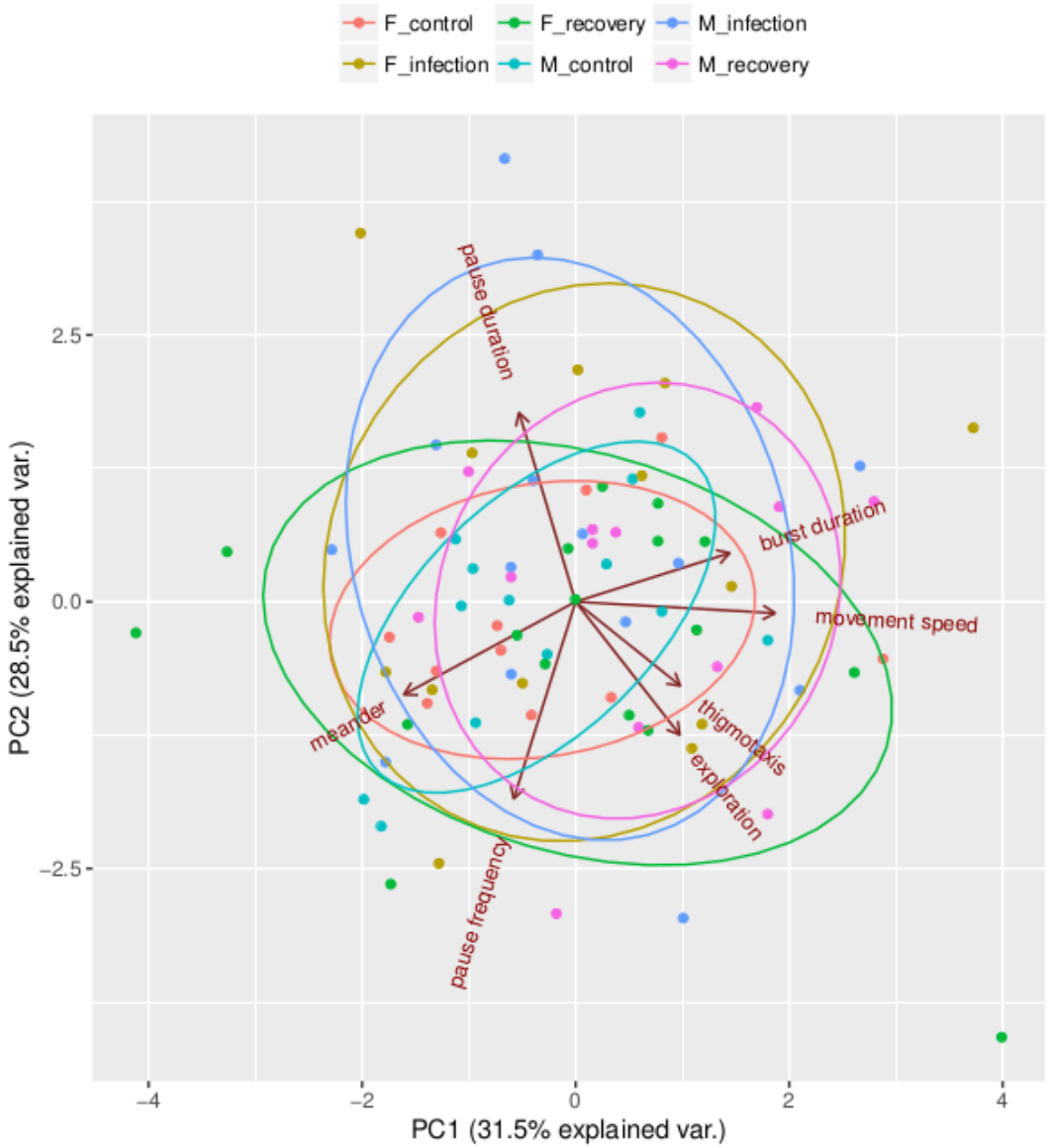


Figure 5.3. A scatter plot of the first two principal components from the PCA of behavioural differences. Each dot represents an individual and ellipses represent the 80% confidence interval for the group. Although PC3 (which explains an additional 13.0% of the variance) is not included here, its inclusion in 3D models provides little additional distinction between groups (data not shown).

5.4. Discussion

This quantitative analysis of behaviour suggests that immune stimulation with LPS does not greatly affect overall patterns of locomotion in either males or females, but may nevertheless cause differences in more subtle behavioural features, such as speed, turning or activity levels, and that these responses may vary between males and females. Importantly, the 'recovery period' included in this study sheds light on short-term temporal changes in behavioural responses to infection, as key behavioural differences only became apparent 7 d after infection. Whilst both males and females exhibited little change in locomotion immediately after immune stimulation, only males showed significant increases in their rate of locomotion and overall levels of activity at 7 d post-challenge. Comparing responses between the sexes, it appeared that males showed a greater level of inactivity and reduction in movement speeds than females immediately after infection, yet greater increases than females in speed, activity and turning at 7 d post-infection. The potential for such behavioural changes to represent sickness behaviours is considered below, as are potential explanations for observed gender differences.

5.4.1. Fitness benefits of sickness behaviours

Immune-induced changes in behaviour are collectively known as 'sickness behaviours' (Hart, 1988). Whilst sickness behaviours were once considered to be a passive byproduct of physical debilitation resulting from infection, there is mounting evidence that many behavioural modifications represent an adaptive response which reduces the costs of parasitism (Adamo, 2006), preserving host fitness by facilitating mechanisms of resistance or recovery, or offering indirect fitness benefits through investment in non-immunological life-history traits such as reproduction (e.g. Polak & Starmer, 1998; Adamo, 1999; Barribeau *et al.* 2010). Illness-induced anorexia is a common sickness behaviour in invertebrates, observed in response to both live infection and artificial immune stimulation (Schneider & Ayres, 2008; Adamo *et al.*, 2010). Although feeding rates were not measured in this study, meandering behaviours, such as greater distances travelled, longer durations of activity, higher levels of exploration and more turning behaviour, may form a proxy for foraging strategies. Increased locomotor activity may be indicative of increased rates of food seeking (Delthier, 1976), suggesting that the reduced overall activity observed in immune-challenged males could indicate a reduced motivation to forage. It is not clear why this appears to be restricted to males, but it may be indicative of gender differences in life-history-based tolerance strategies such as

terminal investment (discussed in section 5.4.3).

Several adaptive explanations have been offered for the reduction of feeding during infection, including the starvation of infective pathogens of key micronutrients such as iron (Hart, 1988; Ong *et al.*, 2006), the prevention of trade-offs between digestion and immunity (which both rely on the same protein, apolipophorin III, for lipid transport [Weers & Ryan, 2006; Adamo *et al.*, 2008; Adamo *et al.*, 2010]), or the restriction of immunopathological damage through limitation of resources available to invest into cytotoxic immune functions (Schneider & Ayres, 2008; Adamo *et al.*, 2010). Alternatively, post-infection anorexia may prevent the continued consumption of contaminated food, preventing an escalation of the parasitic load and thus limiting the intensity and duration of infection. For example, *Caenorhabditis elegans* tend to vacate feeding sites containing the pathogenic bacteria, *Serratia marcescens*, but not sites containing their standard food source, *Escherichia coli* (Pujol *et al.*, 2001). Increased enzymatic activity induced in the gut following pathogen ingestion is thought to induce a systemic 'malaise' response which could influence host behaviour via bidirectional signalling processes between cells in the gut and the brain (Adamo *et al.*, 2006; Zhang *et al.*, 2005).

An alternative explanation is that reduced levels of activity and exploration during immune challenge may represent conservative behaviours which prevent infected hosts from venturing into new and unknown territory, where their weakened state may leave them more vulnerable to predation. Although not observed here, decreased environmental exploration may reduce the risk of encountering additional threats of pathogenesis or aggressive encounters from conspecifics in unknown areas of the local environment, and allow time for recovery and limit horizontal transmission. Fear and anxiety have been suggested to overlap sickness effects in rodents (Dantzer, 2009; Kinoshita *et al.*, 2009), and observations of increased thigmotaxis in LPS-treated mice coincide with overall reductions in locomotory activity (Kinoshita *et al.*, 2009). In support of this, it has been shown that nematodes (*Caenorhabditis elegans*) that are allowed to feed on a bacterial lawn contaminated with *Staphylococcus aureus* are able to clear their infection if transferred to an uncontaminated food source within 8 h (Sifri *et al.*, 2003). This suggests that continual environmental surveillance and behavioural modulation by the host can preserve fitness even after they directly contact a pathogen.

Whilst males exhibited a typical lethargic response after immune challenge, females appeared to increase their level of activity and acceleration. This could result from physiological differences between the sexes during immune challenge, given that

females typically exhibit greater levels of immunocompetence and lower mortality than males in response to infection (Rolff, 2002). Alternatively, differences in life-history strategies may account for these differences, as females that increase their level of activity or exploration may benefit from finding sites for foraging or oviposition which are uncontaminated by pathogens. Although several other studies have found evidence of reduced activity levels and slower speeds of locomotion in insects following pathogenic infection (e.g. Webster *et al.*, 2000; Evans *et al.*, 2009), the use of LPS as an artificial immune elicitor in this experiment eliminates the potential for parasite manipulation. There are numerous studies in the vertebrate literature which find that LPS challenge reduces overall activity and increases resting time (e.g. Yirmiya *et al.* 1994; Engeland *et al.*, 2001; Owen-Ashley *et al.* 2006; Hawley *et al.*, 2007; Burness *et al.* 2010), but only one example exists in the invertebrate literature (Aubert & Richard, 2008); however, this study was conducted in honey bees, in which mechanisms of social immunity may play a prominent role in the expression of sickness behaviour.

5.4.2. Indirect fitness benefits of sickness behaviours

Decreased levels of locomotion have been suggested to decrease the spread of a pathogen in social insect colonies (Traniello *et al.*, 2002; Aubert & Richard, 2008), possibly conferring a net benefit to inclusive fitness by reducing transmission to kin. Sickness behaviours may also offer indirect fitness benefits to kin by providing related conspecifics with a behavioural cue of infection which can inform investment in prophylactic immune defences, such as the induction of physiological immune priming or avoidance behaviour. Recent studies in social insects have found evidence of 'social immunisation', whereby immunologically naïve hosts that are housed with parasitised conspecifics augment their resistance to subsequent infection by the same pathogen, presumably either through the transmission of low-level pathogenic infection or the transfer of host-derived immune factors (Traniello *et al.*, 2002; Ugelvig & Cremer, 2007; Hamilton *et al.*, 2010; Konrad *et al.*, 2012). Termites and ants have been found to increase their rates of social contact with and allogrooming of infected conspecifics (Hughes *et al.*, 2002; Yanagawa & Shimizu, 2009, 2012; Aubert & Richard, 2008), which may facilitate immunisation through a 'controlled exposure' (Hart, 1990).

Whilst *T. molitor* do not exhibit sociality, they are gregarious and share several key ecological features of sociality, such as high population densities, the use of a permanent and confined habitat and the presence of stored food. These factors have been suggested to increase pathogenic susceptibility (Schmid-Hempel, 1998) and may favour

the development of group-level defences (Otti *et al.*, 2014). However, previous chapters found no evidence to support the existence of a similar gregarious immunisation process in *T. molitor* (Chapters 2 & 3). In vertebrate species, sociality is generally reduced during immune stimulation (Dantzer, 2009), although little data is available on the effect of immune challenge upon non-sexual contact rates in non-social insects. It is possible that sophisticated behavioural immune defences can only evolve in eusocial societies, as non-social insects do not exhibit many of the behaviours which are suspected to facilitate social immunisation, such as allogrooming and trophallaxis (Rosengaus *et al.*, 1998; Traniello *et al.*, 2002; Hamilton *et al.*, 2010). Although a behavioural choice experiment yielded no effect of conspecific odour upon avoidance or attraction of challenged vs. unchallenged individuals of either sex (Appendix 4), the potential role of sickness behaviours as a cue which stimulates conspecific investment in immune prophylaxes merits further study.

5.4.3. Gender differences and sexual conflict

The observed differences in behaviour between males and females following immune challenge may be due to differences in life-history strategies between the sexes; males stand to gain more than females from increasing their investment in reproduction during infection, as males can linearly increase their immediate reproductive success through increasing their number of matings (Bateman 1948; Rolff, 2002). Females should invest more in immune defence in order to preserve their future reproductive capacity and thus maximise their lifetime reproductive success (Rolff, 2002), and, in many species, females exhibit greater levels of immunocompetence than males (reviewed in Rolff, 2002).

If the perceived threat to survival is great enough, infected males may preserve fitness by undertaking 'terminal investment' in reproduction (Clutton-Brock, 1984), redirecting resources away from immunity and into reproductive traits such as pheromone production, copulatory activity and sperm production (Polak & Starmer, 1998; Adamo, 1999; Abbot & Dill, 2001; Shoemaker *et al.*, 2006; Sadd *et al.*, 2006; Krams *et al.*, 2011). In male *T. molitor*, quantitative changes in the CHC profile (Nielson & Holman, 2011) and production of sex pheromones (Sadd *et al.*, 2006) during immune challenge increases the attractiveness of immune-challenged males to females (Sadd *et al.*, 2006; Nielson & Holman, 2011; c.f. Worden *et al.*, 2000; Worden & Parker, 2005).

Although we may expect terminally-investing males to increase their level of activity and exploration in order to find more mates, we found that immune-challenged males instead exhibited reduced activity. *T. molitor* produce highly potent volatile (and

non-volatile) sex pheromones which attract the opposite sex (Bryning *et al.*, 2005), meaning that males may be able to remain stationary and secure matings, reallocating resources from non-essential locomotory behaviour into direct reproductive investment (e.g. pheromone production, ejaculate quality). Indeed, one study found that female *T. molitor* prefer males that exhibit reduced locomotory activity following an artificial immune insult (Krams *et al.*, 2011), and another found that the attractiveness of male *T. molitor* was negatively correlated with encapsulation ability (Krams *et al.*, 2014). Furthermore, more attractive males also exhibit higher resting metabolic rates and suffer from reduced longevity (Krams *et al.*, 2014), suggesting that an important trade-off exists between investment in reproduction (through sexual signalling) and other physiologies in males.

Sexual signalling in immune-challenged males can therefore be dishonest with regards to condition (Sadd *et al.*, 2006). Such a false advertisement of mate quality stands to create sexual conflict, as copulating with an infected mate can incur costs to personal fitness in females, as well as the fitness of their offspring, for instance through horizontal transmission of infection (e.g. Knell & Webberley, 2004), reduced reproductive quality of mates (Carver & Hurd, 1998; Pai & Yan, 2003), or if parasite susceptibility is inherited genetically in the offspring (Carazo *et al.*, 2004).

Females may seek to avoid these costs by reducing investment in some reproductive activities. Female mealworm beetles may similarly reduce their reproductive investment restricting the production of sexual signals. 4-methyl-nonanol is a volatile pheromone produced by female *T. molitor* (Tanaka *et al.*, 1986) whose production is downregulated within 12h of mating (Seybold & Vanderwel, 2003), reducing the attractiveness of mated females (Carazo *et al.*, 2004). Alternatively, in a natural mixed-sex population, females could avoid unwanted solicitation by males during immune challenge by physically avoiding them, which could explain the increased locomotory activity observed in females following LPS injection.

Gender differences in the adaptive values of behavioural responses may explain the observed differences between males and females following immune challenge. As females often exhibit greater immunocompetence and greater efficiency of immune responses compared to males (Rolff, 2002), it is possible the greater decreases in overall activity seen here in males are a maladaptive consequence of immunopathological damage and/or resource depletion following immune stimulation. Local histological changes in infected tissue or damage resulting from infection could affect higher-level physiological functions and behaviour such as locomotor activity (Evans *et al.*, 2009).

Insect hosts can also cause immunopathological damage to their own tissues during an artificial immune challenge (Sadd & Siva-Jothy, 2006). Tetracycline treatment has been shown to cause reductions in the density of mitochondria in *Drosophila* and cause damage to sensory mushroom bodies, which alters locomotory activity (Martin *et al.*, 1998).

