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Ecological Immunology:

Immune Defence Strategies of Australian Locusts

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STATEMENT OF AUTHORSHIP

I certify that the research chapters in this thesis have not previously been submitted either in whole or as part of the requirements for a degree.

I also certify that this thesis has been written by me. Any help that I have received in my research and the preparation of the thesis itself has been acknowledged within the text.

In addition, I certify that all information sources and literature used are indicated in the thesis.

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Dr Fleur Ponton

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"You have to give yourself credit, not too much because that would be bragging." Frank M^{c}Court, *Teacher man*

To everyone who helped, supported and encouraged me in the production of this thesis, thank you.

This thesis is dedicated to Roman, Frances and Stefan Pulpitel

ABSTRACT

Immune defence is regulated and implemented through behaviour and physiology, both of which are greatly influenced by a host's environment. When studying fitness traits and immune function it is therefore critical to consider the fundamental ecological factors that contribute to a host's immunity. This idea of ecological immunology is a rapidly expanding field, extending far beyond the direct host-parasite interaction studies classically seen throughout the literature. This thesis investigates the immunology of Australian locusts (Orthoptera: Acrididae) in a broad ecological context.

In Chapter 2, the density-dependent prophylaxis (DDP) hypothesis was tested in the Australian plague locust, *Chortoicetes terminifera*. This theory predicts that animals living in groups will exhibit greater investments in immune function compared to solitarious conspecifics. Accordingly, *C. terminifera* at different population densities were tested for variation in both constitutive and responsive immune function using the fungal pathogen, *Metarhizium acridium*. Interestingly, through a series of survival and conventional immune assays, different immune traits were found to both support and oppose the DDP theory. This ultimately accounts for the contradictory literature that currently exists in DDP in locusts.

The effect of cannibalism on the transmission of disease was investigated in Chapter 3. In choice experiments, starved locusts were found to cannibalise uninfected victims significantly more than fungal-infected victims. In the absence of alternative resources, locusts succumbed to starvation rather than ingest infected victims, revealing the striking adaptation of *C. terminifera* to avoid fungal infected conspecifics.

Given these results, Chapter 4 tested for predictive immune activation in *C. terminifera*. After measuring several immune traits in locusts, exposed to both directly and by proximity to live or dead infected conspecifics, physiological immune anticipation in this species could not be detected.

In Chapter 5, gene expression assays were developed in *Locusta migratoria* as a molecular technique to detect small differences in immune gene expression, both across different tissue types and between different immune states. These assays found distinct tissue-specificity in expression levels across genes, highlighting the importance of tissue selection when conducting such assays.

Finally, gene expression assays were applied to *L. migratoria* in Chapter 6 to identify possible changes in constitutive immunity in response to the introduction of commensal bacteria to the gut. Conventional immune assays were also performed and used to compare detection sensitivity of all approaches. These experiments arose fortuitously, following the search for a potential locust pathogen observed to cause a mass epizootic of locusts in the field in 2010. Although this was a preliminary study, data suggest that the alteration of gut microbiota communities can induce subtle immune responses within hosts.

By looking at some of the behavioural and physiological phenomena commonly used to describe (and in some cases define) locusts, this work has been able to tie together some of the mechanisms that contribute to the survival and success of locusts in the field. As one of the world's most destructive agricultural pests, a wider insight into the immune function of locusts is of particular ecological and economic interest, particularly in the context of potential biocontrol strategies.

TABLE OF CONTENTS

Statement of Authorship ..... **i**

Abstract ..... **iii**

Chapter 1 ..... **1**
General Introduction **1**
1.1 Living with microbes 2
1.2 Ecological immunology 2
1.3 Evolution of immune function 3
1.4 Types of immune function 4
1.4.1 Pathogen defence: behaviours and barriers 4
1.4.2 Infection response: innate and adaptive immunity 5
1.5 Trade-offs and resource allocation 7
1.6 The value of microbial communities 8
1.7 Insect immunology: a model for innate immunity 10
1.8 Techniques in invertebrate immunology 11
1.8.1 Conventional immune measurements 11
1.8.2 Molecular techniques 12
1.9 Locusts as a model for integrating ecological immunology 12
1.9.1 Locust ecology 12
1.9.2 Behavioural phase polyphenism in locusts 13
Adaptive polyphenism 13
Density-dependent phase polyphenism in locusts 14
Influences and implications of phase change in locusts 15
1.10 Locust immunity 17
1.11 Applied importance 18
1.12 Study species 19
1.13 Project aims 20

Chapter 2 ..... **22**
Density-dependent Prophylaxis in the Australian Plague Locust **22**
2.1 Summary 23
2.2 Introduction 24
2.2.1 Group living and effects of density 24
2.2.2 Density-dependent prophylaxis 25
DDP in insects 25
DDP and phase polyphenism in locusts 27
2.2.3 Aims 29
2.3 Materials and methods 30
2.3.1 Insect cultures, experimental setup and treatments 30
Solitary-reared insects 30
Gregarious culture 31
2.3.2 Infection treatments with *M. acridium* 31
2.3.3 Phase treatments 32
2.3.4 Dose-dependent LD_{50} survival assay 33
2.3.5 Density-dependent immune assays and haemolymph collection 33
2.3.6 Second-generation behavioural phase assays 34
2.3.7 Immune and host condition assays 34
Total haemocyte counts (THCs) 34
Lysozyme activity 35
Prophenoloxidase activity 36
Haemolymph protein assays 36
2.3.8 Analyses 37
2.4 Results 38

2.4.1 Dosage-dependent survival assays: lethal dose 50 (LD_{50}) 38
2.4.2 First-generation solitarious versus long-term gregarious locusts 39
Total haemocyte counts 39
Lysozyme concentration 39
Prophenoloxidase activity 40
Haemolymph protein concentration 40
2.4.3 Second-generation solitarious versus long-term gregarious locusts 40
Total haemocyte counts 40
Lysozyme concentration 41
Prophenoloxidase activity 41
Haemolymph protein concentration 41
2.5 Discussion 42
2.5.1 Density-dependent prophylaxis in *C. terminifera* 42
2.5.2 Resistance to fungal infection: locust phase and fungal dose 43
2.5.3 Phase duration and immune function 44
Total haemocyte counts 44
Lysozyme activity 46
Prophenoloxidase activity 48
Haemolymph protein concentration 50
2.5.4 Density-dependent phase polyphenism and locust immunity 51
2.5.5 Conclusions and applied significance 53

Chapter 3 55
Cannibalism and Immunity 55
3.1 Summary 56
3.2 Introduction 57
3.2.1 Pathogen transmission and other costs of cannibalism 57
3.2.2 Cannibalism and collective movement in Orthoptera 58
3.2.3 Vulnerability increases the risk of cannibalism in locusts 59
3.2.4 Aims 61
3.3 Materials and methods 61
3.3.1 Insect cultures and treatments 61
3.3.2 Victim generation 62
Pathogen handling and locust inoculation 62
Victim generation: infection stage treatments 62
3.3.3 Cannibal generation and choice behavioural assays 64
Choice assays 64
Video data collection 65
3.3.4 Starvation and no-choice assays 65
Starvation curve generation 65
No-choice assays 66
3.3.5 Analyses 66
3.4 Results 67
3.4.1 Behavioural choice assays: victim selection 67
First victims visited 67
Number of visits 67
Visit duration 68
Physical evidence of cannibalism 69
Cannibal survival: pathogen transmission 70
3.4.2 Starvation and no-choice assays 70
3.5 Discussion 70
3.5.1 Locusts avoid ingestion of fungal-infected conspecifics 70
3.5.2 Infection avoidance eliminates pathogen transmission through cannibalism 73
3.5.3 Cannibalism, infection and plague control 75
3.5.4 Conclusions and future research 76

Chapter 4 ..... **78**
Predictive Immune Function **78**
4.1 Summary 79
4.2 Introduction 80
4.2.1 Infection prediction and detection 80
Immune anticipation in response to ecological factors 80
Response, protection and immune priming 81
Group and social immunity in insects 83
4.2.2 Pathogen recognition demonstrated in locusts 84
4.2.3 Aims 84
4.3 Materials and methods 85
4.3.1 Test and stimulus insects: control, infected, alive and dead 85
4.3.2 Proximity exposure: live infected and uninfected stimuli 86
4.3.3 Direct contact exposure: live and dead uninfected stimuli 86
4.3.4 Direct contact exposure: infected and uninfected dead stimuli 87
4.3.5 Haemolymph collection and assays 88
4.3.6 Analyses 88
4.4 Results 89
4.4.1 Proximity exposure to live infected, live control or no stimulus groups 89
4.4.2 Stimulus insects: fungal infected versus uninfected controls 90
4.4.3 Direct contact exposure: effects of live and dead uninfected stimuli 91
4.4.4 Direct contact exposure with infected and uninfected dead stimuli 92
4.5 Discussion 93
4.5.1 Fungal infection and detection 94
4.5.2 Time-dependent increases in immune function 96
4.5.3 Immune assay sensitivity 97
4.5.4 Conclusions and further research 98

Chapter 5 ..... **100**
Tissue-specific Immune Gene Expression **100**
5.1 Summary 101
5.2 Introduction 102
5.2.1 Immune specificity in insects 102
5.2.2 Tissue-specific immune responses 103
5.2.3 Study species and applied importance 104
5.2.4 Aims 105
5.3 Materials and methods 106
5.3.1 Insect cultures and injection treatments 106
5.3.2 Insect bleeding, dissections and tissue collection 106
5.3.3 Gene expression assays 107
RNA isolation 107
Reverse transcription and cDNA synthesis 108
Primers 108
Reverse transcription quantitative polymerase chain reactions (RT-qPCR) 109
5.3.4 Data mining and statistical analyses 110
5.4 Results 110
5.4.1 Immune gene expression after LPS injection 111
5.4.2 Immune gene expression across tissue types 111
5.5 Discussion 112
5.5.1 Tissue-specific immune gene expression 112
5.5.2 Immune gene significance 113
Peptidoglycan recognition protein SA (PGRP-SA) 113
Gram-negative binding protein 1 (GNBP1) 114
Prophenoloxidase (ProPO) 115
5.5.3 Conclusions and future work 116

Chapter 6 ..... **118**
Locusts and their Bacteria **118**
6.1 Summary 119
6.2 Introduction 120
6.2.1 Host infection outbreaks 120
Plague in a plague: field locusts die in migratory band epizootic 120
6.2.2 Microbial communities in the gut 121
The benefits of host-microbe symbiosis 122
6.2.3 Insect gut microbiota 123
Density versus diversity of gut microbiota in locusts 124
6.2.4 Aims 124
6.3 Materials and methods 125
6.3.1 Field locust bacteria isolation, purification and identification 125
Genomic DNA extraction 126
Bacterial PCR and sequencing 126
6.3.2 Determination of bacterial doses 127
Bacterial growth assays 127
Colony counts: concentration determination 127
6.3.3 Experimental insects and gut bacteria treatments 128
6.3.4 Gut bacteria inoculation 128
6.3.5 Haemolymph and tissue sampling 129
6.3.6 Immune function and gene expression assays 129
6.3.7 Data analysis 130
Gene expression assays 130
Conventional immunological assays 130
6.4 Results 131
6.4.1 Immune gene expression 131
6.4.2 Conventional immune assays 132
6.5 Discussion 132
6.5.1 Experimental considerations 133
6.5.2 Assay diversity: technical sensitivity and subtle clues 135
6.5.3 Ecological relevance of microbial diversity 136
6.5.4 The lost pathogen 137
6.5.5 Conclusions and future work 137

Chapter 7 ..... **139**
General Discussion **139**
7.1 Overview 140
7.2 Locust ecology and immunity 140
7.3 Summary of findings 141
7.4 Plague management and locust biocontrol 144
7.5 Conclusions and future work 147

References **151**

Chapter 1.

General Introduction

"A very large percentage of illnesses are the expressions of inadequate responses to the environment." René Dubos, *Man Adapting*

1.1 Living with microbes

In a game of numbers, microorganisms will always win. Through abundance and adaptability alone, microbes are considered among the most diverse and influential groups of all living organisms (McFall-Ngai*, et al.*, 2013). In many natural systems, they are also considered necessary. Interactions between microbial communities and their environment are extremely complex. Whether harmful, helpful or neutral to their neighbours and hosts, understanding the significance of these organisms is crucial for many aspects of biology and medicine (Sadd and Schmid-Hempel, 2009a; Hawley and Altizer, 2011).

For most organisms, interactions with microbial communities are a never-ending challenge (Sheldon and Verhulst, 1996). Whether exposed to pathogenic or mutualistic microbes, the protection and regulation of host fitness is highly dependent on a stable immune function (Schulenburg*, et al.*, 2009). Classically, host-pathogen studies typically investigate the underlying mechanisms by which individuals maintain and activate various immune responses to prevent and fight parasite invasion (O'Connor, 1985; Scott and Trinchieri, 1995; Sorci*, et al.*, 1997). Similar aims investigating symbiotic interactions have also become a recent focus (Round and Mazmanian, 2009; Tremaroli and Bäckhed, 2012). However, microbes are not the only factor impacting host immunity. Ecological circumstance and environmental variation are of equal importance (Sandland and Minchella, 2003).