5.4.4. Opportunity costs and recovery

Although behavioural responses to infection can be significantly less costly than physiological immune defences (Siva-Jothy *et al.*, 2005; Schulenburg & Ewbank 2007), they may still incur fitness costs for the host. However, the primary currency in which these costs are paid may differ; whereas physiological immune effectors tend to have directly detrimental effects upon immediate host condition through immunopathological damage and resource expenditure, behavioural responses tend to carry more indirect 'opportunity costs' (de Roode & Levefre, 2012), such as reduced ability to forage or fewer reproductive prospects. Not all behavioural strategies are necessarily intended to improve host condition through the action of immunological resistance or tolerance mechanisms, and hosts may also ameliorate the fitness costs associated with infection and immune activation by investing in other beneficial life-history traits, such as reproduction. Such non-immunological responses to parasitism have been referred to as 'life-history escape attempts' (van Baalen, 1998; Minchella, 1985).

In the period immediately after infection, males exhibited no significant changes in behaviour compared with baseline levels before infection; there was a trend for a decreased rate of acceleration, although a similar decrease was also exhibited in males which remained unchallenged in both observation periods, suggesting this change was more likely a result of habituation or age-related changes, such as senescence. However, when males were observed after a recovery period of 7 d, individuals showed increases in several metrics of activity (greater distance travelled, less time spent stationary, longer periods of movement, and greater levels of turning). Females, on the other hand, showed no significant changes in behaviour in either of the two observation periods following immune stimulation, although there was some weak evidence for increased activity (increased acceleration and less pauses) in females immediately after challenge.

It is possible that these gender differences were due to differential latencies in the induction of behavioural defences between males and females. As beetles were only observed for a period of 1 h immediately following immune challenge, it is possible that sickness behaviours which took longer to be expressed may not have been observed. An

alternative explanation is that such late-acting behavioural changes could function as 'recovery behaviours', which repay opportunity costs incurred during sickness, when defensive strategies, such as anorexia, lethargy and reduced libido, were prioritised over other life-history traits such as feeding, social interaction and reproduction. LPS stimulated males may have: (i) became lethargic immediately after challenge in order to focus their investment of resources into reproduction, and increased their rate of locomotion afterwards in order to forage and recover their physical condition, or (ii) became lethargic in order to maximise investment in physiological immune defence following challenge, and increased their rate of locomotion in order to search for mates and recover lost reproductive fitness. Given that overall activity and acceleration were significantly higher in females immediately after immune stimulation, it is possible that females did not have such an opportunity debt to repay, and therefore had no measurable recovery period.

Future work would benefit from investigating this temporal element in more detail in order to identify if (and when) there is a peak expression of sickness behaviour. This could provide information on the latency of behavioural immune responses, determining whether challenged hosts reach a nadir of sickness, and how long it takes them recover fully and return to their normal baseline behaviour. Furthermore, temporal changes were apparent within the 60 minute observation period, as distance travelled, velocity and exploratory behaviour showed a tendency to decline in both males and females (Appendix 3). Habituation is a classically observed trait in rodents, which display decreased locomotion and increased inactivity around 15-20 min after being placed in an open field arena (e.g. Kinoshita et al., 2009), and has also recently been observed in honeybees (Sokolowski et al., 2012).

5.4.5. Context-dependence and behavioural plasticity

Given that behavioural responses are inherently labile, we may expect behavioural immune defences to be plastic with regards to environmental and social factors which modify the risk of pathogenic transmission (such as host population density, pathogen presence in the environment or seasonal variation), the dynamics of infection (e.g. general condition, coinfection status or exposure history of host), or the ability of the host to regain fitness through investment in non-immune life-history traits, particularly reproduction (e.g. gender, quality, availability and breeding status of surrounding conspecifics).

In this experiment, behaviour was monitored in a relatively small arena which

contained no food or water and in which individuals were kept in social isolation, meaning it is possible that the observed behavioural responses to immune challenge were not representative of a natural context in which individuals are able to mate, feed or escape the confines of the observation arena. The absence of potential mates may have altered the expression of sickness behaviours in immune-challenged males and females.

In *Drosophila melanogaster,* males and females both maximised investment in immunity when given *ad libitum* access to food but deprived of sexual partners (McKean & Nunney, 2005), suggesting that males will only terminally invest in reproduction when they stand to gain fitness returns from doing so. Following artificial immune stimulation, male rats (Yirmiya *et al.*, 1995) and birds (Lopes *et al.*, 2013) have also been shown to behave similarly to uninfected males when allowed access to females, presumably trading-off reproductive investment against immunity. We may expect males to suppress sickness behaviours when females are present, as hosts that behave as if they were sick may be less likely to attract mates (Knell & Webberley, 2004). Furthermore, receiver-dependent signal transmission may evolve in order to reduce the costs of maintaining or transmitting a costly signal. For example, female burying beetles (*Nicrophorus vespilloides*) advertise their breeding status via the emission of methyl geranate (a compound similar to juvenile hormone), but do so only when a male partner is present (Steiger *et al.*, 2011).

5.4.6. Non-adaptive explanations for behavioural modifications

It is possible that the observed differences in behaviours were a maladaptive artifact of the immune challenge or experimental design used in this study. Firstly, LPS challenge may not have constituted a survival threat sufficient enough to provoke true behavioural sickness. Whilst LPS is effective at enhancing the level of activity of certain immune effectors in insects (Moret *et al.*, 2003), it confers a relatively low cost in terms of mortality in *T. molitor* (Moret & Siva-Jothy, 2003; Vainikka, 2007; c.f. crickets [Jacot *et al.*, 2004], bumble bees [Moret & Schmid-Hempel 2000]), and may not be perceived as a serious survival threat by the host. Secondly, a terminal investment in reproduction is expected only when the costs of responding to infection outweigh the benefits of successful resistance, and we may thus expect a relatively minor immune challenge with a low risk of mortality such as LPS to be insufficient to stimulate a terminal investment in males. Finally, as discussed above, the absence of conspecifics in the observation arena may have prevented the expression of non-immunological defensive strategies, such as escape behaviour or increased reproductive investment.

5.4.7. Summary

This chapter provides some evidence for subtle behavioural modifications in a non-social insect host induced by challenge with a non-pathogenic immune elicitor. Gender differences in behavioural responses are attributed to dimorphic life-history investment strategies between males and females, which may also explain differences between behaviours modifications observed immediately after immune challenge and those observed after a one week 'recovery' period. Future work on sickness behaviours in insects may benefit from taking concurrent measures of behavioural responses and physiological immune effectors from the same individuals in order to examine the trade-offs between these two lines of immune defence. Although the tracking software used in this chapter is only capable of measuring the movement of one insect per observation arena, it would be interesting to examine the expression of sickness behaviours in a more natural population consisting of multiple, mixed-sex individuals. This would allow us to investigate whether behavioural cues of infection can be used to stimulate investment in immune prophylaxes such as immune priming or behavioural avoidance.

In this chapter, I have:

- Found some evidence for the expression of subtle sickness behaviours which are not a consequence of parasite manipulation
- Found a potential role for a behavioural 'recovery period', in which delayed behavioural changes following immune challenge (here, 7 days post-challenge) may help mitigate certain opportunity costs occurred during actual challenge
- Identified gender differences in behavioural responses to immune stimulation

CHAPTER SIX
GENERAL DISCUSSION

6.1. Summary of thesis

Physiological and behavioural prophylaxes can reduce the risk of hosts becoming infected, enhance their ability to resist invading pathogens, or help them tolerate the fitness costs of infection. In this thesis, I have investigated the potential for a 'social transfer of immunity', which I term 'gregarious immunisation', in a non-social but group-living insect, *Tenebrio molitor*. Gregarious insects are exposed to many similar pathogenic risks as social insects, yet lack many of the sophisticated group-level immune defences which characterise the eusocial insects, suggesting that hosts should benefit from responding to social cues of pathogenic threat in order to inform a suitable investment in prophylactic immune defence.

I first investigated whether naïve female beetles exhibited enhanced resistance to subsequent infection following cohabitation with a bacterially-infected female conspecific (Chapter 2). There was little support for gregarious immunisation in this experiment as naïve individuals that were paired with a neighbour suffering from a live *Staphylococcus aureus* infection showed no increase in antibacterial activity following subsequent infection by the same pathogen, and actually suffered from decreased longevity (relative to naïve individuals that were housed with a healthy neighbour). Interestingly, however, naïve individuals that were paired with neighbours given an injection of heat-killed *Staphylococcus aureus* exhibited a significant decrease in their level of antibacterial activity following subsequent bacterial infection, yet showed no decrease in their longevity. Despite the lack of evidence for gregarious immunisation, the data do suggest that cohabitation with an immune-stimulated, but non-contagious, conspecific could promote enhanced tolerance of infection by the same pathogen in naïve individuals. This could be an advantageous strategy when the risk of pathogenesis is high, as hosts would be better able to preserve fitness by circumventing survival costs of infection and immune activation. Although the specific mechanisms remain to be investigated, similar studies in eusocial insects (Hamilton *et al*., 2010; Konrad *et al*., 2012) suggest that the naïve host may become primed through direct contact with the pathogen or pathogenic material. In this experimental design, live transmission is a possibility from neighbours with a live

bacterial infection, but not from neighbours with a heat-killed infection. It is possible that the direct transfer of live bacteria between hosts leads to non-adaptive pathology in naïve hosts, but that contact with dead bacterial material produces an advantageous form of immune priming which helps the host to combat the same pathogen or its effects during subsequent full-scale infection.

I then went on to investigate the role of gender differences in gregarious immunisation through the effect of cohabitation with a conspecific suffering from only a non-transmissible immune challenge (Chapter 3). By using both male-male and female-female pairs of naïve/infected individuals, and measuring both constitutive (before a subsequent immune challenge) and inducible (post-challenge) defences of naïve individuals, I could assess whether cohabitation-induced priming occurs via the immediate induction of an immune response following the cohabitation period, or via a sensitisation to a secondary danger signal (i.e. priming against future full-scale infection).

The data showed obvious differences between the immune responses of naïve males and females following cohabitation. There was little effect of cohabitant immune status upon either constitutive or induced immunity in females, but in males, there was a significant effect which was opposite to that predicted by the gregarious immunisation hypothesis. Naïve males housed with nylon-challenged conspecifics exhibited lower levels of constitutive phenoloxidase activity and haemolymph protein concentrations (pre-challenge), and significantly lower levels of induced nylon encapsulation (post-challenge). This was corroborated by data from a separate experiment, whereby males, but not females, showed a reduction in phenoloxidase activity after being housed in the presence of bacterial volatiles (Appendix 5). Together, these findings implicate a role of chemosensory cues of pathogenesis in altering host immune investment, as opposed to direct pathogen transfer between hosts. In support of this idea, the data also indicate a positive correlation between the degree of cuticular melanisation in immune-challenged individuals and immune activity in their paired naïve cohabitants, although exactly how cuticular darkness may affect the production of cues of infection by immune-challenged hosts remains to be determined.

I developed an automated tracking system that is capable of recording the movement of insects and extracting a range of metrics to describe locomotion quantitatively (Chapter 4). I then used this software to investigate whether male and female *T. molitor* engage in sickness behaviours following an immune challenge (Chapter 5), which allowed me to

determine whether behavioural cues of infection produced by infected individuals may play a role in stimulating gregarious immunisation.

Here, there was some evidence for subtle changes in behaviour following immune stimulation, and evidence that these responses differed between males and females. Both sexes exhibited few behavioural changes in the first 1 h following infection challenge, but when observed after a 7 d 'recovery period' post-challenge, males exhibited a general increase in overall rates of movement and activity, whilst females showed no difference in behaviour. This suggests that females do not exhibit any major behavioural shifts during or after immune challenge, unlike males, which appear to experience delayed behavioural changes in response to challenge. One explanation for this finding is that males, which typically have lower immunocompetence and life-history strategies which focus more on reproduction than self-preservation, increase their investment in locomotory activities (e.g. mate searching, foraging) for some time after infection to help mitigate the costs of missed opportunities incurred during infection (e.g. less mating, anorexia). In the context of gregarious immunisation, it seems unlikely that such subtle behavioural changes during artificial immune challenge could provide a reliable signal of conspecific immune status, although true pathogenic infection seems more likely to result in more noticeable changes in both locomotion and physiology by causing pathology and more exhaustive resource expenditure.

Finally, a choice experiment found no evidence that naïve individuals (either male or female) showed avoidance of immune-challenged conspecifics (either same-sex or opposite sex) (Appendix 4). Naïve individuals did not appear to exhibit any aversion towards the odour of immune-challenged conspecifics, and naïve females actually showed some evidence of being preferentially attracted towards males that had received an immune-challenged 48 h previously.

6.2. Discussions arising from this thesis

6.2.1. Gender differences

Fundamental life-history differences between the sexes mean that males are able to gain a linear increase in reproductive success by increasing their number of matings, unlike females (Bateman 1948; Rolff, 2002). In order to maximise their lifetime reproductive success, females are generally predicted to invest more in immunity to preserve their capacity for future reproduction (Rolff, 2002), and females tend to exhibit a greater level of immunocompetence than males in many insect species (Nigam *et al.* 1997; Radhika *et al.* 1998; Kurtz *et al.* 2000; Kurtz & Sauer 2001; Siva-Jothy *et al.* 2001).

I found consistent differences in immunity and behaviour between *T. molitor* males and females throughout this thesis. Naïve males housed with an infected conspecific downregulated their investment in constitutive immunity (Chapter 2), unlike females (Chapters 2 and 3), and displayed more noticeable modifications in locomotory behaviours than females following immune stimulation (Chapter 5). Such gender differences may stem from sexually dimorphic life-history strategies, which are predicted to modulate the role of sexual selection on host responses to a pathogenic threat.

6.2.2. The effect of sexual selection upon host-pathogen dynamics

If the perceived costs of current or future infection are sufficiently high, hosts may benefit from changing their investment strategies away from immunological resistance mechanisms, which kill the invading pathogen and/or limit its growth, into tolerance mechanisms which allow the host to preserve fitness without actively attacking the pathogen. Strategies like terminal investment represent "life-history escape attempts" (van Baalen, 1998; Minchella, 1985) which allow the host to tolerate the negative effects of infection through investment in non-immunological life-history traits. In this way, terminally investing hosts trade-off self-preservation, driven by natural selection, against reproduction, driven by sexual selection.

While terminally investing hosts may gain fitness through increased reproductive effort following infection, healthy individuals choosing to mate with an infected partner may suffer several costs. There may be direct fitness costs for mating partners via the transfer of sexually transmitted infections (STIs), which are widespread in insects (Burand *et al.*, 2012) and often detrimental to fertility (Knell & Webberley, 2004). Direct reductions in reproductive fitness are also common during infection, as infected males can have poorer sperm quality (Carver & Hurd, 1998; Pai & Yan, 2003) and infected females may lay eggs of poorer quality, depending on the type of immune challenge (Adamo *et*

al., 1999; Shoemaker *et al.*, 2006). Furthermore, there may be indirect fitness costs to offspring if parasites are vertically transmitted (e.g. Goodacre & Martin, 2012), or if parasite susceptibility is genetically inherited by the offspring (e.g. Carazo *et al.*, 2004). The costs for offspring are also likely to become compounded if both parents are infected at the time of mating. However, the costs are predicted to be greater for healthy females mating with infected males than healthy males mating with infected females, as males invest less resources per offspring and their reproductive potential is ultimately constrained only by their number of mates (Bateman, 1948).

Despite these potential costs, there is little evidence that many insects avoid mating with infected conspecifics (Fedina & Lewis, 2008; c.f. Carver & Hurd, 1998; Worden *et al.*, 2000; Worden & Parker, 2005; Vainikka *et al.*, 2007). Elevated reproductive effort in invertebrates during infection is well documented (Thornhill *et al.*, 1986; Polak & Starmer, 1998; Adamo, 1999; Abbot & Dill, 2001; Shoemaker *et al.*, 2006; Sadd *et al.*, 2006; Krams *et al.*, 2011), and there are, in fact, numerous examples of immune-challenged males being able to attract more mating partners than healthy individuals (Knell & Webberley, 2004; Goodacre & Martin, 2012), including in *T. molitor* (Sadd *et al.*, 2006; Krams *et al.*, 2011; Nielson & Holman, 2011). My experiments similarly suggested that immune-challenged males became more attractive to females (Appendix 4), and neither males nor females appeared to avoid immune-challenged conspecifics. There may be several explanations for this apparent lack of mate choice.

Firstly, sexual signalling by infected males may be dishonest with regards to condition (Sadd *et al.*, 2006), as males may enhance their attractiveness through increased investment in sexual signalling despite being in an unhealthy condition (Sadd *et al.*, 2006; Krams *et al.*, 2011; Nielson & Holman, 2011). Hosts that are obviously sick should be less likely to attract mates (Knell & Webberley, 2004), and infected hosts may be under selective pressure to suppress signs of sickness in order to combat the fitness costs of parasitism with reproduction. This should be especially true in non-social species, which are likely to gain more in direct fitness from increasing their level of reproduction during infection than they stand to lose in terms of indirect (kin) fitness through increasing the rate of disease transmission in the population.

Secondly, the benefits of mating with additional partners may outweigh the costs of missing a mating opportunity, even if the sexual partner is infected. Polyandry is highly beneficial for female *T. molitor* (Drnevich *et al.*, 2001; Worden & Parker, 2005), as multiple mating increases larval viability of offspring (Pai *et al.*, 2005), enhances

insemination success of sons (Pai & Yan, 2003) and increases egg viability in F1 offspring (Pai & Yan, 2003). Female tenebrionids appear to possess peri-mating and post-mating choice mechanisms (see below) which may allow them to retrospectively exercise mate choice when mating with infected males of poor quality.

Thirdly, mating with an infected male may provide indirect fitness benefits for potential offspring. Males that are able to increase their attractiveness during infection (e.g. Sadd *et al.*, 2006; Nielson & Holman, 2011) may produce sons that inherit the same trait and thus also possess the ability to mitigate the fitness costs of parasitism through increased investment in reproduction. Such a trait should become more advantageous as the risk of infection in the population increases. Healthy males may also gain from mating with infected females; for example, female crickets (*Gryllus texensis*) infected with a potentially lethal pathogen (*Serratia marcescens*) increase their rate of egg laying when environmental resources are favourable, offering fitness gains for the healthy male (Adamo *et al.*, 1999; Shoemaker *et al.*, 2006).

Finally, offspring may benefit through transgenerational immune priming (TGIP), whereby infected mothers (and fathers) produce offspring that are more resistant to the same pathogen as their parents (Little *et al.*, 2003; Sadd *et al.* 2005; Moret 2006; Sadd & Schmid-Hempel, 2007; Freitak *et al.*, 2009; Tidbury *et al.*, 2011; Roth *et al.* 2009b; Zanchi *et al.*, 2011). In insects with short generation times and low dispersal rates which experience little environmental change, such as *T. molitor*, offspring are likely to face the same pathogenic pressures as their parents. TGIP can therefore be effective way for infected hosts to indirectly mitigate fitness costs of parasitism, especially if this strategy is paired with an increase in reproductive activity.

Whilst the direct transfer of bacterial pathogens from the maternal gut lumen to developing eggs has been shown following oral infection in female *Galleria mellonella* (Freitak *et al.*, 2014), such a mechanism of vertical pathogen transmission seems unlikely to occur via infected males, as bacterial deposition is suggested to occur at an early stage of egg development. Mating with an infected male may even be advantageous by better informing appropriate immune priming in offspring, augmenting their defence against contemporary pathogenic threats in the environment. The efficacy of transgenerational immune priming may be further enhanced by multiple mating, as mothers who 'sample' more males should acquire more information regarding current environmental challenges, and should be more likely to produce optimally primed offspring.

It is possible that the benefits of multiple mating therefore outweigh the benefits of screening mates prior to copulation, and could explain why pre-copulatory mate choice

processes appear to be largely absent in tenebrionids (Fedina & Lewis, 2008). Indeed, the cost to female *T. molitor* of assessing male signalling honesty has been suggested to outweigh the costs of mating with a parasitised male (Nielson & Holman, 2011). Nevertheless, tenebrionids may utilise cryptic female choice processes in order to vet their mating partners. For example, female *Tribolium castaneum* have been shown to actively block spermatophore transfer from starved males of poor phenotypic condition (Fedina & Lewis, 2006), and even eject their spermatophores shortly after mating (Qazi *et al.*, 1996; Fedina, 2007).

An intriguing new study by Peuss *et al.* (in press) suggests that *Tribolium castaneum* decrease their expression of two heat-shock proteins (Hsp83 and Hsp90) following cohabitation with wounded conspecifics. The authors suggest that depletion of Hsp90, which is a suppressor of mutagenic transposon activity, acts to generate novel genetic diversity in offspring and increase evolvability of the next generation. This could represent a bet-hedging strategy by the host, which revolves around a life-history tolerance of pathogenesis as opposed to immunological resistance through personal immune defence. Such a strategy is likely to be more beneficial when combined with an increased reproductive effort by the parental host.

For these reasons, the driving force of sexual selection in tenebrionids can often run counter to natural selection pressures which should favour self-preservation mechanisms, such as the deployment of physiological immune responses against pathogenic threat. Nevertheless, the effects of cohabitation treatment upon immunity reported in this thesis (Chapters 2 and 3), and the findings of Peuss *et al.* suggest that the ability to discriminate against conspecific immune status does exist in tenebrionids.

6.2.3. Effects of host tolerance upon epidemiology and host-parasite coevolution

The downregulation of immunity shown by males exposed to an environmental pathogenic threat (Chapter 3, Appendix 5) and the apparent suppression of sickness behaviours shown by males experiencing actual immune challenge (Chapter 5) suggest that male *T. molitor* prioritise reproduction above immune defence when facing costs of parasitism. As well as creating a potential for sexual conflict due to differing life-history strategies between the sexes, this strategy may have important impacts on host-parasite coevolution.

In many insect species, individuals undergo immunosuppression after mating, which likely prevents the diversion of resources away from reproduction and into immunity and should be particularly favourable for terminally investing hosts (Sheldon &

Verhulst 1996). Mating has been shown to reduce phenoloxidase titres in *Tenebrio molitor* (Rolff & Siva-Jothy 2002), reduce encapsulation ability in damselflies (Siva-Jothy *et al.* 1998), suppress antibacterial activity in *Drosophila melanogaster* (McKean & Nunney, 2001), and decrease encapsulation ability, lytic activity and haemocyte number in the cricket, *Allonemobius socius* (Fedorka *et al.* 2004). In some species, males may benefit from the induction of post-mating immunosuppression in females, which boosts female reproductive output (Lawniczak *et al.* 2007; Fedorka *et al.* 2007), although post-mating female immunosuppression in *Drosophila melanogaster* has been suggested to be a predominantly female-driven trait that has not evolved through sexual conflict (Short & Lazzaro, 2010). It is therefore not always clear whether such responses to infection represent an adaptive strategy which benefits the male host, an adaptive strategy which benefits the female host, or even a maladaptive strategy which benefits the parasite. Communicable parasites may benefit from affecting host condition in such a way so as to suppress sickness behaviours, particularly those which are transmitted through physical or sexual contact, as hosts that behave as if they were sick should be less likely to attract mates (Knell & Webberley, 2004).

For example, the cricket *Gryllus texensis* typically exhibits sickness behaviours during infection, such as anorexia and reduced sexual activity (Gerhardt & Huber, 2002; Jacot *et al.*, 2004). However, when infected with the iridovirus IIV-6/CrIV, which induces sterility in females and reduces sperm motility to the point of effective sterility in males (Adamo *et al.*, 2014), male and female crickets both continue to mate as normal, and infected males are quicker to court females than healthy males (Adamo *et al.*, 2014). Infected crickets also undergo immunosuppression, exhibiting lower levels of phenoloxidase activity and total haemolymph protein (Adamo *et al.*, 2014). In this case, continued reproduction is unlikely to be beneficial for infected hosts, as female crickets have few eggs and are incapable of storing sperm from previous matings, and the sperm motility of infected males is extremely low.

Similarly, *Drosophila melanogaster* infected with Drosophila C virus (DCV) upregulate expression of a pheromone binding protein, pherokine-2 (Phk-2), which is not directly involved in viral resistance (Sebatier *et al.*, 2003). A molecule with similar sequence structure to Phk-2, MbraAOBP2, has been shown to bind the pheromone cis-vaccenyl-acetate (Bohbot, *et al.*, 1998), which is transferred by male *Drosophila* during copulation and has an anti-aphrodisiac effect on future female courtship (Brieger & Butterworth, 1970). Interestingly, Phk-2 is expressed in the ejaculatory bulb of *Drosophila* males, and its upregulated expression following infection could represent a

reproductive defence strategy for infected males, especially as DCV is not transmitted vertically (Sebatier *et al.*, 2003). This could explain why DCV-infected flies have been shown to have higher fecundity and fertility than uninfected hosts (Gravot *et al.*, 2000).

Whilst DCV infection in *Drosophila* therefore appears to offer a fitness benefit to both the host and parasite (and may thus even be considered mutualism), IIV-6/CrIV iridovirus infection in crickets may more likely be a form of parasitic manipulation of host behaviour (e.g. Poulin, 1995; Adamo, 2012). Regardless of whether the aphrodisiac effect induced by infection is the result of a targeted attack by the virus, a biochemical byproduct of the site of infection, or an intentional behavioural response by the host, terminal investment strategies can be under positive selection in both host and parasite. Tolerance strategies (both immunological and life-history based) can thus have important effects upon the coevolutionary dynamics between hosts and parasites, and the sexually dimorphic effects uncovered in this thesis may in part be explained by such complexities in coevolution between *T. molitor* and its natural parasites.

6.2.4. Proximate mechanisms of immune cue production and detection

The social transfer of immunity may not involve the direct transfer of pathogens or immune molecules between hosts, but instead hinge upon the detection of social signals that reliably indicate infection (e.g. chemosensory cues, visual symptoms of infection; Konrad *et al.*, 2012). Infection can alter the chemosensory profile of insect hosts, and qualitative changes in cuticular hydrocarbon (CHC) profile, which has many important functions in communication in insects (Singer, 1998; Wyatt, 2003), have been documented following immune stimulation in several eusocial insects species (Trabalon *et al.*, 2000; Salvy *et al.*, 2001; Richard *et al.*, 2008; Evans & Spivak 2010), as well as in *T. molitor* (Nielson & Holman, 2011). Pheromone production in *T. molitor* is also known to alter according to reproductive status (Seybold & Vanderwel 2003; Carazo *et al.*, 2004), condition (Rantala *et al.*, 2003) and possibly immune status (Sadd *et al.*, 2006).

Whilst the social transfer of immune factors between hosts may play a role in social immunisation in eusocial insects (Rosengaus & Traniello, 1999; Hamilton *et al.*, 2010), the social behaviours of trophallaxis and allogrooming, which are thought to mediate this process, are largely absent in non-social insects. However, chemosensory cues may still be emitted passively via exocrine glands or in waste products. For example, antimicrobial factors in the haemolymph can be readily transported to the epicuticular surface (Schal *et al.*, 1998; Tzou *et al.*, 2002), and infected hosts may excrete pathogenic materials (live pathogens, dead pathogens or pathogen-derived compounds) into the

environment which could be capable of eliciting an immune response in naïve individuals that contact them.

Insects are able to eavesdrop on microbial volatile organic compounds (MVOCs) released by bacteria and fungi in the environment (Davis et al., 2012; Farag *et al.*, 2013), which provide the host with a reliable indicator of microbial presence in the environment and inform a range of behavioural prophylaxes, including behavioural avoidance, reduced feeding and reduced oviposition at sites with detectable MVOCs (Tasin *et al.*, 2012; Hussain *et al.*, 2010; Yanagawa *et al.*, 2011; Ormond *et al.*, 2011; Mburu *et al.* 2012; Sun *et al.*, 2008; Stensmyr *et al.*, 2012; Lam *et al.*, 2010; Villani *et al.*, 1994; Myles, 2002; Zhang *et al.*, 2005). In support of a role of chemosensory cues in influencing immunity, I found evidence that male, but not female, *T. molitor* that were exposed to MVOCs from a bacterial pathogen exhibited reduced phenoloxidase activity in response to subsequent immune challenge (Appendix 5). This is intriguing, as the invertebrate literature documents more instances of females responding to environmental MVOCs, perhaps because the most commonly observed responses involve the modification of oviposition strategies

Alternatively, it is possible that cohabitating males (in Chapter 3) did not detect immunological cues of infection *per se*, but rather responded to other cues elicited by an infected male, such as sickness behaviours. As male *T. molitor* often invest more in sexual signalling during immune challenge to increase their attractiveness (Sadd *et al.*, 2006; Nielson & Holman, 2011), it is possible that naïve males also trade-off reduced investment in immunity in favour of reproduction as a response against increased competition for mates. However, the similar response observed following exposure to MVOCs (Appendix 5) suggests that males are able to detect environmental chemosensory cues of infection directly. It could be that males are more sensitive to such infection cues, having an increased propensity to detect and/or respond to them, which would also support the findings of reduced immunity in naïve males following cohabitation with an immune-challenged conspecific (Chapter 3).

6.2.5. Communication or eavesdropping?

Even if insects are capable of producing signals of infection, such as chemosensory cues or sickness behaviours, with the intended purpose of intraspecific communication with conspecifics, there may be costs of maintaining or transmitting such signals. For example, female burying beetles (*Nicrophorus vespilloides*) advertise their breeding status via the emission of the hormone methyl geranate, and increase their emission when a male

partner is present (Steiger *et al.*, 2011). It seems likely that this hormone is produced passively as a byproduct of mating, rather than intended as a form of 'true communication' (i.e. where information benefits both sender and receiver), as females stand to gain from multiple mating (Drnevich *et al.*, 2001). This is an example of specialisation restricted to the receiver, or 'eavesdropping' (Stowe *et al.*, 1995; Bradbury & Vehrencamp 1998; Sorensen & Stacey 1999; Wyatt 2003).

Such a form of signalling has been suggested to influence mate choice in *T. molitor*, as males show a preference for virgin over mated females, mediated by the nonvolatile pheromone, 4-methyl-1-nonanol, whose production is downregulated in females following mating (Tanaka *et al.*, 1986; Carazo *et al.*, 2004). Passive cues produced during infection may play a similar role in the social signalling of immune status given parsimonious evolution of chemosensory signalling. Whilst some eusocial insects engage in corpse management behaviours as a social immune defence (reviewed in Cremer *et al.*, 2007), non-social insects instead tend to show avoidance of conspecific corpses, for example in cockroaches (Rollo *et al.*, 1994), springtails (Yao *et al.*, 2009) and solitary bees (Abbott, 2006). Attraction to or avoidance of corpses appears to be mediated by the fatty acids oleic acid and linoleic acid (Wilson *et al.*, 1958; Howard & Tschinkel, 1976), which have been referred to as 'necromones' (Yao *et al.*, 2009). These compounds are produced passively by injured or dying cells through altered enzymatic or microbial processes, and are thus byproducts of infection as opposed to communicatory signals.

Interestingly, social immune priming in plants via green leafy volatiles (GLVs) produced by neighbouring plants during herbivory or pathogenesis (Bate & Rothstein, 1998; Karban *et al.*, 2000; Arimura *et al.* 2002; Engelberth *et al.*, 2004; Karban *et al.*, 2006; Heil & Ton, 2008) has been suggested to be an example of eavesdropping, as opposed to true communication (Conrath *et al.*, 2006). These volatiles likely initially evolved in order to prime distal parts of the plant more rapidly than vascular signals within the plant could, priming these parts of the plant to augment their resistance during subsequent attack (Heil & Ton, 2008). However, since these volatiles are a reliable cue of environmental threat, neighbouring plants should benefit from being able to eavesdrop on the signal and use it to inform their own immune prophylaxis. Such immune volatile production could therefore benefit indirect (kin) fitness in populations with high relatedness (e.g. plant species with short-range dispersal), where they could be classified as a social immune defence (Cotter & Kilner, 2010) despite only direct fitness benefits initially favouring the evolution of the trait. Furthermore, production of such an interceptable signal is unlikely to be selected against unless the costs of priming nearby

neighbours (e.g. by offering them a competitive advantage) outweigh the benefits offered to the acting individual.