1.2 Ecological immunology

As the name suggests, ecological immunology encompasses an integration of both the environmental and mechanistic aspects of host immune defence (Sadd and Schmid-Hempel, 2009a; Schulenburg*, et al.*, 2009). A host's physiology is well understood to change and adapt to environmental variation. Immune function is no

different. Season, reproductive status, developmental stage and nutrition represent a mere fraction of the biotic and abiotic factors that are likely to vary within a host's lifetime (Joop and Rolff, 2004; Long and Nanthakumar, 2004; Nelson, 2004; Ponton*, et al.*, 2011a). Some such variables (*e.g.,* season and development) can be anticipated and, to a degree, prepared for (Nelson and Drazen, 2000; Moret*, et al.*, 2010). Others are inconsistent and beyond host control.

The field of ecological immunology aims to describe the interactions between host organisms, their pathogens and their symbionts in a wide environmental context (Sadd and Schmid-Hempel, 2009a). Until recently, the literature has largely focused on the identification and direct responses of host-parasite interactions under controlled environmental or pathogenic treatments (French*, et al.,* 2009; Lazzaro and Little, 2009). Whilst this is useful for identifying the variation and capacity of host immune function, many studies are shifting focus toward broader descriptions of the behavioural, physiological and ecological influences of immunity (Tauber, 2008).

1.3 Evolution of immune function

Hosts are constantly being exposed and re-exposed to novel and longstanding pathogens and parasites. In many circumstances microbes can become opportunistic, exploiting niche vacancies and posing additional unpredictable threats to hosts (Blinkhorn*, et al.*, 1989; Lyczak*, et al.*, 2000). The co-evolution of microorganisms and hosts has led to immense pressures on organism fitness and immunity. Short generation times and large population sizes enable microbial communities to evolve rapidly and adapt to new environments, including the internal ecologies of hosts themselves (Schulenburg*, et al.*, 2009). Ultimately, the success and survival of microbes depends upon their ability to develop, reproduce and effectively transmit to new hosts (Sandland and Minchella, 2003; Sadd and Schmid-Hempel, 2009a).

The capacity for many hosts to counter-adapt to such rapid changes is often slower and less efficient than for microbes. In this way hosts can suffer a handicap, with a constant need to detect and respond to new and evolving parasites (Tauber, 2008). Despite these challenges, organisms possess a wide range of adaptations and responses to prevent the infection and proliferation of pathogens (Baucom and de Roode, 2011). Host immune function is therefore plastic and constantly evolving based on new microbial and environmental encounters.

Immune function is additionally impacted by factors such as temperature, nutrition and changes in development and life stage. Hence, new defence traits can be adaptively generated throughout an individual's lifespan. Others are genetically fixed (Schulenburg*, et al.*, 2009). Nonetheless, the heterogeneity of host immune function is highly influenced by pathogen diversity and environmental variation (Lazzaro and Little, 2009).

1.4 Types of immune function

1.4.1 Pathogen defence: behaviours and barriers

With a common functional aim to limit the costs of parasite burden, host immune defence occurs on many levels in an intricate network of interacting traits (Schulenburg*, et al.*, 2009). Behavioural defence strategies in animals are known to occur both pre- and post-infection, either reducing the likelihood of infection or controlling the spread of pathogens once infected (Sheldon and Verhulst, 1996). As a preventative measure, many animals (*e.g.,* social insects and crustaceans) will avoid interactions (including mating) with infected conspecifics (Behringer*, et al.*, 2006; Ali and Tallamy, 2010). Behaviour and olfactory cues often enable conspecifics to identify infection and act by removing its source (Adamo*, et al.*, 2007; Arakawa*, et al.*, 2010). Some individuals will withdraw from a population when they themselves

are infected (Bos*, et al.*, 2012). Microbial transfer between individuals, as a form of self-immunisation, is another strategy observed in social communities (Koch and Schmid-Hempel, 2011a; Konrad*, et al.*, 2012).

Physical boundaries (*e.g.,* skin or cuticle) are a fundamental and effective form of protection against foreign particles (Elias, 2007). This includes the gut epithelia, with a large number of pathogens coming into contact with hosts through feeding and diet (Ashida*, et al.*, 2012). If, however, the physical barriers of defence are breached, the infection must be detected and a suite of other responses put into action. Pathogen type, environmental setting and resource availability often determine the efficiency of such responses.

1.4.2 Infection response: innate and adaptive immunity

Conserved across nearly all multicellular organisms are innate cell-based immune defences, which provide the first line of attack against foreign invaders (Beutler, 2004). The innate immune system is conserved across vertebrates and invertebrates. This incorporates constitutive, non-specific cellular and molecular defences (including the anatomical barriers mentioned above). With a wide range of defence functions, innate cellular networks are thought to have evolved well before the additional adaptive systems seen only in vertebrates (Pancer and Cooper, 2006). Consequently, cell-mediated responses have been well studied in invertebrates. Here, immune cells (haemocytes) attack foreign particles through several mechanisms including melanisation, phagocytosis, encapsulation, inflammation, clotting, and nodule formation (Gillespie*, et al.*, 1997; Lavine and Strand, 2002).

Signalling cascades transmit non-self recognition events from receptors through pathways of different elements (known as signal transduction) to generate an appropriate response (Schmid-Hempel, 2011). Each cascade has a characteristic

function and is triggered by particular types of pathogens. In insects, the Toll pathway generally responds to bacteria and fungi, whilst Imd primarily responds to gram-negative bacteria (Schmid-Hempel, 2011). Such cascades are suggested to be precursors for similar pathways in the complement humoral systems of vertebrates (*e.g.,* the Toll-like receptor pathway) (Pancer and Cooper, 2006). Initiated by the release of stored enzymes, these systems are responsible for phagocytosis and direct cell lysis, ultimately inhibiting pathogen replication (Janeway*, et al.*, 2005; Schmid-Hempel, 2011).

In vertebrates, specialised cells (*e.g.,* lymphocytes, monocytes and macrophages) interact directly with invading microbes (Schmid-Hempel, 2011). Pathogen suppression, non-self recognition, inflammation and phagocytosis are just some of the functions carried out by these immune cells, which overlap considerably between innate and adaptive systems (Akira*, et al.*, 2006).