6.2.6. Ecological relevancy and plasticity of immune priming

The importance of sexual selection as a mediator of immune defence could not be accounted for in this experimental design as only same-sex pairs were used. As such, the absence of breeding partners precludes understanding investment in reproduction as a non-immunological, life-history escape response to ameliorate the costs of infection.

It is also possible that reliable signals of infection are only produced in the presence of members of the opposite sex. For example, in the burying beetle, *Nicrophorus vespilloides*, breeding females release methyl geranate, a substance which indicates breeding status, but only when in the presence of a male partner (i.e. a signal receiver; Steiger *et al.*, 2011). Such receiver-dependent chemical signalling is expected to evolve when costs are involved in the production or transmission of the signal (Steiger *et al.*, 2011), and has been shown previously. For example, female *Drosophila melanogaster* upregulate expression of antimicrobial peptides and phenoloxidase activity following mating (Fedorka *et al.* 2004), and recently mated female crickets exhibit increased resistance to subsequent parasitism (Shoemaker *et al.* 2006). Additionally, both male and female *D. melanogaster* maximise their immune investment when given *ad libitum* access to food but deprived of sexual partners, yet only males demonstrate immune suppression when given access to mates (McKean & Nunney, 2005). This suggests that terminal investment in reproduction is a phenotypically plastic defence, and suggests that males modulate their immune strategy based upon social cues in their environment (i.e. presence of females).

Given the inherent plasticity of behaviour and comparatively low costs of behaviour defences versus physiological (Siva-Jothy *et al.*, 2005; Schulenburg & Ewbank 2007), it is possible that sickness behaviours form the most important part of an effective immune response, offering flexibility with regards to unpredictable environmental factors shaping infection, recovery and host fitness (e.g. host resource availability, immunological history and current coinfection, mating opportunities). Also, considering that males are a more likely source of disease transmission to females, particularly regarding sexually transmissible infections, mixed sex effects on immune defence warrant further study. It may also be interesting to investigate whether sickness behaviours are expressed in a pathogen-dependent and/or dose-dependent manner; for example, terminal investment may be only be expected in the case of particularly damaging infections, such

as those caused by highly virulent pathogen or by high infection titres.

6.2.7. Implications for other non-social insects

Similar immune phenomena to social immunisation are know outside of the eusocial insects. Some plants are able to prime themselves against future attack by priming their immune system in response to airborne volatile organic compounds (VOCs) produced by conspecifics whom are experiencing herbivory or pathogenesis (Bate & Rothstein, 1998; Karban *et al.*, 2000; Arimura *et al.* 2002; Engelberth *et al.*, 2004; Karban *et al.*, 2006; Heil & Ton, 2008; Erb *et al.*, 2015). In the vertebrate literature, rats have been shown to induce both behavioural and immune prophylaxes following cohabitation with immune-stimulated conspecifics, demonstrating behavioural avoidance of their challenged nestmates (Kavaliers & Colwell, 1995; Penn & Potts, 1998; Arakawa *et al.*, 2011) or altering several physiological features associated with stress and immunity, such as noradrenaline turnover and neutrophil activity (e.g. Penn & Potts, 1998; Alves *et al.*, 2006).

Comparing immunity in invertebrates and plants is problematic due to vast mechanistic differences between the groups, but parallels between invertebrates and vertebrates should also be cautioned for several reasons. Inverteberates are generally more short-lived, have higher fecundity, faster gestation periods, and typically invest less in their offspring, in terms of immediate resource expenditure (e.g. oviparity versus viviparity) and parental care, than vertebrates. Long-lived species should favour the protection of future reproductive success in the face of pathogenic threat. Furthermore, species that live longer should encounter more parasites (a quantitatively greater number and a qualitatively more diverse selection) within their lifespan, increasing the importance of an adaptive immune system (within a single generation). On the other hand, species with a short generation time and the ability to upregulate fecundity massively during infection (e.g. by laying large clutches of eggs) may benefit more from maximising their immediate reproduction, especially when such a strategy is combined with mechanisms of transgenerational immune priming which prime immunity in offspring, whom are likely to face the same pathogenic threats as their parents (van Baalen, 1998; Schmid-Hempel, 1998). Such differences in life-history suggest that non-immunological forms of tolerance like terminal investment in reproduction may represent a much more feasible strategy to counter the costs of parasitism in non-social insects, and may explain why this thesis found no evidence for gregarious immunisation in *T. molitor* (Figure 6.1).

Externalised antimicrobial secretions are known to be produced by many

tenebrionid species, such as volatile benzoquinones in *T. molitor* and *Tribolium spp.* (Tschnikel, 1974). Whilst the primary purpose of these secretions may be to manage unwanted microbial growth in food stores (Yezzerski *et al.*, 2000; Otti *et al.*, 2014), benzoquinones also have a repellent effect on conspecifics and predators in *Tribolium* (Suzuki, 1980), and their secretion is also upregulated in *T. molitor* during parasitism by the rat tapeworm, *Hymenolepis diminuta* (Yan & Phillips, 1993).

This suggests that these compounds may serve a secondary defensive function, perhaps as a group-level defence. Defences enacted at the level of the individual can become co-opted to act at the group-level under certain ecological conditions (Elliot & Hart, 2010; Brown & Taylor, 2010), which Otti *et al.* (2014) suggest may include large group sizes, use of permanent and confined nests, and permanent food storage; all traits shared by *T. molitor*. For example, salicylaldehyde-based volatiles secreted by the brassy willow leaf beetle (*Phratora vitellinae*) have strong antimicrobial action, and when synergistically amplified in beetle aggregations, form a fumigant cloud which protects against fungal and bacterial pathogens in the microenvironment (Gross *et al.*, 1998, 2008).

The externalised secretion of antimicrobial compounds such as benzoquinones or production of detectable pathogenic cues which may stimulate conspecific investment in immunity are unlikely to have evolved primarily for their function as a group-level, socialised defence in *T. molitor*. Any benefits these defences confer to kin fitness are therefore predicted to be of secondary importance in their selection. Furthermore, the potential for dishonest signalling in populations with low relatedness is high, as the direct fitness benefits of securing matings despite infection are likely to be greater than the indirect (kin) fitness costs of facilitating pathogen transmission in the population.

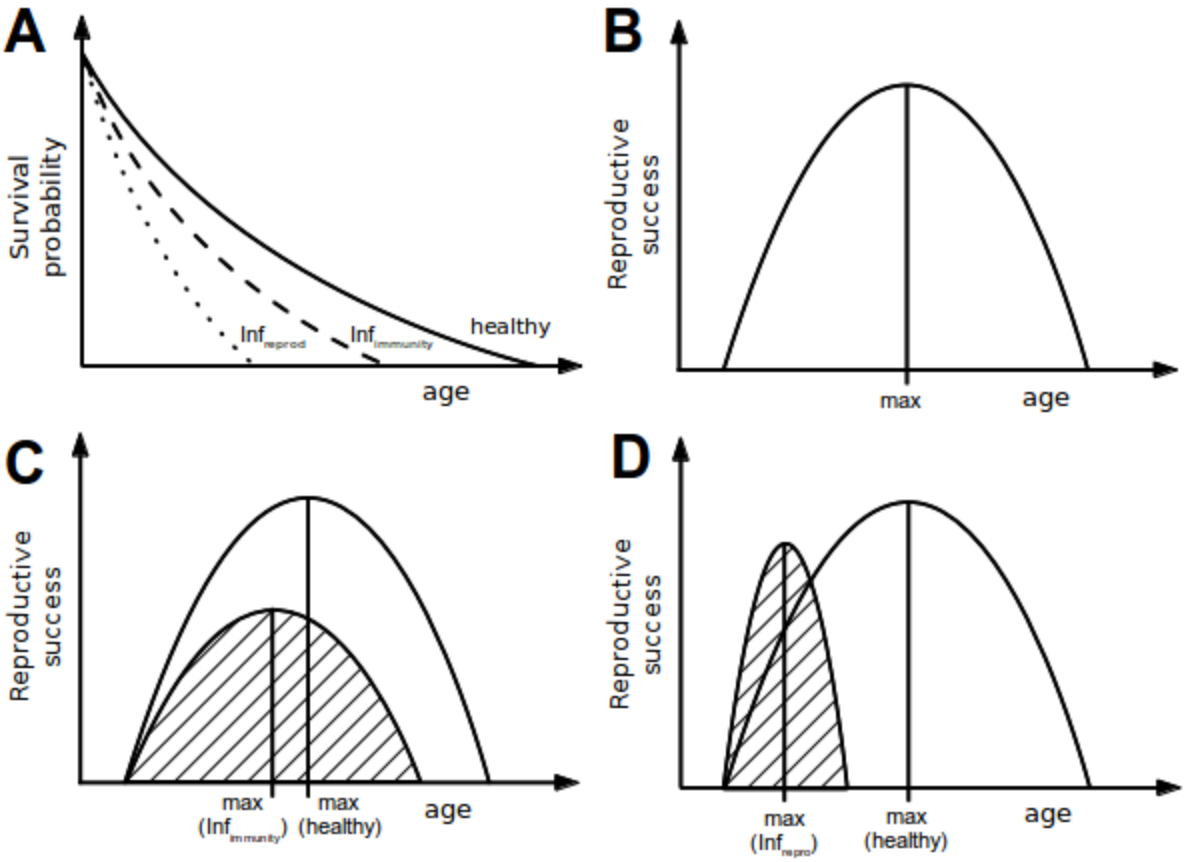


Figure 6.1. Theoretical considerations of terminal reproductive investment strategies upon host fitness during pathogenesis. (**A**) depicts the probability of survival during the lifespan of a healthy individual, which naturally declines as a function of age (solid line). Infection generally acts to shorten longevity, although hosts which successfully defend against infection by resisting the invading pathogen or physiologically tolerating its damage (dashed line) are predicted to live longer than hosts which allow themselves to succumb to infection in order to invest in other (non-immunological) life-history traits, such as reproduction (dotted line).

(**B-D**) depicts reproductive success as a function of age. (**B**) Healthy hosts begin mating once reaching sexual maturity and usually achieve peak reproductive output some time before natural senescence. (C) Infected hosts are likely to suffer decreased reproductive success as a result of reduced longevity and lower mating success resulting from pathogenic and/or immunopathological damage, reduced resource availability, reduced attractiveness, and reduced quality of gametes and/or offspring. (**D**) Infected hosts which tolerate the effects of infection through a terminal investment in reproduction are likely to achieve peak reproductive success sooner than hosts investing in immune defence, but are likely to die sooner.

Even if host strategies (**C**) and (**D**) both yield the same level of lifetime reproductive success for the infected host (shaded areas), immediate changes which allow the host to more rapidly reach their maximum reproductive output should be more beneficial for fitness given the uncertain conditions of infection (e.g. pathogenic virulence, risk of reinfection, resource availability). If the perceived threat to survival posed by a current infection, or the perceived threat posed by detected pathogens in the environment, is sufficiently high, hosts may thus be predicted to favour investment in life-history escape attempts over investment in immune defence. Figure modified from Agnew *et al.* (2000).

6.3. Future research

6.3.1. Chemical identification of immune-related chemosensory cues

Although this thesis suggests that chemosensory cues (host-derived semiochemicals and/or parasite-derived MVOCs) may play a role in modulating the immune investment of naïve individuals, the exact compounds involved are unknown. Mass spectrometry could identify semiochemicals that are altered in males and females through immune challenge; both airborne volatiles, such as sex pheromones (collected by headspace sampling), and contact-based non-volatiles, such as cuticular hydrocarbons, such as CHCs (collected by immersion). Use of isolated concentrates of these compounds in behavioural choice experiments could then reveal their resultant effects upon attraction or avoidance displayed by same-sex and opposite-sex conspecifics, and further experiments could more thoroughly determine their role in the induction of immunological and behavioural investment strategies.

6.3.2. Identify role of behavioural immune-related cues in immune priming

Whilst I found evidence for locomotory changes (sickness behaviours) in response to immune stimulation, and found no evidence that volatile chemosensory cues produced by immune-challenged conspecifics induce aversion in naïve individuals, I did not investigate whether conspecific sickness behaviours can alter immune investment in nearby naïve hosts. The development of a behavioural tracker (or modification of the software developed in Chapter 3) which is capable of tracking two or more insects in the same arena simultaneously, would allow investigation of how pathogenic threat affects behaviour in naïve hosts, as well as shedding light on the behavioural interactions between naïve and immune-challenged conspecifics (e.g. number of physical contacts, physical distance between hosts).

6.3.3. Additional experimental considerations

Although same-sex cohabiting pairs were used in my experiments to exclude the potentially confounding effects of mating upon immunity (Rolff & Siva-Jothy, 2002), the responses undertaken by naïve hosts may not have been representative of those in a more natural setting due to a lack of mating partners. This may have prevented hosts from the use of strategies aimed at tolerating the potential costs of pathogenesis, such as terminal reproductive investment (e.g. McKean & Nunney, 2005). Furthermore, whilst my results suggest that male *T. molitor* may undergo terminal investment following direct immune challenge and indirect pathogenic threat, additional experiments could demonstrate this

explicitly by taking measurements of reproductive investment and reproductive output in males and females (e.g. mating frequency, sperm quality/quantity, egg count, offspring fitness). Measurement of other non-immunological traits that affect the dynamics of infection, such as feeding rate (which was beyond the scope of behavioural tracking software), may also quantify the importance of non-physiological responses in immune tolerance.

Finally, the use of one-on-one pairs of naïve/immune-challenged beetles in my cohabitation experiments may have been ineffective for the investigation of a gregarious immunisation process. It is possible that the quantity of immune-related chemosensory cues produced by a single immune-challenged host are undetectable, or that its concentration is insufficient to induce an immune priming response in nearby naïve hosts. Indeed, in a gregarious species such as *T. molitor*, a single immune-challenged conspecific is a relatively small infection threat for the population. Further experiments may use more than one immune-challenged individual to impose an infection threat for naïve hosts, or even vary the perceived infection threat through a titration (i.e. varying the number of challenged individuals, or varying the ratio of challenged:unchallenged conspecifics) to determine whether there danger signals only stimulate investment in immune priming above certain thresholds.

6.3.4. Investigation of socially-induced immune priming with greater temporal resolution

Similar experiments to those conducted in this thesis that incorporate a greater degree of temporal resolution could allow investigation of time-dependent elements of gregarious immune priming. While the cohabitation experiments in this thesis examined immunity in naïve individuals after being housed with an immune-challenged conspecific for 72 h (Chapters 2 and 3), it is possible that this was an inappropriate exposure duration for the elicitation of immune prophylaxes. It is possible that immunological response is initiated rapidly in naïve hosts upon exposure to an infection threat, and that this response peaks sometime before 72 h. Conversely, a long-term exposure of >72 h may be required before immune prophylaxes, which are typically long-lasting and may be costly to maintain, are invested in by naïve hosts. In the vertebrate literature for example, one study found that mice exhibited changes in locomotion and noradrenaline turnover only after 7 days of cohabitation with an immune-challenged nestmate (Tomiyoshi *et al.*, 2009), and another study found that behavioural changes and hyperalgesia only occurred after a cohabitation period of 14–21 days (Langford *et al.*, 2006). Furthermore, this thesis found evidence of

behavioural modifications 7 days after immune stimulation, although the use of only two observation periods means that the latency period in the induction of these responses is unknown. Observations conducted over a longer time period and with greater frequency could reveal exactly when certain sickness behaviours are induced and how long for.

6.4. General conclusions

In conclusion, the work conducted in this thesis provides little evidence for the existence of gregarious immunisation in a non-social but gregarious insect. However, socialised cues of infection produced by immune-challenged conspecifics do appear to influence immune investment in immunologically naïve males at least, causing a downregulation of an important constitutive immune effector which may represent a non-immunological form of parasitic tolerance. Behavioural responses and life-history modifications are overlooked in many immunological studies, and are often assumed to be simply traded-off against immunity as opposed to forming an adaptive anti-parasite strategy in their own right, as well as a synergistic effect with immunity. Although immunity is a highly plastic trait, it seems that relatively inflexible host life-history traits and ecology may be equally important influences on the undertaken responses to infection threat. Further work on other non-social insect species which differ from *T. molitor* in their ecology and life-history (e.g. higher rates of dispersal, more parental care, lower levels of polygamy, longer lifespans) must be conducted before we are able to conclude that sociality is necessary trait for the development of socialised forms of immunity.

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APPENDICES

APPENDIX 1:
CALCULATION OF *Staphylococcus aureus* LD_{50}

In order to determine an appropriate dosage of *Staphylococcus aureus* with which to infect hosts in Chapter 2, the median lethal dose (LD_{50}) was calculated from a survival analysis which used a range of infective doses, from 10^2 to 10^8 CFUs (Figure A1.1). The LD_{50} after 14 days was estimated to be 5.33 x 10^7 CFUs per 5µL inoculate (Figure A1.2). Beetles were cultured, treated and housed in the same manner described in Chapter 2.

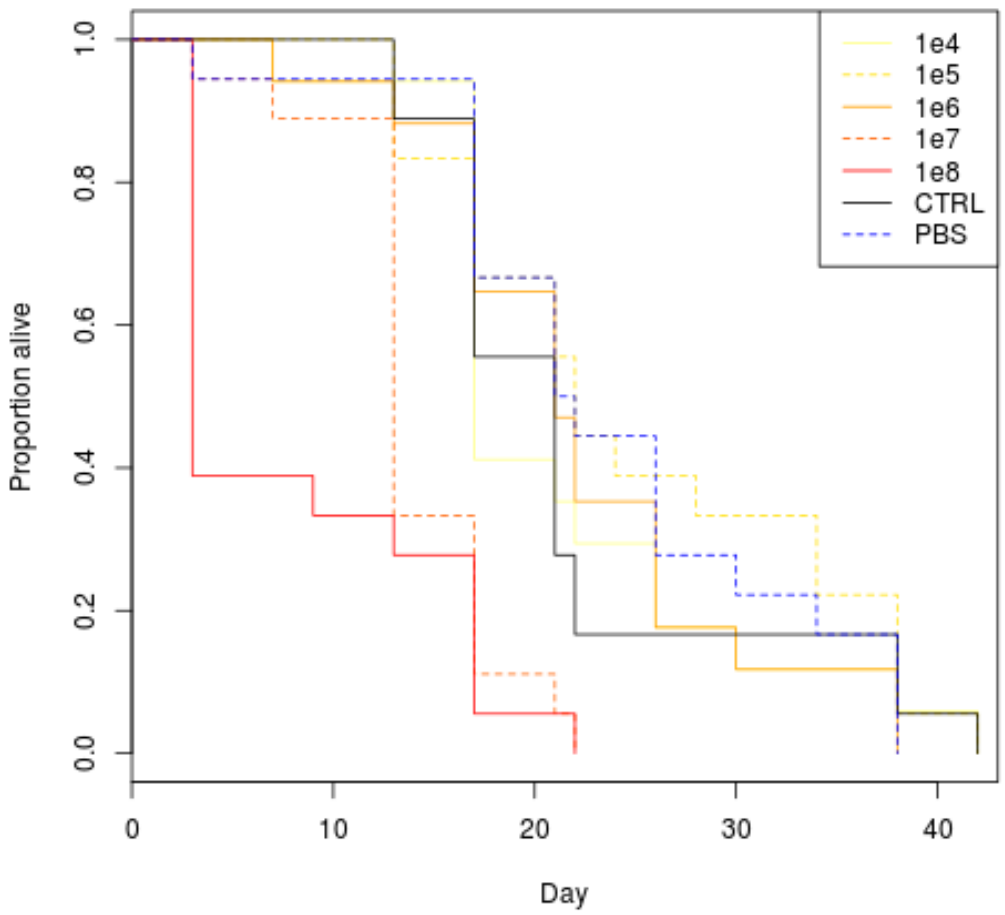


Figure A1.1. Survival analysis of naïve individuals infected with varying dosages *Staphylococcus aureus*, as well as a procedural control (PBS; blue dashed line) and a no treatment control (CTRL; black line).

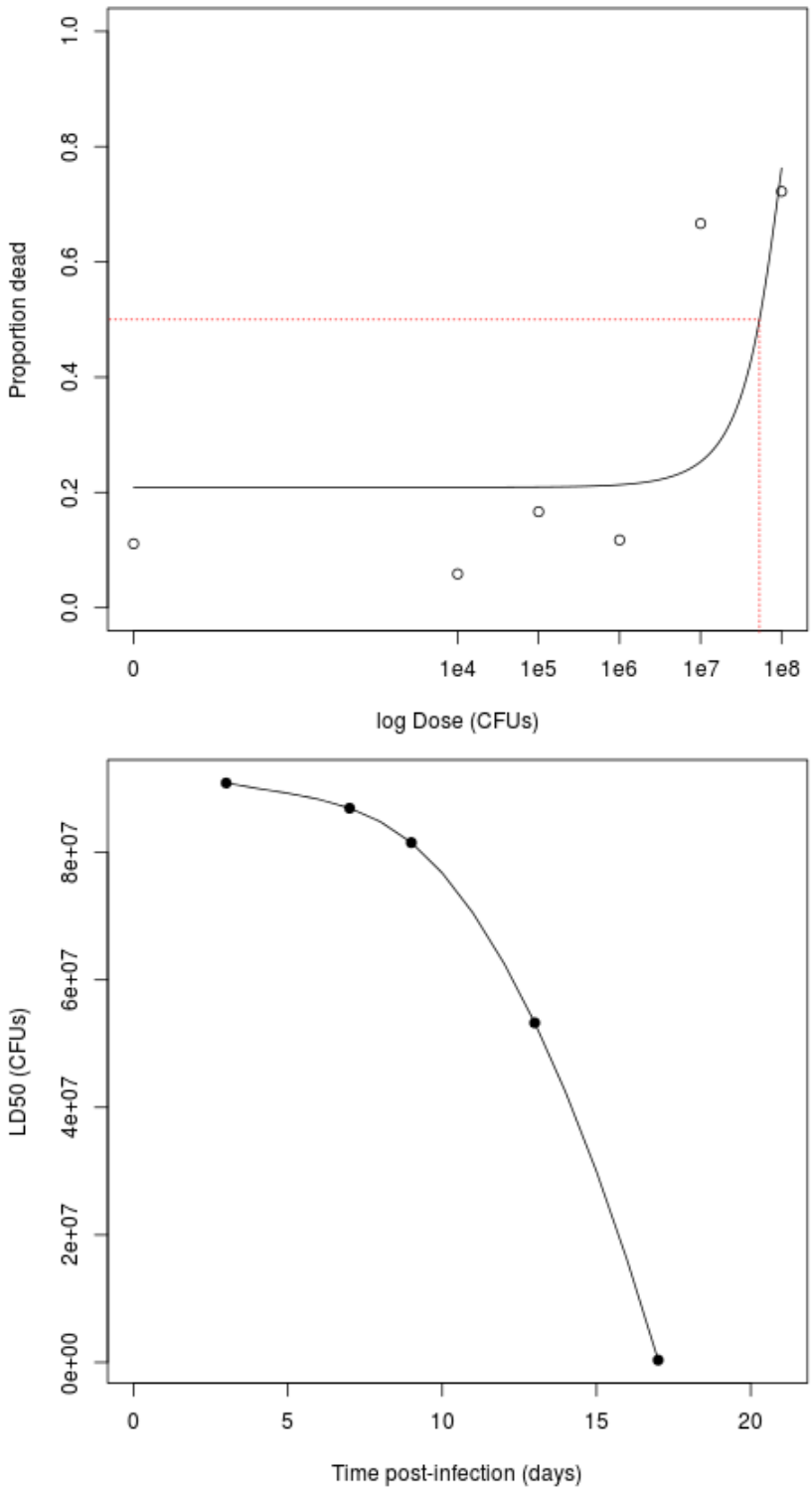


Figure A1.2. (a) Proportion mortality of individuals 14 days after being infected with varying dosages of live *S. aureus*. Red line indicates median lethal dose (LD_{50}) at $5.33x10^7$ CFUs. (b) LD_{50} similarly calculated for different time points post-infection, from 3 days to 17 days.

APPENDIX 2:
IMAGE ANALYSIS OF NYLON ENCAPSULATION AND CUTICULAR MELANISATION

Sample images and calibration data from the image analysis processes described in Chapter 3. The image analysis script, calibration tool and sample images are documented online (https://github.com/JoGall/nylon-encapsulation/).

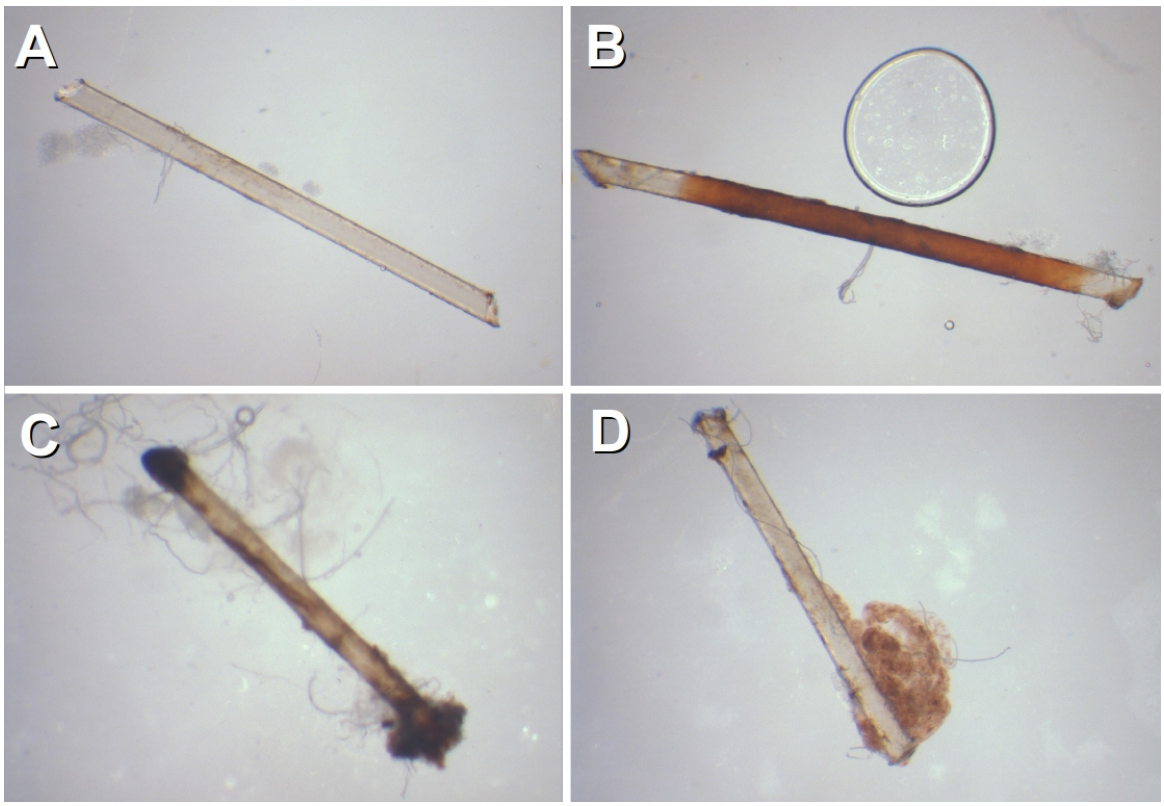


Figure A2.1. Sample images showing the various states of extracted nylon monofilaments. (a) shows low levels of both encapsulation and melanisation as the nylon filament has retained its light colour and has little cellular material attached, (b) shows a high level of melanisation (brown areas) but relatively low encapsulation, (c) shows amount of encapsulation (black areas), (d) shows low levels of encapsulation and melanisation, though with a mass of Malpighian tubules attached that could be mistaken for encapsulating material (light brown area).

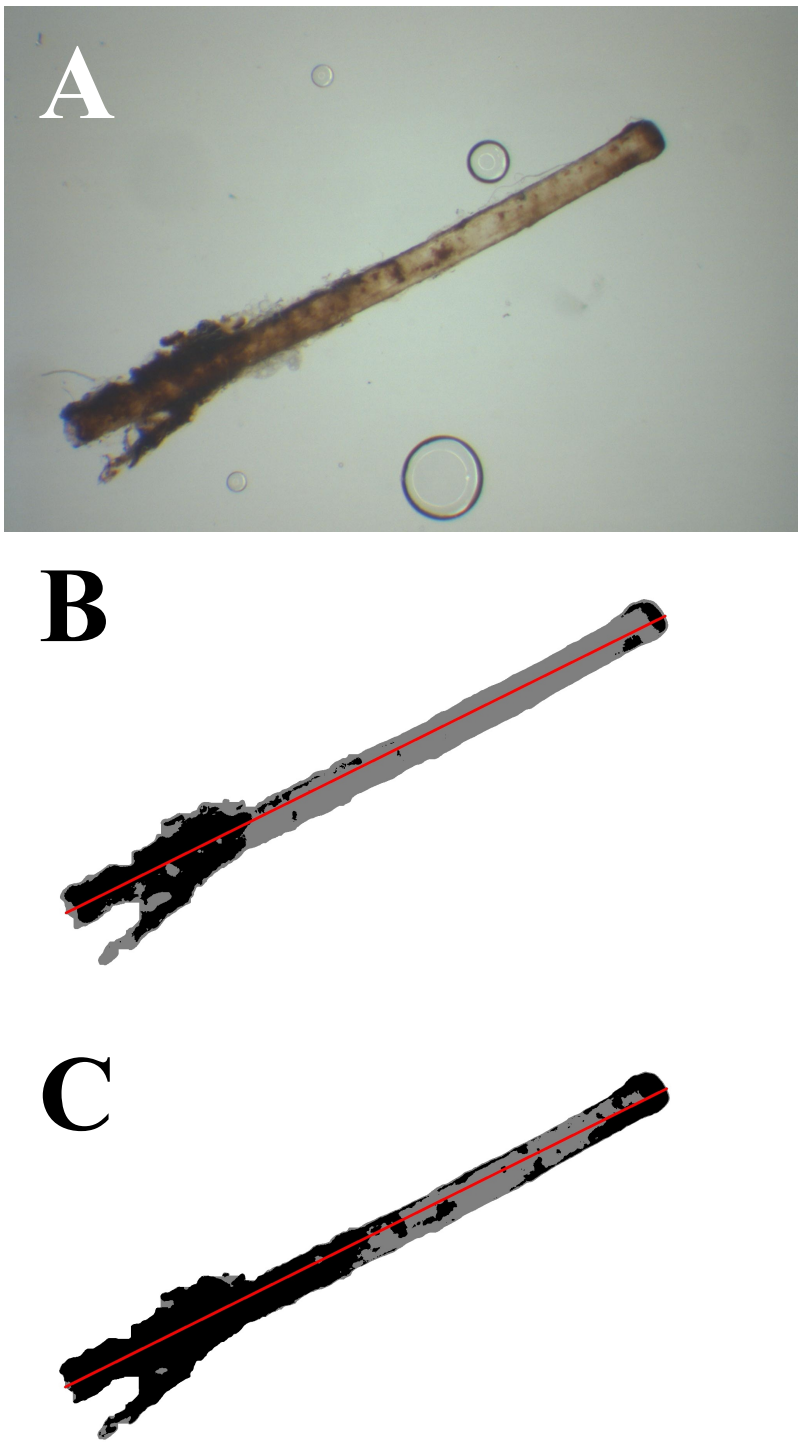


Figure A2.2. (a) Raw image and (b, c) processed image masks of an encapsulated nylon monofilament. In both image masks, grey areas show the identified foreground, in which mean pixel saturation is calculated to produce a melanisation score. Black areas show pixels that are below the user-defined brightness threshold within the foreground area. These pixels are enumerated to give an encapsulation score. A stringent brightness threshold (70) was used to define encapsulating material in (b), whilst a more lenient threshold (110) was used in (c). The red line depicts the calculated length of the monofilament.