It is within the vertebrate system that adaptive immunological memory is also produced, enabling long-lasting protection from recurring infection (Janeway*, et al.*, 2005; Siva-Jothy*, et al.*, 2005; Akira*, et al.*, 2006). Adaptive immunity is predominantly a macromolecule-mediated system. Here, synthesised antibodies, proteins and antimicrobial peptides act against infection in several ways. By binding to specific antigens, these molecules can prevent pathogens from entering host cells thereby neutralising their toxic effects (Schmid-Hempel, 2011). In this way adaptive systems have the capacity to acquire information about infections such that responses can be tailored to particular pathogens and parasites. The more frequent the encounters, the faster, stronger and more specific the infection response (Janeway*, et al.*, 2005).

With the addition of immunological memory, vertebrates are suggested as being better protected against repeated infection with the same pathogen (Pancer and Cooper, 2006). Exactly how it became a permanent addition to the vertebrate immune system, however, is still unknown and remains a topic of great interest (Beutler, 2004; Kurtz, 2005).

1.5 Trade-offs and resource allocation

Maintaining favourable life-history traits (*e.g.,* high reproduction and survival) requires stable energetic investment into a variety of physiological processes (French*, et al.*, 2009). Unsurprisingly, immune function regulation is also resource intensive (Sadd and Schmid-Hempel, 2009a). With a continuous risk of infection, investment toward constitutive immunity is necessary for most organisms. Immune reactions to specific infection or environmental stimuli require further energetic investment still (Sheldon and Verhulst, 1996).

The availability of resources required to sustain the immune system, and a suite of other physiological and metabolic functions, is a chief limiting factor. Physiological constraints can also limit an individual's ability to exploit existing resources. With finite access to nutrients, yet an imperative to maximise immune protection and overall fitness, hosts are often forced to concede energetic allocation to particular life history, metabolic or immunological traits (Long and Nanthakumar, 2004). In addition to the trade-offs between life history and physiology, nutritional allocations toward specific immune traits are being increasingly quantified in a range of study systems (Wilson*, et al.*, 2003; Cotter*, et al.*, 2004b). Reproduction and longevity, for example, have been frequently demonstrated to incur fitness trade-offs throughout the lives of many organisms (Lochmiller and Deerenberg, 2000).

When mounting a response to infection, the energetic demands of immune activation increase in a way that depends upon both the infection type and its complexity (Long and Nanthakumar, 2004). Hosts often initiate sickness behaviour, where infected animals reduce their activity and increase resting periods in an effort to conserve and reorganise their metabolic priorities (Konsman*, et al.*, 2002; Segerstrom, 2007). This too has its costs, with lethargic and less alert individuals increasing their likelihood of falling victim to predation (Behringer and Butler, 2010). Such risks, however, are often necessary, particularly where the probability of death from infection is high (Rolff and Siva-Jothy, 2003).

As mentioned above, immune trade-offs are influenced by the extent and type of infection present, enabling the activation of specific and targeted responses (Adamo, 2004a). With such a complex (and largely unknown) set of interactions between immunity and ecology, appropriate selection and measurement of traits within ecoimmunological investigations becomes difficult. Many studies report host immunity as a function of just one or two measured variables, leaving many of the underlying mechanisms untouched. This approach has several drawbacks, highlighting the potential bias associated with the assumption that individual traits will accurately represent a host's overall immunocompetence (Martin*, et al.*, 2006).

1.6 The value of microbial communities

As mentioned above, microbial adaptations have imposed many selective pressures upon host populations and their immune systems (Medzhitov and Janeway Jr, 1997; Beutler, 2004). Not all microbes, however, are a burden to hosts. Many, in fact, serve vital roles in maintaining host fitness and survival (O'Hara and Shanahan, 2006). Consequently, the capacity for hosts to differentiate between "friend and foe" is a critical adaptation (Pamer, 2007; Cerf-Bensussan and Gaboriau-Routhiau, 2010).

Commensal microbiota have been identified on the skin and integument of almost every species of animal studied (Fredricks, 2001; O'Hara and Shanahan, 2006). By far the most diverse (and arguably most important), however, are the symbiotic flora that colonise within the gastrointestinal tract (Sekirov*, et al.*, 2010). A recent surge in studies on gut microbiota has revealed just how important these organisms are in the maintenance and regulation of animal immune function (O'Hara and Shanahan, 2006; McFall-Ngai*, et al.*, 2013). Interactions between host and enteric microbiota are a prime example of symbiosis in nature.

As a system, host tolerance of established microbes within the gut can be crucial in the protection against harmful invaders (Dillon*, et al.*, 2005; Cerf-Bensussan and Gaboriau-Routhiau, 2010). This is particularly seen through colonisation resistance, where resident microbes produce antimicrobial compounds to eradicate pathogenic or competing introduced species (Dillon and Charnley, 2002; Stecher and Hardt, 2008). Hosts also gain other immunological, nutritional and metabolic benefits. In return, commensal microbes profit with optimal growth conditions and consistent resources that facilitate successful reproduction and survival (Hooper*, et al.*, 2012).

The majority of animals on the planet would probably not survive in the absence of microorganisms, particularly those in the gut (Maczulak, 2010). Transfer of commensal microbes between conspecifics is commonly observed (*e.g.,* through coprophagy or transgenerational priming), providing a means of acquiring and maintaining these symbiotic relationships (Moret, 2006; Koch and Schmid-Hempel, 2011a; Konrad*, et al.*, 2012). From individual organisms to communities and ecosystems, current studies are still only scratching the surface of how microorganisms contribute to ecological systems.

1.7 Insect immunology: a model for innate immunity

Current estimates suggest that only a small fraction of insect species in existence have been discovered, yet they are likely to account for approximately 90% of all multicelled species on the planet (Hoffmann*, et al.*, 1996; Snaddon and Turner, 2007). The widespread interest in insect behaviour, physiology, immunity and ecology should therefore come as no surprise (Adamo, 2011). The discipline of insect immunology continues to provide great insight into current understandings of the mechanistic underpinnings of innate immunity (Siva-Jothy*, et al.*, 2005). The Nobel Prize for Physiology or Medicine in 2011, for instance, was awarded to three scientists for discoveries in innate and adaptive immunity, one of whom, Jules Hoffmann, worked on insects (Beutler and Steinman were the others).

Despite the contribution of insects to innate immunological research, however, the majority of immunological studies to date are still performed using vertebrate models (Rolff and Siva-Jothy, 2003). With a focus on their adaptive immune systems, the complexities of innate immunity in vertebrates are far less understood. Fortunately, the tendency to describe invertebrate immunity as a simplistic precursor to vertebrate function is fast being overshadowed (Lackie, 1980; Gillespie*, et al.*, 1997). It is now generally accepted that much of the innate immunity of insects is much more analogous to vertebrates than previously thought, providing the opportunity to gain new insight into ancestral defence mechanisms and immune adaptations (Vilmos and Kurucz, 1998).

Similar to the microbes they harbour, insects demonstrate a phenomenal capacity to evolve and adapt to new environments (Siva-Jothy*, et al.*, 2005; Adamo, 2011). Such flexibility provides great potential for insect immune studies, allowing the diversity and capacity of innate defences to be uncovered (Rolff and Siva-Jothy, 2003). The

addition of complete genomic information of several model insects (*e.g., Drosophila*) further enables the genetic underpinnings of conventional immune measures to be investigated (Hoffmann and Reichhart, 2002). Another advantage offered by insect systems is apparent in the field of ecological immunology, where insects offer a tractable model for studying the environmental determinants of host immune function (Lackie, 1988; Gillespie*, et al.*, 1997).

1.8 Techniques in invertebrate immunology

Since immune trade-offs may change the detection and reliability of measured responses, many studies have stressed the importance of employing a multifaceted approach to ecoimmunological research (Sheldon and Verhulst, 1996; Cotter*, et al.*, 2004b). Coinciding with this has been the development and application of new and sensitive techniques, allowing the identification of many new aspects of host immune function (Schulenburg*, et al.*, 2009). The application of new methods to insect models in particular, has become an essential step toward increasing current knowledge about the invertebrate world (Vilmos and Kurucz, 1998; Rolff and Siva-Jothy, 2003).

1.8.1 Conventional immune measurements

When quantifying immune function in invertebrates, an array of traits and measurements can be exploited. Classical methods such as total haemocyte density reveal cellular proliferation, giving insight into immediate immune reactions (Gilbert and Miller, 1988). Differential haemocyte counts, a technique that compares individual cell-type abundance, can be valuable indicators of specific pathogen responses. In some species, however, conflicting ideas on the classification of different cell types mean that the implications of differential counts may vary. This can depend on both the type of infection and predicted role of enumerated cells

(Ribeiro and Brehélin, 2006). In such cases a deeper examination of individual cell function and role diversity is critical.

Other commonly used measures include encapsulation response, phagocytosis, nodule formation, wound healing and various enzyme activity assays (Schmid-Hempel, 2011). Parasite clearance and survival assays are also frequently used to represent an organism's ability to resist pathogens or environmental challenges. Studies that use an integrative approach, by measuring multiple traits, provide a more comprehensive view of the interactions, trade-offs and contribution that each trait has toward overall immune function (Martin*, et al.*, 2006).

1.8.2 Molecular techniques

Rapid developments in molecular biology and medicine have greatly contributed to the expanding field of ecological immunology (Schulenburg*, et al.*, 2009). Conventional immune assays are well complemented by molecular techniques, providing a more detailed view of the underlying molecular and genetic function of host immunity (Martin*, et al.*, 2006; Schulenburg*, et al.*, 2009). Gene expression assays, ELISAs, flow cytometry and sequencing assays are at the forefront of technologies commonly used in this field (Svensson*, et al.*, 1998; Cárdenas*, et al.*, 2000; Jenner and Young, 2005). For studies using some invertebrate models (*e.g., Drosophila*) access to complete genome sequences is another key benefit (Hoffmann and Reichhart, 2002).

1.9 Locusts as a model for integrating ecological immunology

1.9.1 Locust ecology

The lifespan of a locust is a complex integration of development, adaptation and resilience, with individual development from hatchling to adult shaped largely by

local environmental conditions (Uvarov, 1957; Dadd, 1960; Raubenheimer and Simpson, 1999; Pener and Simpson, 2009). Locusts face several ecological challenges throughout their lives. Environmental pressures often lead to extreme changes in population density, nutritional deficits, acts of cannibalism and variation in commensal gut communities (Uvarov, 1927; Dillon and Dillon, 2004; Simpson and Sword, 2008). Because of their global socio-economic impact (F.A.O, 2014), as well as their intriguing biology, locusts are a focal study system for many research groups around the world. With a large body of existing work on locust nutrition, physiology, behaviour and population dynamics (Pener and Simpson, 2009), this thesis focuses on the impacts that many of these aspects have on locust immunity.

1.9.2 Behavioural phase polyphenism in locusts

Adaptive polyphenism

Animals occurring in variable environments often benefit from the ability to exhibit behavioural, physiological and immune plasticity. Environmental stressors include inadequate nutrition, rapid shifts in local or seasonal climate and changes in pathogen type or prevalence. Interactions between such variables result in complex systems of host response (Lazzaro and Little, 2009). For organisms unable to adapt quickly, the effects of environmental variation can be drastic, impacting both individual reproduction and overall survivorship (Sheldon and Verhulst, 1996).

Polyphenism, where a single genotype expresses more than one distinct phenotype, is a common evolutionary strategy that enables species to cope with rapid changes in their environment (Mayr, 1963; West-Eberhard, 1989). Phenotypic plasticity is observed across many taxa and is often influenced by changes in food quality, sexual selection, developmental stage and climate (Nijhout, 2003; Simpson*, et al.*, 2011). Variation of phenotypic traits in response to external stimuli is often exhibited

through changes in behaviour, physiology, morphology or any combination of these factors (Nijhout, 2003; Sword, 2003). Since polyphenism is an expression of complex gene-environment interactions, external cues (*e.g.,* photoperiod, population density or food availability) enable animals to phenotypically adapt in order to maximise individual fitness (Nijhout, 2003).

Polyphenism has been identified as a large contributor to the success of insects over evolutionary time. Separate developmental stages and phenotypes, for example, allow individuals to adapt their physiology and behaviour to best exploit changes in their environment (Simpson*, et al.*, 2011). For insects in particular, local changes in resource availability, microclimate, predation and intraspecific competition all coalesce to make polyphenism a favourable evolutionary trait (Evans and Wheeler, 2001; Nijhout, 2003).