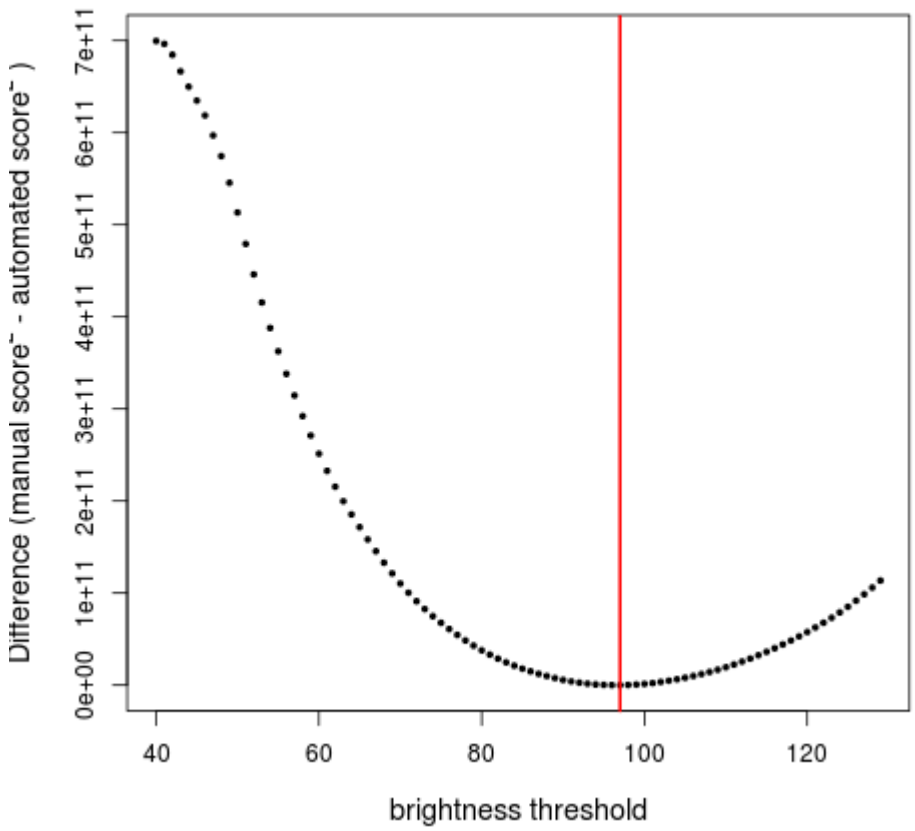


Figure A2.3. Calibration of the user-defined brightness threshold used to define encapsulation score. Iteration of the automated image analysis script across a series of calibration images generated encapsulation scores for all possible brightness thresholds. Automated scores were then compared to manually calculated scores to find the closest matching brightness threshold, as shown by the red line.

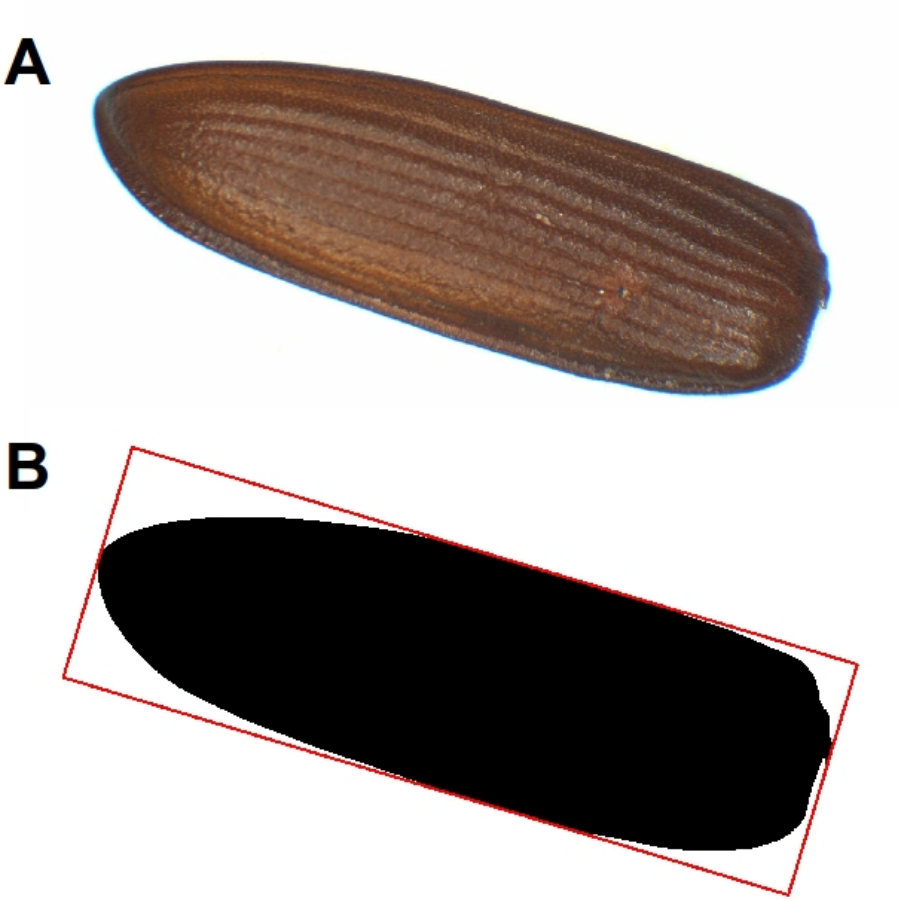
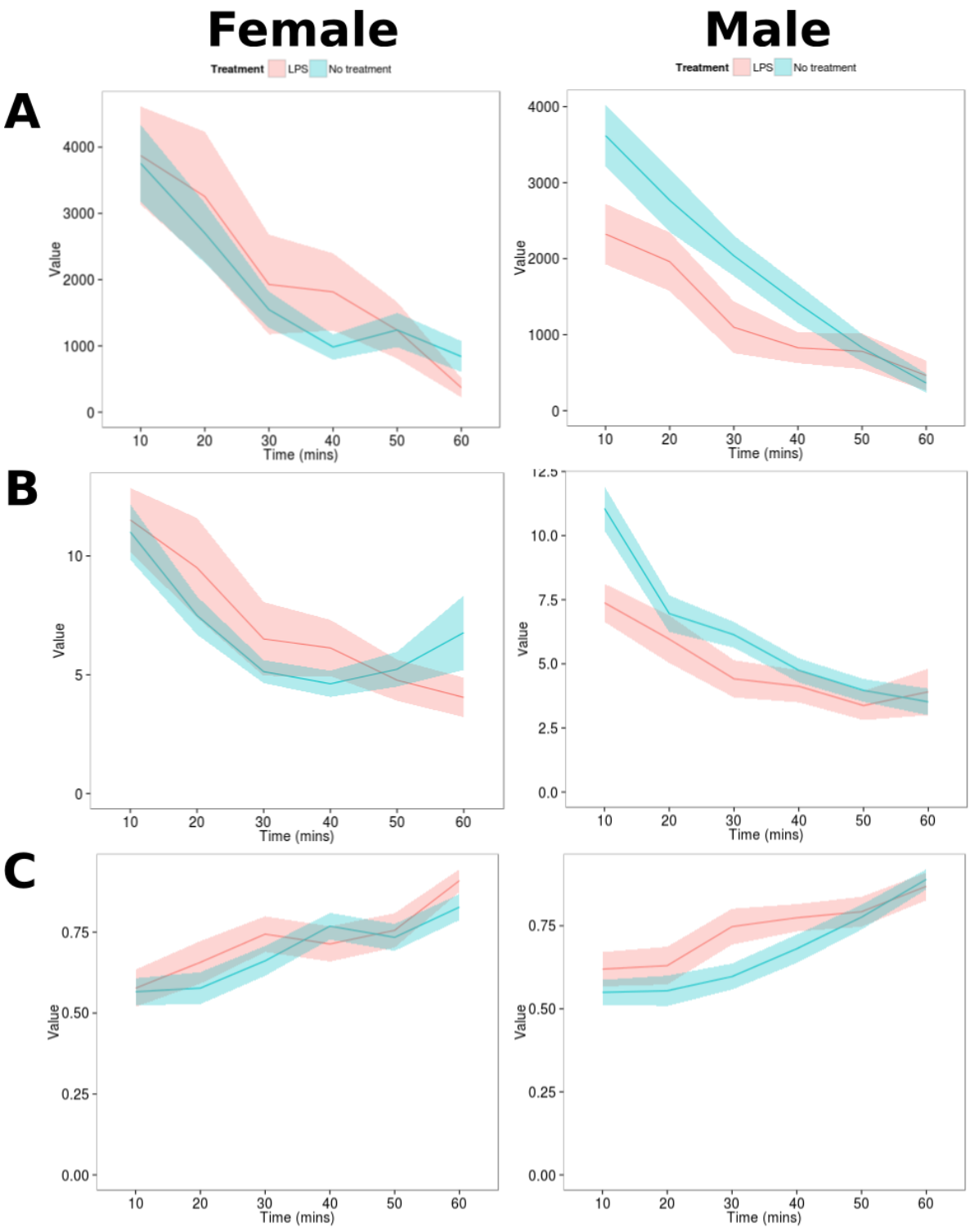


Figure A2.4. (a) Raw image and (b) post-processed image of a *T. molitor* elytron. (b) The black area shows the fitted mask, from which cuticular melanisation was estimated as the mean pixel saturation. Red lines show the minimum bounding rotated rectangle fitted to the mask, from which elytron length was estimated as the length of the rectangle's longest side.

APPENDIX 3:
TEMPORAL PATTERNS IN *Tenebrio molitor* BEHAVIOUR

Additional figures and analyses related to Chapter 5 are presented here, showing differences in various behavioural metrics in *Tenebrio molitor* males and females during non-pathogenic immune stimulation.

[starts overleaf]


Female
Male
Treatment LPS No treatment
A
B
C
Value
Time (mins)

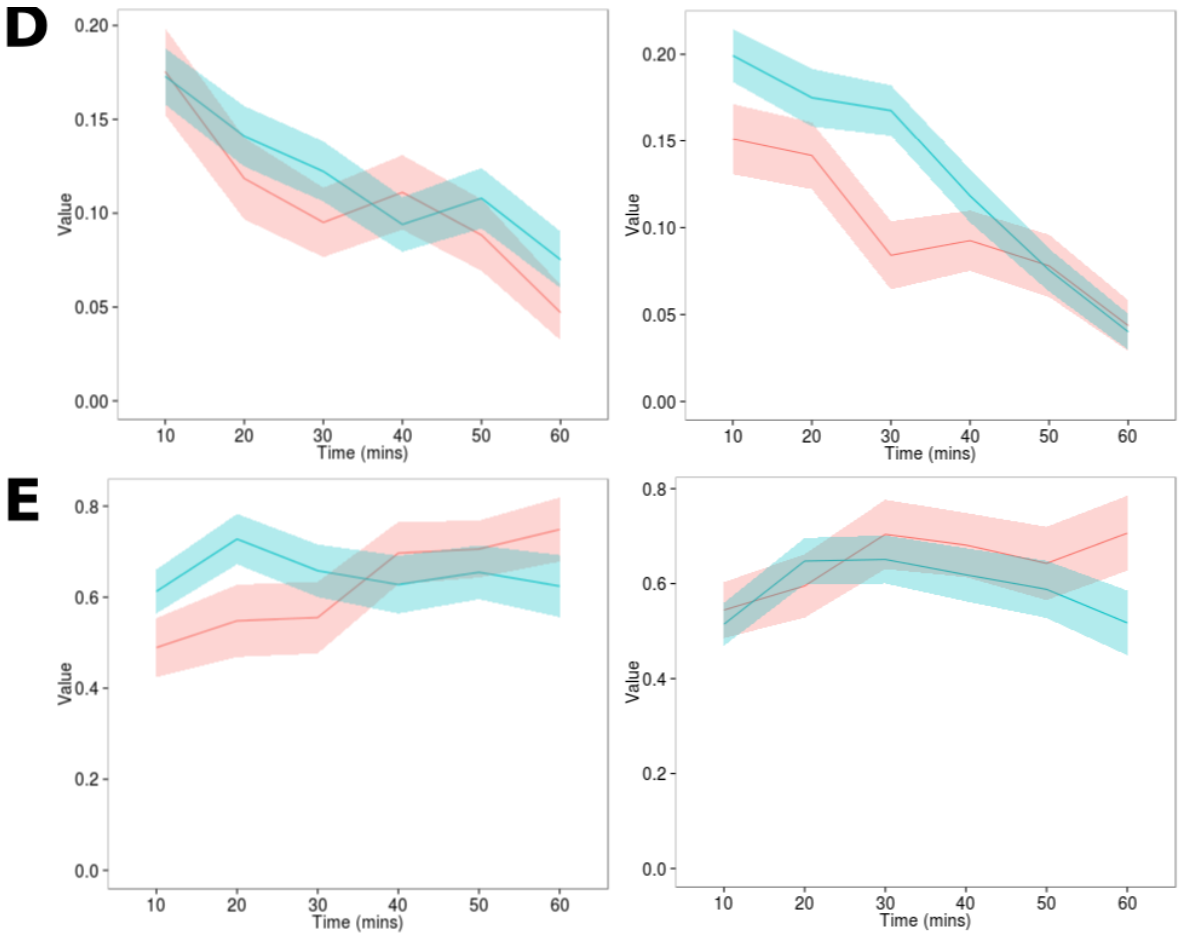


Figure A3.1. Temporal changes in behaviour in males and females during the course of the 60 min observation period, with behaviour following LPS challenged depicted in red and unchallenged baseline behaviour depicted in blue. Behavioural metrics shown are: (A) total distance travelled (mm), (B) mean speed of locomotion (mm/s), (C) proportion of time spent stationary, (D) exploration behaviour (proportion of arena cells visited and (E) thigmotaxis (proportion of time spent in perimeter of arena). Data are binned into 10 min intervals. Lines depict means and shaded areas depict mean ± S.E.M..

APPENDIX 4:

BEHAVIOURAL PREFERENCE OF IMMUNOLOGICALLY NAÏVE *Tenebrio molitor* TOWARDS CHEMOSENSORY CUES PRODUCED BY IMMUNE-CHALLENGED CONSPECIFICS

A4.1. Introduction

Although avoidance of parasitised individuals is predicted to benefit non-social insects by allowing them to reduce the risks of acquiring an infection, there is conflicting evidence on behavioural avoidance in *Tenebrio molitor*, with some studies reporting aversion of parasitised conspecifics and others reporting increased attraction (Carver & Hurd, 1998; Worden *et al.*, 2000; Worden & Parker, 2005; Sadd *et al.*, 2006; Vainikka *et al.*, 2007; Krams *et al.*, 2011; Nielsen & Holman, 2011). This experiment investigated behavioural preference of immunologically naïve *T. molitor* towards immune-challenged conspecifics. The use of an artificial immune elicitor, lipopolysaccharide (LPS), was used to stimulate immunity whilst excluding the potential for parasite manipulation upon any observed changes in attraction. I accounted for the interacting effects of sexual selection on behavioural preference by examining choice in both males and females vs. same-sex and opposite-sex conspecifics.

A4.2. Methods

A.4.2.1. Insect treatments

Insects were cultured as described in Chapter 2, and used in trials between 8-10 days after imaginal eclosion. Behavioural preference was tested using a Y-maze olfactometer (Figure A4.1). In each trial, naïve beetles were presented with a choice of two conspecific treatments; an immune-challenged conspecific, injected with 5µL of 0.5mg/mL lipopolysaccharide (LPS) (as described in detail in Chapter 5), or an unchallenged conspecific (no treatment). In order to investigate whether behavioural choices varied over the course of a conspecific's immune response, separate trials were conducted at 0h, 24 h and 48 h post-challenge. A factorial design was used to test the preference of naïve males and females towards both same-sex and opposite sex conspecifics. However, both treated conspecifics presented in each trial were always of the same sex (i.e. challenged male vs. unchallenged male, or challenged female vs. unchallenged female). Whilst

procedural controls were not conducted here, previous work conducted in *T. molitor* has shown that males and females prefer the odours of healthy and immune-challenged conspecifics over blank odours (i.e. they prefer conspecific presence over absence, regardless of conspecific immune status) (Nielsen & Holman, 2011). Treated beetles were placed in glass vials which were affixed to each arm of the olfactometer. The arm position (left vs right) of treatments was randomised for each trial. Purified air was passed over each vial (flowing towards the olfactometer entrance) at a rate of 1 L/min using a charcoal filtered air delivery system (Analytical Research Systems; OLFM-4C-ADS+V). Airflow was allowed to proceed for at least 1 minute before naïve beetles were introduced into the olfactometer and their position in the arena recorded over 5 minutes.