Density-dependent phase polyphenism in locusts

Possibly the best-studied example of polyphenism in insects occurs in locusts (Orthoptera; Acrididae). Termed phase polymorphism in 1966 (Uvarov), phenotypic plasticity in locusts was first described to occur as a result of shifts in population density some 45 years earlier (Uvarov, 1921). Since then, this phenomenon has been studied in several species, ultimately characterising locusts from other Orthoptera as having the capacity to undergo what is today referred to as density-dependent phase polyphenism (DDPP) (Faure, 1933; Ellis, 1951; Staal, 1961; Pener, 1991; Simpson*, et al.*, 1999; Sword, 2003; Gray*, et al.*, 2009). Among the suite of traits that comprise DDPP, behaviour is the most labile and important at the population level (Pener and Simpson 2009). Whilst many insect species are known to exhibit phenotypic plasticity in response to rearing density (Pener, 1991; Cotter*, et al.*, 2004a), the

economic and ecological influences that locusts contribute toward agriculture, society and the environment makes them a hugely important study system.

Increased population densities result in heightened frequencies of tactile, olfactory and visual interactions between locusts. It is such interactions that trigger the transition between the solitarious and gregarious behavioural phases that occur in these insects (Roessingh*, et al.*, 1998; Cullen*, et al.*, 2010). Locusts living in isolated conditions, with limited or no stimulation from conspecifics, have been found to exhibit low levels of activity; avoiding interactions with other locusts except to mate. This is known as the solitarious condition. Conversely, locusts found in crowded conditions (gregarious), are generally more active and have been typically found to orient towards each other (Simpson*, et al.*, 1999).

Behaviour is one of many traits known to change in locusts during DDPP. Physiology, morphology and metabolism are also known to shift between phases at different rates alongside population density (Roessingh and Simpson, 1994). In 2004, Kang *et al.* identified 532 phase-related genes that were differentially expressed between solitarious and gregarious phases of the migratory locust (*Locusta migratoria*). Many of these genes were found to contribute to the physiological changes that occur during phase transition, the majority of which were shown to be downregulated in gregarious treatments (Kang*, et al.*, 2004).

Influences and implications of phase change in locusts

Depending on the species of locust under study, different stimuli have a greater weighted impact than others in triggering DDPP (Simpson*, et al.*, 2001; Cullen*, et al.*, 2010). Both degree and latency of phase transition have been shown to vary across species (Sword, 2003; Gray*, et al.*, 2009). That species respond differently to the same stimuli reflects the independent evolution of polyphenism in locusts

(Uvarov, 1966; Pener and Simpson, 2009). What's more, locusts have the capacity to transition between solitarious and gregarious phases multiple times throughout their lives, a process that can take hours to days, depending on the direction of phase change as well as the species in question (Ellis, 1963; Roessingh and Simpson, 1994; Roessingh*, et al.*, 1998).

Locust phase polyphenism provides the driving force in migratory band and swarm formation in the field (Figure 1.1). When resources are limited, solitary locusts (Figure 1.1a) are forced into close proximity in areas of available food and other resources such as favourable microclimatic and perching sites (Figure 1.1b) (Bouaichi *et al*., 2003). Here, sensory stimulation from conspecifics sets in motion the transition from solitary to gregarious phase (Figure 1.1c) (Pener and Simpson, 2009). Once food becomes scarce, nutrient deficiencies drive locusts to swarm or march, thereby increasing the likelihood of locating new resources.

Previous studies have identified protein as the nutrient of highest priority in Mormon crickets and locusts (Simpson*, et al.*, 2006; Bazazi*, et al.*, 2008). In the absence of other resources, the most available means of obtaining protein is by cannibalism (Figure 1.1d) (Simpson*, et al.*, 2006; Bazazi*, et al.*, 2008). Consequently, in an attempt to satiate nutritional requirements by moving toward potential prey (victims) and simultaneously avoid being eaten from behind, locusts find themselves migrating in parallel on a forced, cannibalistic march for protein (Figures 1.1e) (Simpson*, et al.*, 2006; Simpson and Sword, 2008; Bazazi*, et al.*, 2011; Hansen*, et al.*, 2011).

Marching bands and swarms (Figure 1.1f) can sometimes contain billions of individuals, where locusts migrate until new resources are encountered (Simpson and Sword, 2008). It has been estimated that in a single day, a plague of 1 billion desert locusts are capable of consuming the same amount of food as 2.5 million people

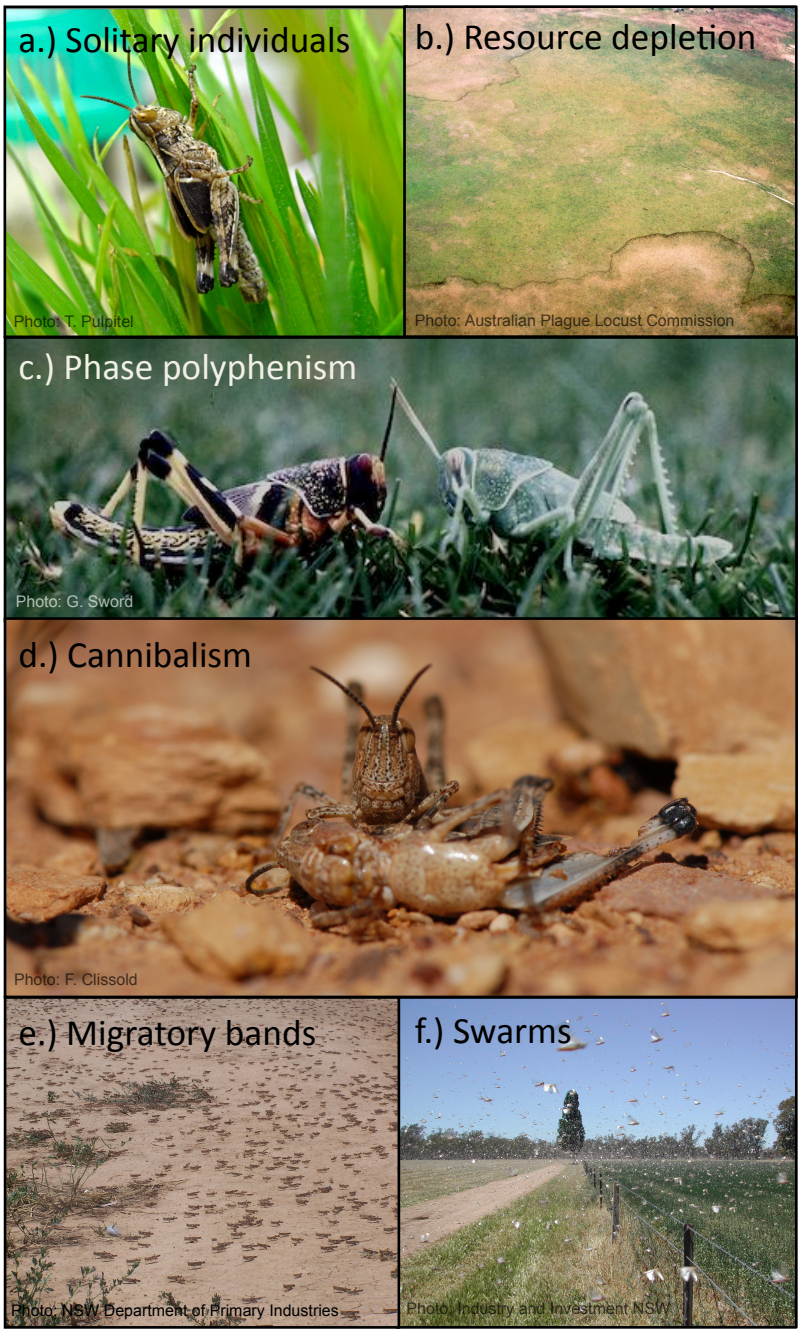

Figure 1.1 Typical ecology encountered within a locust's lifespan. Solitarious locusts (a) generally avoid conspecific interactions. Diminishing resources, however, force individuals into close proximity (b). This leads to density-dependent phase polyphenism (c), where locusts transition between solitarious and gregarious phases. Close proximity and nutritional deficiency can also lead to cannibalism (d), which in turn aids in the formation of marching bands (e) that migrate until new resources are found. Winged adults migrate in swarms by following wind patterns (f).

(F.A.O, 2010). Not surprisingly, the agricultural destruction caused by locusts generates worldwide economic devastation. Density-dependent phase polyphenism both defines locusts as a phylogenetically heterogeneous group of otherwise typical grasshoppers (Family Acrididae), and lies at the heart of their status as an agricultural pest (Pener and Simpson, 2009).

1.10 Locust immunity

The immune system of locusts is typical of other invertebrates as well as some aspects of the vertebrate innate system, making them fitting models for immune studies (Hoffmann*, et al.*, 1996; Goldsworthy*, et al.*, 2003). Locust immunology has been a widely investigated topic throughout the literature, with several studies now considering more than just the underlying mechanisms of their immune function and physiology (Goldsworthy*, et al.*, 2003; Dillon*, et al.*, 2005). Surprisingly, however, the number of antimicrobial peptides and proteins that have been identified in locusts are few, particularly in comparison to other insects such as *Drosophila* (Lemaitre and Hoffmann, 2007).

Like most organisms, locusts are continuously exposed to pathogens and infection (both naturally or through applied infectious agents and insecticides by humans). Pathogen exposure levels, however, are not constant, nor are the environmental variables commonly associated with locust habitats. Consequently, many of the behaviours and physiologies that locusts have evolved to tolerate and respond to infection are still unclear. Shifts in population density, opportunities of cannibalism and variation in commensal microbiota may all influence infection risk and therefore immune function investment.

Given their biology as a phenotypically plastic outbreaking insect, immunological studies of locusts serve an important role in modelling how ecological factors

influence immune plasticity in a range of other hosts. The abundance of knowledge encompassing physiology and environmental influences means that locusts provide a solid foundation for ecoimmunological studies.

1.11 Applied importance

The efficient and safe control of swarms and marching bands is a core priority in locust research (Krall*, et al.*, 1997). Insecticides and general pesticides are used frequently in agriculture, yet heightening insect resistance to such chemicals means that liberal amounts and high concentrations are often required for effective results (Symmons, 1992; Showler, 2002). Impacts on other non-harmful organisms, including long-term residual effects of sprayed areas, are also often unclear (Krall*, et al.*, 1997). Consequently, the development and use of biological agents in the control of pest species has become a shared goal in agricultural and biological studies (Zelazny*, et al.*, 1997; Milner, 2000; Lomer*, et al.*, 2001; Hunter, 2005). By exploiting the natural pathogens of locusts, such as bacteria and fungi, there is potential for a more targeted and localised approach to plague control.

Investigations seeking to understand the primary mechanisms and responses of locust immunity are critical for the development and application of new biological controls. With a greater insight into how individuals respond to infection, such studies provide an increased capacity to tailor pathogens to pest weaknesses (Thomas and Blanford, 2003; Wilson, 2005). One currently used biocontrol formulation (Green Guard®) uses the acridid-specific fungus, *Metarhizium acridium*, as its pathogenic agent (Milner, 2000; Hunter*, et al.*, 2001). While the mechanisms of how *M. acridium* functions within locusts are well known, its wider impacts on non-target species are, once again, less understood. In addition, long incubation times of *M. acridium* delay the impact on insect mortality, rendering this agent less than ideal for immediate

plague control (Hunter*, et al.*, 2001; Hunter, 2005). Nonetheless, the potential for its use as a biocontrol agent early in the locust outbreak cycle is promising.

As emphasised above, understanding how a locust's ecology impacts its constitutive and responsive immune function is of huge agricultural, economic and ecological concern. With extensive devastation caused by these organisms worldwide, investigations into the interactions between ecology and immunity are essential for integrated applications toward plague control (Hunter, 2005; Wilson, 2005).

1.12 Study species

This project focuses on two of the three main locust species that occur in Australia. Owing to its high frequency of outbreaks and widespread distribution (covering almost the entire continent) the Australian plague locust, *Chortoicetes terminifera*, is by far the most destructive pest species of agriculture and pasture across the country (A.P.L.C, 2014). Migratory bands of *C. terminifera* can stretch several kilometres wide and hundreds of metres deep, causing enormous agricultural devastation in their wake (A.P.L.C, 2014).

Locusta migratoria is a larger species that, despite its capacity to travel long distances, demonstrates fewer outbreaks in Australia than *C. terminifera*. Though occupying a smaller range within Australia, *L. migratoria* are also found in Africa, Eastern Europe and Asia, making them a major agricultural pest worldwide (A.P.L.C, 2014). Comparable in biology, both species fledge within a month of hatching and soon after become reproductive. All stages of the locust life cycle, however, have the potential to be destructive (Simpson and Sword, 2008).

A large proportion of studies performed within the field of locust immunology have used *L. migratoria* as a focal species (alongside the similarly sized *Schistocerca*

gregaria). Immunological studies on *C. terminifera*, however, are few in number and, coupled with a wide distribution across highly variable habitats, is the primary reason that this species was chosen for the majority of this work. Experiments requiring larger insects, with more familiar physiological background, used *L. migratoria*. It is highly anticipated that many of the outcomes of this work will be applicable to other species.