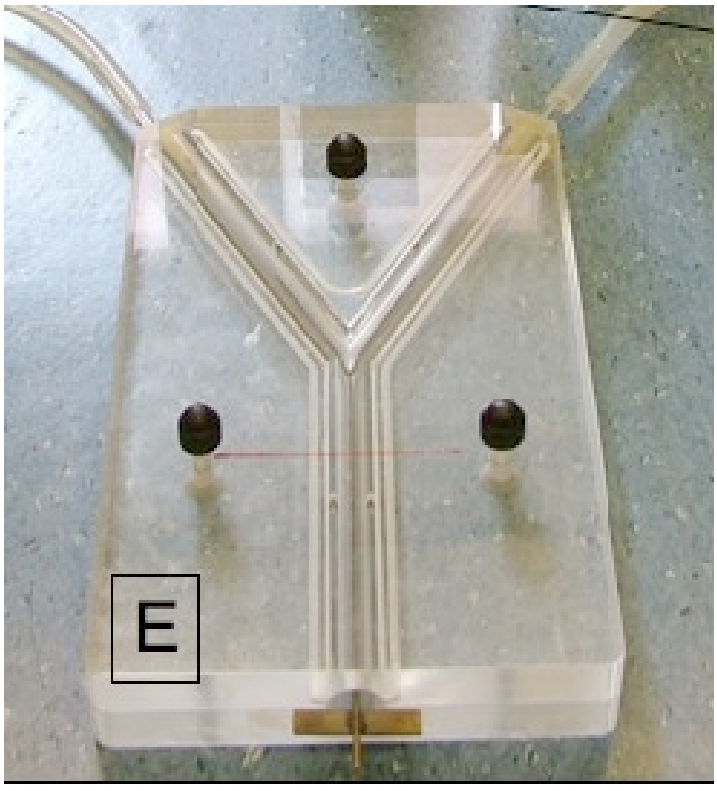


Figure A4.1. Y-maze olfactometer set-up. A vacuum pump blows purified air through each of the sample tubes (top left and top right), which both contain an insect, and downstream towards the olfactometer entrance, E. A focal beetle whose preference is to be tested is introduced into this entrance and its position in the arena recorded as either left arm, right arm, or neutral (central part of tube before bifurcation).

A.4.2.2. Statistical analysis

Two choice metrics were used to measure preference: duration of time spent by naïve beetles in each zone of the olfactometer (challenged, unchallenged and neutral), and first zone chosen by naïve beetles (challenged vs. unchallenged). Duration data were analysed using a general linear model. First choice data were analysed using Chi-squared tests to assess whether choice of treatment arm by naïve beetles significantly differed from random (50%). All analyses were conducted in R (R Core Development Team, http://r-project.org).

A4.3. Results

A.4.3.1. No zone choice

95.6% of individuals made a choice between one of the two arms of the arena during the 5 min observation period.

A.4.3.2. Duration of time spent in each zone

There was an effect of conspecific immune status on male preference for females ($F = 4.15$; $df = 2, 282$; $p = 0.017$) and on female preference for females ($df = 2, 282$; 9.92; $p < 0.001$), but not for either male or female preference regarding males ($p > 0.12$) (Figure A4.2). There was also a significant interaction effect between conspecific treatment and the duration of their infection upon preference in each sex pairing ($p < 0.05$ for all). Post-hoc t-tests conducted within each group showed only one significant difference, with naïve females preferring to remain in the unchallenged zone when choosing between other females at 0 h post-challenge (Table A4.1).

A.4.3.3. First zone choice

In most trials, conspecific treatment (challenged vs unchallenged) had no effect upon preference in naïve beetles (Table A4.2). However, naïve females exhibited a significant avoidance of challenged female conspecifics immediately after immune challenge (infection time = 0), and naïve females exhibited a significant attraction to challenged male conspecifics 48 h after immune challenge (infection time = 48).

A.4.3.4. Time taken to make first zone choice

Males and females did not differ in the length of time taken to make their first choice ($t = 0.81$, $df = 1$, $p = 0.417$), and the mean length of time taken to make a choice for both

sexes combined was 28.19 ± 1.18 s (mean ± S.E.). On average, males and females took longer to make a decision when they first chose the olfactometer arm containing an immune-challenged conspecific (30.36 ± 2.02 s) than the arm containing an unchallenged individual (26.29 ± 1.41 s), although this difference was not significant (t = 1.65, df = 1, p = 0.099).

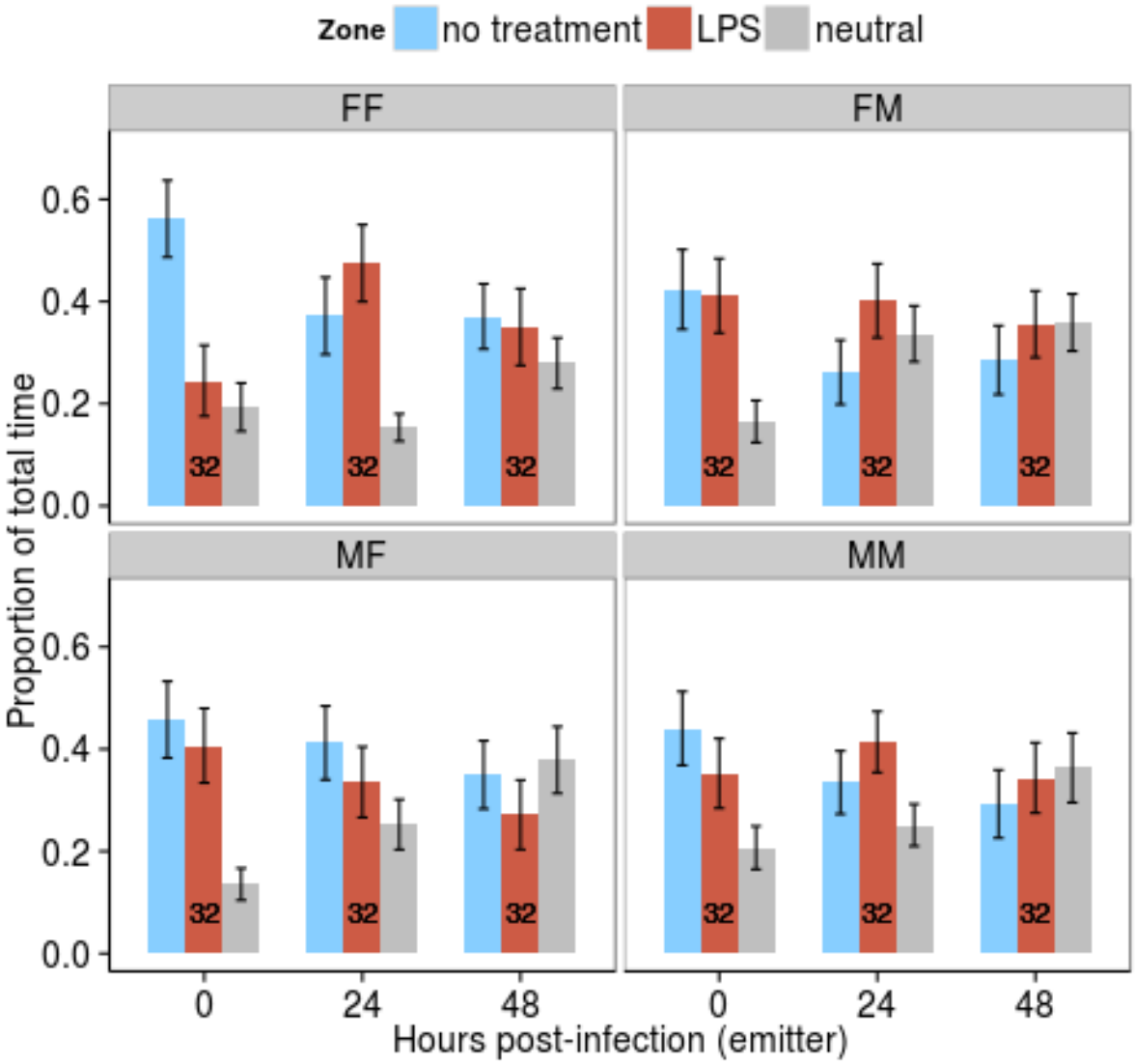


Figure A4.2. Proportion of the total observation period (5 minutes) spent by naïve beetles in each zone of the Y-tube olfactometer (immune-challenged conspecific zone [LPS; red], unchallenged conspecific zone [no treatment; blue], neutral zone [neutral; grey]). (a) naïve females choosing between treatment females, (b) naïve females choosing between treatment males, (c) naïve males choosing between treatment females, (d) naïve males choosing between treatment males. Bars indicate mean ± S.E.

Table A4.1. Results of t-tests on time spent in zone data (time spent in neutral zone excluded). Significant results ($p<0.05$) are highlighted in bold.

Naïve sex	**Challenged sex**	**Infection time**	**Pref?**	**Statistics**
Female	Female	0	**yes**	**t = 3.17, df = 61.71, p = 0.002**
Female	Female	24	no	t = 0.966, df = 62, p = 0.3377
Female	Female	48	no	t = 0.211, df = 60.44, p = 0.8334
Female	Male	0	no	t = 0.115, df = 61.693, p = 0.9087
Female	Male	24	no	t = 1.46, df = 60.873, p = 0.1486
Female	Male	48	no	t = 0.752, df = 61.931, p = 0.4549
Male	Female	0	no	t = 0.488, df = 61.953, p = 0.6274
Male	Female	24	no	t = 0.768, df = 61.878, p = 0.4455
Male	Female	48	no	t = 0.822, df = 61.975, p = 0.4144
Male	Male	0	no	t = 0.883, df = 61.788, p = 0.3804
Male	Male	24	no	t = 0.924, df = 61.931, p = 0.3592
Male	Male	48	no	t = 0.533, df = 61.933, p = 0.5957

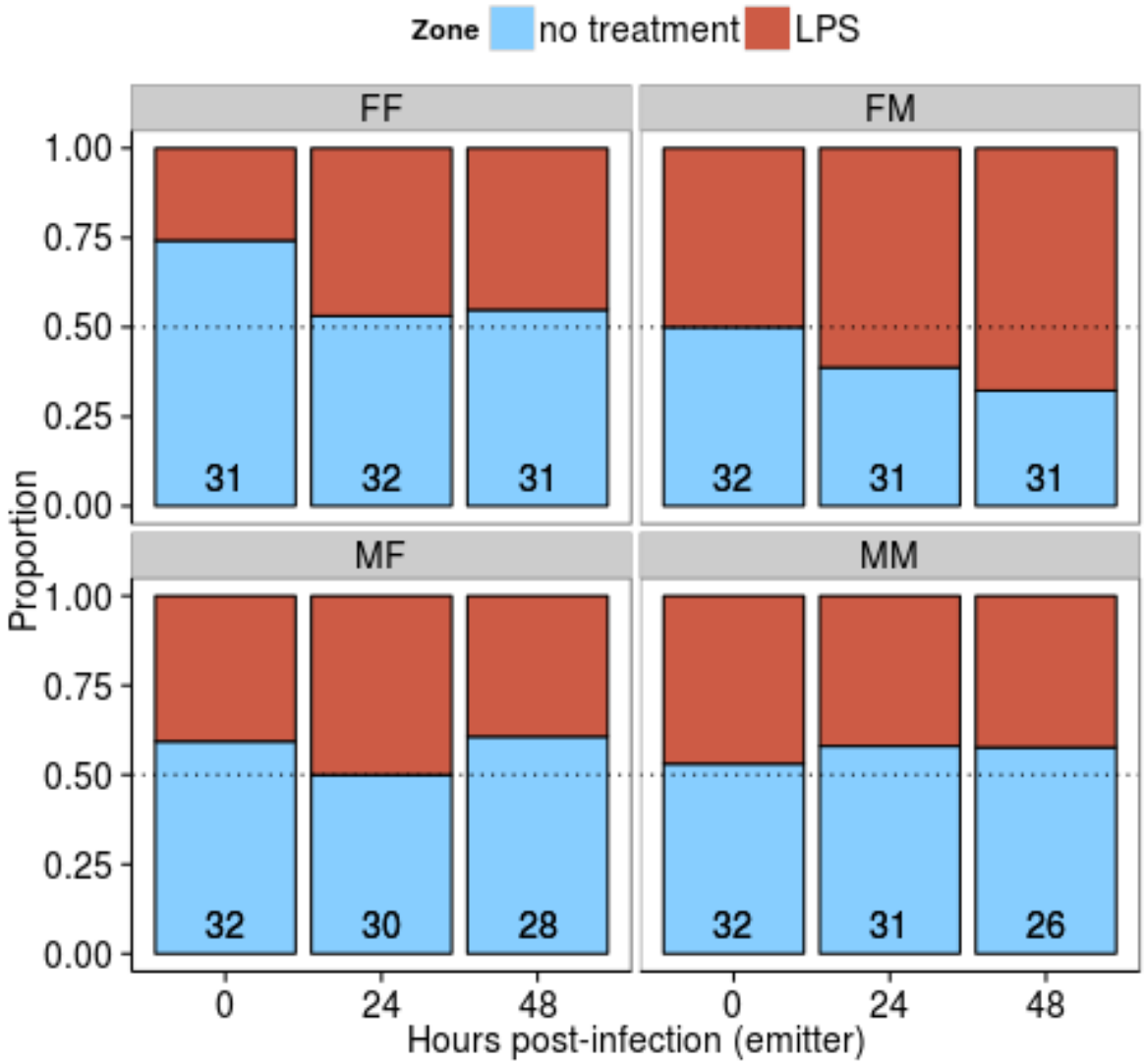


Figure A4.3. First treatment zone of the Y-tube olfactometer entered by naïve beetles (immune-challenged conspecific zone [LPS; red], unchallenged conspecific zone [no treatment; blue]). (a) naïve females choosing between treatment females, (b) naïve females choosing between treatment males, (c) naïve males choosing between treatment females, (d) naïve males choosing between treatment males. Dashed line reflects random zone choice (50%).

Table A4.2. Results of chi-squared tests on first zone chosen data. Significant results ($p<0.05$) are highlighted in bold.

Naïve sex	Challenged sex	Infection time	Pref?	Statistics
Female	**Female**	**0**	**yes**	**$\chi2 = 7.258$, df = 1, p-value = 0.007**
Female	Female	24	no	$\chi2 = 0.125$, df = 1, p-value = 0.724
Female	Female	48	no	$\chi2 = 0.290$, df = 1, p-value = 0.590
Female	Male	0	no	$\chi2 = 0.00$, df = 1, p-value = >0.999
Female	Male	24	no	$\chi2 = 1.58$, df = 1, p-value = 0.209
Female	**Male**	**48**	**yes**	**$\chi2 = 3.90$, df = 1, p-value = 0.048**
Male	Female	0	no	$\chi2 = 1.13$, df = 1, p-value = 0.289
Male	Female	24	no	$\chi2 = 0.00$, df = 1, p-value = >0.999
Male	Female	48	no	$\chi2 = 1.29$, df = 1, p-value = 0.257
Male	Male	0	no	$\chi2 = 0.13$, df = 1, p-value = 0.724
Male	Male	24	no	$\chi2 = 0.81$, df = 1, p-value = 0.369
Male	Male	48	no	$\chi2 = 0.62$, df = 1, p-value = 0.433

A4.4. Discussion

These data suggest that *Tenebrio molitor* do not exhibit aversion towards the volatile odours of immune-challenged conspecifics, either of same of the sex or opposite sex. Instead, naïve females actually appeared to become preferentially attracted towards immune-challenged males that had been insulted 48 h earlier, supporting data from the literature which suggests that immune-challenged males become more attractive due to terminal investment in reproduction through an increased investment in sexual signalling (Sadd *et al.*, 2006; Nielson & Holman, 2011; Krams *et al.*, 2011). This experimental design relied on the detection of volatile (airborne) cues of infection by naïve individuals, but it is possible that less volatile semiochemical cues may be involved in the detection of conspecific immune status. For example, cuticular hydrocarbons (CHCs) are known to change quantitatively during immune insult in *T. molitor* (Nielson & Holman, 2011), but are long-chained and poorly volatile, and necessitate direct physical contact to be detected. The role of visual cues of infection, such as sickness behaviours, upon induction of immune defence (either physiological or behavioural) also remains to be investigated. It is possible that olfactory and visual cues of infection act synergistically to inform a process of gregarious immunisation in *T. molitor*.

APPENDIX 5:

ALTERED IMMUNE INVESTMENT OF NAÏVE *Tenebrio molitor* IN RESPONSE TO THE ENVIRONMENTAL PRESENCE OF MICROBIAL VOLATILES

A5.1. Introduction

Many bacteria and fungi release microbial volatile organic compounds (MVOCs) which diffuse readily through the environment (Davis et al., 2012), and can be detected by a diverse range of host species (Farag *et al.*, 2013). Several studies in insects have found that hosts exhibit a range of behavioural responses following the detection of MVOCs in the environment, including physical avoidance, reduced feeding and reduced oviposition (Tasin *et al.*, 2012; Hussain *et al.*, 2010; Yanagawa *et al.*, 2011; Ormond *et al.*, 2011; Mburu *et al.* 2012; Sun *et al.*, 2008; Stensmyr *et al.*, 2012; Lam *et al.*, 2010; Villani *et al.*, 1994; Myles, 2002; Zhang *et al.*, 2005). Here, I investigated whether *Tenebrio molitor* modulates its investment in physiological immunity as a form of prophylactic defence against a perceived pathogenic threat in the environment.

A5.2. Methods

A5.2.1. MVOC preparation

Erythromycin-resistant *Staphylococcus aureus* were cultured as described in Chapter 2. Briefly, a single *S. aureus* colony was suspended in 30mL Luria broth (2% LB, 10µg/mL erythromycin, 5.6µg/mL amphotericin-B) and cultured for 48 h in a shaking incubator (37°C, 110rpm) until reaching a stationary phase. Bacterial population density was estimated turbidometrically by measuring the optical density (OD650nm) of the bacterial suspension using a microplate reader (VersaMax) and referencing a previously calculated calibration curve, before diluting the suspension down to a final solution of $5x10^6$ CFUs/mL. 200µL of this suspension was then plated onto erythromycin-infused agar (2% LB, 1.5% agar, 10µg/mL erythromycin, 5.6µg/mL amphotericin-B) and incubated at 37°C for 24 h to yield a plate with approximately 10^6 CFUs. Bacterial MVOCs were then extracted by adding 5mL hexane to bacterial plates and agitating for 1 min. MVOCs have been shown to be extractable in hexane (Crespo *et al.*, 2008). To ensure that beetles would not become primed through direct infection with live bacteria which may be present in the

hexane solution, the presence of live bacteria was tested for by plating out 200µL of undiluted hexane solution on control agar (2% LB, 1.5% agar, 5.6µg/mL amphotericin-B) and incubating for 48 h at 37°C. No CFUs were detected on any plate (n=8).

A5.2.2. Insect treatments

Beetles were cultured as described in Chapter 2, and used in the experiment at 8-10 days post-imaginal eclosion. Immunologically naïve (untreated) beetles were housed in a small Petri dish (50mm diameter) with *ad libitum* access to Progrub, to which 1mL of hexane suspension was added. Beetles were housed together for 72 h, and then randomly assigned to one of two sub-treatments; one which measured constitutive (pre-challenge) defence through immediate immune assay, and one which measured inducible (post-challenge) defence by challenging beetles and assaying them 24 h later.

Beetles in the pre-challenge group had their haemolymph extracted and analysed immediately after the hexane exposure period. Those in the post-challenge group were wounded through the insertion of a sterile stainless steel pin (0.15mm diameter) between the third and fourth abdominal sternites, before having their haemoylmph extracted for analysis 24 h later. Haemolymph extraction, phenoloxidase (PO) activity analysis and haemolymph protein concentration analysis were all performed as described in Chapter 3.

A5.2.3. Statistical analysis

Linear regressions were built for each response variable using R (v3.1.2; R Development Core Team, 2014), with PO activity and total PO activity log-transformed for normality. For each regression, statistical models were optimised using a stepwise procedure, working backwards from maximal models that included all main effects (sex [male or female], treatment [MVOC hexane or non-MVOC hexane], and challenge status [pre-challenge or post-challenge]) and all possible interactions. Additional models were built to test for the effects of MVOCs within each gender independently by omitting sex and its interactions as fixed effects from the models.

A5.3. Results

A5.3.1. Overall linear model

The interaction between hexane treatment and challenge status had a significant effect upon total PO activity ($F_{5,212} = 5.90$, $p = 0.016$), as well a marginally non-significant effect upon haemolymph protein concentration ($F_{5,212} = 3.93$, $p = 0.051$; Table A5.1). There was

a marginal effect of treatment upon observed PO activity ($F_{5,212}$ = 3.26, p = 0.072) and protein concentration ($F_{5,212}$ = 3.67, p = 0.059), but there were no effects of gender upon any immune response.

A5.3.2. Male immune response

In males, the type of hexane exposure had a significant effect upon PO activity ($F_{3,212}$ = 2.83, p = 0.005) and proPO activity ($F_{3,212}$ = 2.15, p = 0.033). Males exposed to bacterial volatiles exhibiting significantly lower levels of induced total PO (combined PO and proPO) activity than those exposed to the blank hexane control, although this difference in induced PO activity between treatments was non-significant. There was no interaction effect between the presence of bacterial volatile and immune challenge status upon either PO or proPO activity (p>0.1). There was also a significant effect of bacterial volatile presence on haemolymph protein concentrations in males ($F_{3,70}$ = 2.56, p=0.014), and a non-significant interaction effect between bacterial volatile presence and immune challenge challenge status ($F_{3,70}$ = 1.98, p = 0.052).

A5.3.3. Female immune response

In females, there was no significant effect of bacterial volatile presence or immune challenge status (pre-challenge vs. post-challenge) upon either PO or proPO activity, nor any interaction effects between the two variables (p > 0.7 for all). Haemolymph protein concentration was not measured in females pre-challenge, although analysis of post-challenge females revealed no effect of bacterial volatile presence on post-infection protein concentrations (p > 0.2).

Table A5.1. Parameters for linear models fitted to each of the immune measures taken from naïve beetles. Significant effects (p<0.05) are highlighted in bold. The parameters for main effects are excluded in models where their interactions are significant.

	PO activity		Total PO activity		Haemolymph protein	
Term	F	P-value	F	P-value	F	P-value
sex	0.578	0.448	0.294	0.588	0.539	0.465
treatment	3.257	0.072	-	-	3.674	0.059
challenge	2.315	0.129	-	-	0.145	0.705
sex x treatment	0.153	0.696	0.673	0.412	0.496	0.483
sex x challenge	0.008	0.929	0.003	0.954	-	-
treatment x challenge	0.532	0.466	**5.899**	**0.016**	3.929	0.051
sex x treatment x challenge	0.495	0.482	0.404	0.526	-	-

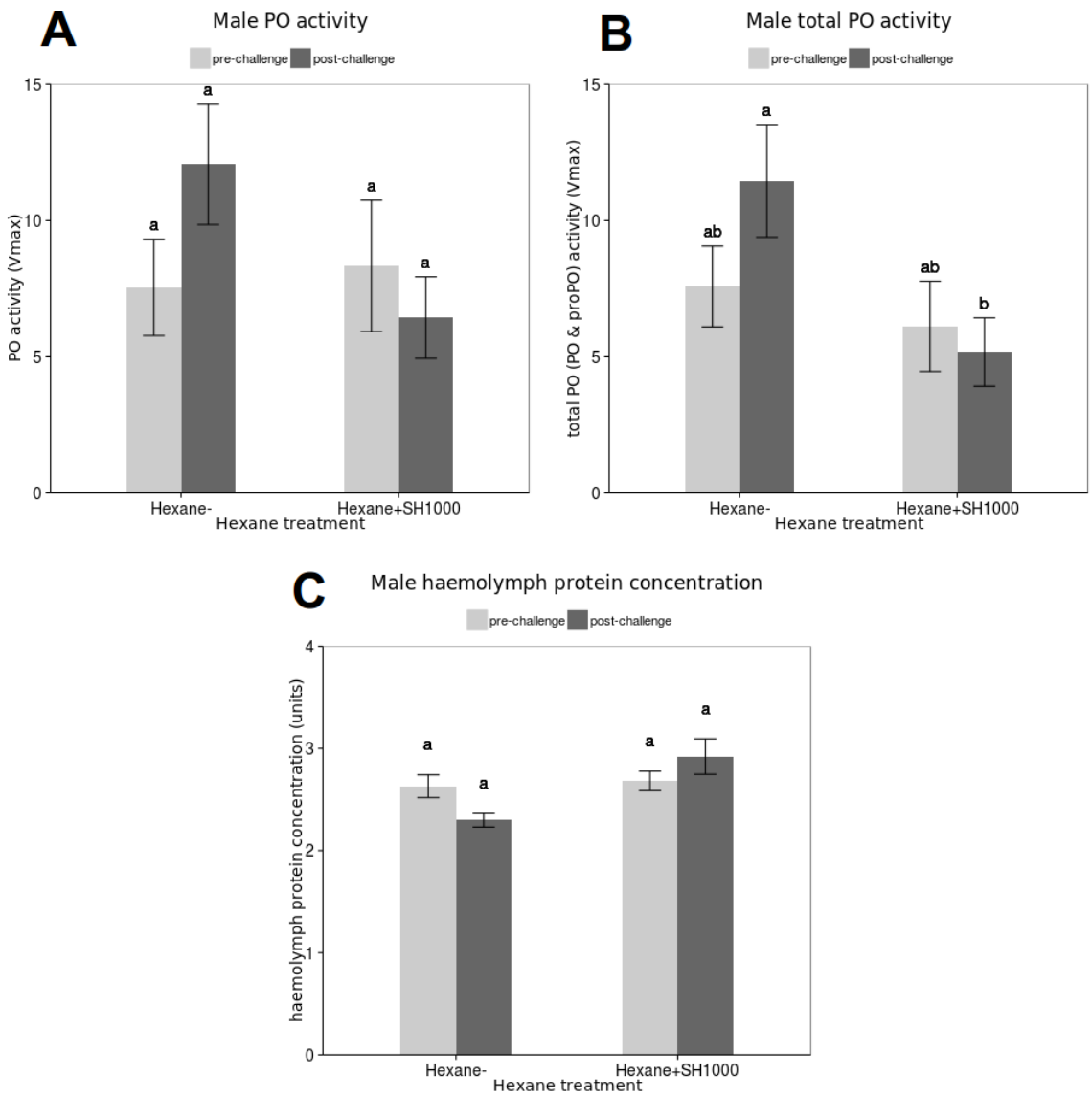


Figure A5.1. Immune activity of male *T. molitor* following 72 h exposure to the environmental presence (Hexane+SH1000) or absence (Hexane-) of bacterial volatiles, and either before (light bars) or after (dark bars) a subsequent immune challenge (sterile stab). (A) shows PO activity, (B) shows total PO activity (PO and proPO activity combined), and (C) shows haemolymph protein concentrations. Bars that do not share a letter differ significantly ($p < 0.05$; Tukey's Honest Significant Differences Test).

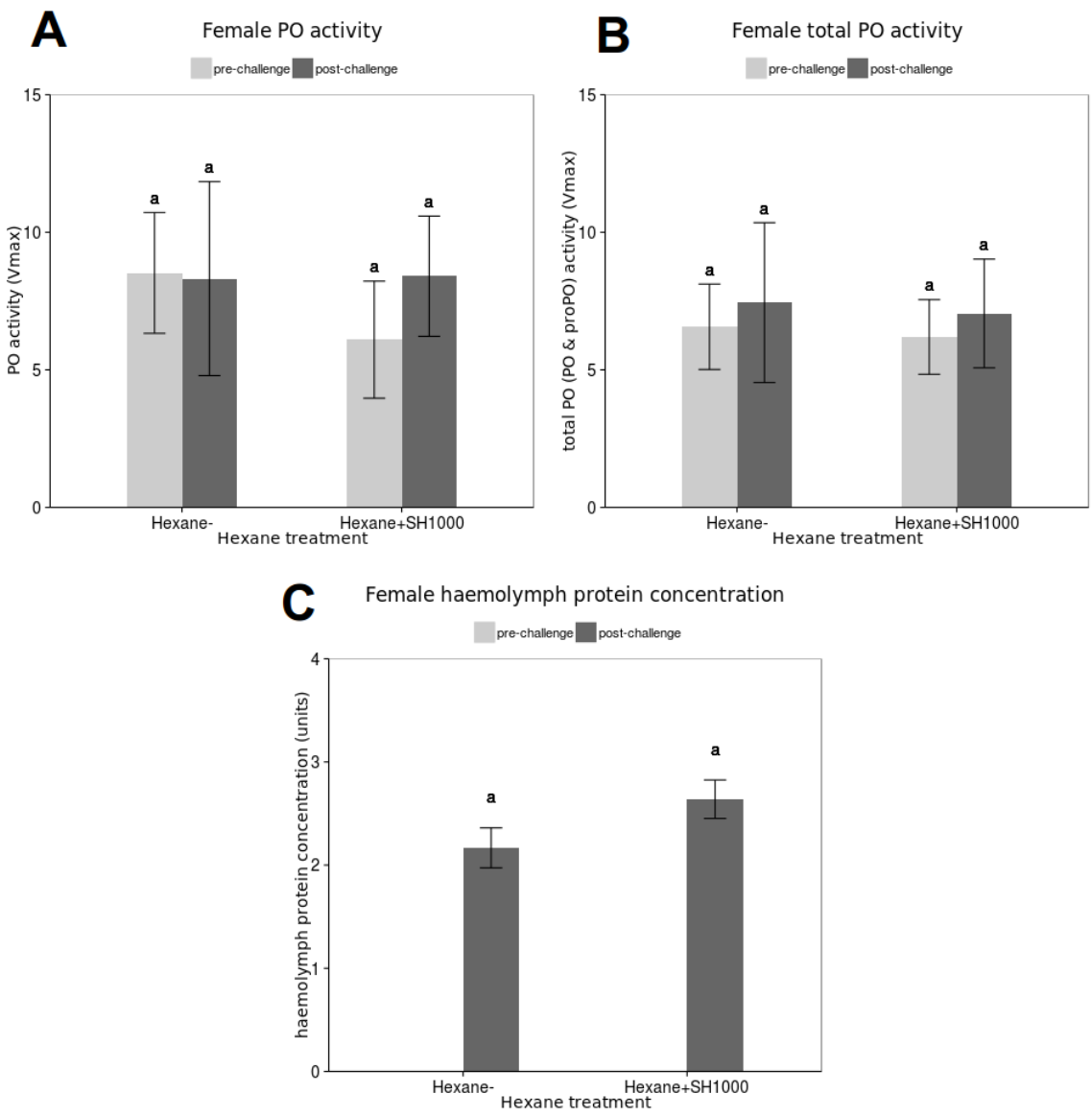


Figure A5.2. Immune activity of female *T. molitor* following 72 h exposure to the environmental presence (Hexane+SH1000) or absence (Hexane-) of bacterial volatiles, and either before (light bars) or after (dark bars) a subsequent immune challenge (sterile stab). (A) shows PO activity, (B) shows total PO activity (PO and proPO activity combined), and (C) shows haemolymph protein concentrations, although data are only available for females post-challenge. Bars that do not share a letter differ significantly ($p < 0.05$; Tukey's Honest Significant Differences Test).

A5.4. Discussion

Naive males, though not females, appeared to downregulation production of an important humoral immune effector following exposure to chemosensory cues of pathogenic threat, as provided by bacterial MVOCs. This complements data showing reduced immune investment in males, but not females, following cohabitation with an immune-challenged conspecific (Chapter 3). Combined, these data suggest that *Tenebrio molitor* are able to detect cues of infection threat from their environment, and that these cues may be chemosensory in nature. Terminal investment in reproduction as a life-history escape response as a prophylactic response to the perceived costs of parasitic threat. However, future work will benefit from gathering measures of reproductive success from naïve individuals during environmental MVOC exposure to explicitly define a role for terminal investment as a preventative form of infection tolerance.