1.13 Project aims

The primary goal of this thesis was to investigate the underlying mechanisms of Australian locust immunity, using their typically encountered ecological influences as a primary foundation. Rather than focusing on a single environmental parameter, experiments tested a range of conditions and defence strategies in an attempt to gain a wider view of overall immune function. Using ecologically relevant microorganisms, the behavioural, physiological and symbiotic interactions that occur within locusts were experimentally manipulated and measured. A combination of gene expression and conventional immune assays allowed identification and comparison of the intricate machinery that underlies host defence.

Each chapter within this thesis looks at a specific aspect of locust ecology and life history, beginning in Chapter 2 by exploring the immune components of phase polyphenism in *C. terminifera.* These experiments used a multifaceted approach to test the effects of density-dependent phase change on survivorship and examine the relationship between immune function, population density and rearing phase. A behavioural study was conducted in Chapter 3 to identify outcomes of individuals faced with the choice of infection versus starvation, notably in relation to cannibalism. Similar conditions were investigated in Chapter 4, to determine the presence or absence of anticipatory immune responses within insects exposed to

infected conspecifics. In Chapter 5, gene expression assays were developed using *L. migratoria*. This technique was used to demonstrate tissue-specificity in immune response on a molecular level. Along with conventional measures, these gene expression assays were used in Chapter 6 to investigate variation in constitutive immunity in response to the introduction of commensal bacteria to the gut.

In total, this thesis presents a comprehensive view of the way locusts maintain and regulate their immunity in response to pathogens, symbionts and ecological variation. Armed with a new understanding of how different tissues and immune traits respond to unpredictable environments, the importance of ecological immunology in the application of biological control strategies for controlling locust outbreaks is discussed. Through consideration of a suite of pathogenic and ecological factors, this study promotes the importance of trait and treatment selection in future studies within ecological immunology.

Chapter 2.

Density-dependent Prophylaxis in the Australian Plague Locust

Collaborative statement:

The experiments presented in this chapter were performed in collaboration with Dr Robert Graham of the University of Lancaster.

2.1 Summary

Population density plays an important role in the life of individuals, their probability of forming groups, and the dynamic behaviour of such groups. From collective decision making to migration and predatory defence, many studies have shown the benefits of living at high densities. However, there are also associated costs, including increased competition for mates and resources as well as an increased risk of infection. Animals are often found to alter their physiology and behaviour to offset such costs of group living. The density-dependent prophylaxis (DDP) hypothesis predicts that animals living in groups invest more in immune function than conspecifics at low population densities. This is proposed to be a result of the increased risk of exposure to pathogens and higher rates of transmission between infected individuals. Whilst DDP has been demonstrated in several species, whether the hypothesis is supported in locusts remains uncertain. Indeed, conflicting results from studies attempting to answer this question have revealed a new level of complexity to locust immune function, particularly in the context of population dynamics. This chapter presents an in-depth analysis of the DDP hypothesis in the Australian plague locust, *Chortoicetes terminifera*. Through the measurement of several conventional immune traits in both fungal-infected and uninfected locusts, these experiments found evidence to both support and refute DDP in this species. This outcome helps unite conflicting results from other published studies and highlights the importance of a multifaceted approach in all immune studies. The likelihood that trait responses are differentially prioritised with respect to density and infection are discussed.

2.2 Introduction

2.2.1 Group living and effects of density

Population density can have an enormous impact on the ecology and success of a species. There are several key benefits to group living including protection from predators, increased feeding efficiency, access to mates, social thermoregulation and division of labour (Molvar and Bowyer, 1994; Krause and Ruxton, 2002). Individuals within groups, however, may also concede fitness costs such as those associated with resource competition, suppressed reproduction, social conflicts, cannibalism and elevated parasite burden (Wilson*, et al.*, 2003; Shen*, et al.*, 2014). Many studies on such costs and benefits have been useful in identifying the impact of population density on individual fitness across a range of taxa. How animal species adapt and compromise their individual immune function in response to population density is a particularly important question aimed at better understanding the evolution of group living (Beauchamp and Fernandez-Juricic, 2005).

In *Drosophila*, larvae reared at high densities are known to decrease in body size and suffer higher mortality before maturity, yet exhibit an increased longevity once eclosed to adults (Sørensen and Loeschcke, 2001). Across several generations of group rearing, however, individuals can better withstand starvation and exhibit a heightened lipid content (despite a persistent increase in eclosure time) (Borash and Ho, 2001). Some mammals similarly demonstrate inverse relationships between local abundance, body size, fecundity and survival (Damuth, 1981; Kruuk*, et al.*, 1999). Adverse effects of crowding stress in fish have also been shown to impact growth, development and disease resistance (Yin*, et al.*, 1995).

Variability in population density can quickly offset both the costs and benefits of group living. Such events are often caused by competition, predation, climate or

resource availability and may require rapid shifts and adaptive responses in individual behaviour, physiology and immune function. Phenotypic plasticity, an adapted trait that enables organisms to alter their biology in response to changes in their environment, is extremely beneficial in this context (see Chapter 1). Polyphenisms to variation in population density are well studied, particularly in insects, with many species exhibiting rapid density-dependent responses in physiology and immunity (Simpson*, et al.*, 2011).

2.2.2 Density-dependent prophylaxis

The density-dependent prophylaxis (DDP) hypothesis predicts that animals living at high population densities exhibit higher immunocompetence than isolated conspecifics due to an increased risk of pathogen exposure and transmission between individuals (Wilson and Reeson, 1998). Within the field of ecological immunology, immunocompetence is defined broadly as the ability of an organism to respond to pathogens while minimising the fitness costs of infection (Owens and Wilson, 1999). Variation in immune investment is therefore critical for hosts living in unpredictable environments, particularly those subject to rapid differences in population density. However, although the DDP hypothesis has been supported in several species, for others the relationship between immune function and population density is more ambiguous.

DDP in insects

The adaptations of density-dependent immune responses have been widely studied across a range of insect genera including the Lepidoptera, Orthoptera, Coleoptera, Hymenoptera and Diptera (Wilson and Reeson, 1998; Barnes and Siva-Jothy, 2000; Sørensen and Loeschcke, 2001; Wilson*, et al.*, 2002; Cotter*, et al.*, 2004a). With the

capacity for many species to exhibit phenotypic plasticity when exposed to variable environments, insects serve as ideal model systems for the study of DDP.

Pathogen resistance studies in Lepidoptera have found a widespread consensus in support of the DDP hypothesis, suggesting that species reared under high densities express greater pathogen resistance than those that occur in solitary (Wilson and Reeson, 1998). Recent works on *Anticarsia gemmatalis* (Lepidoptera: Noctuidae), for instance, have shown strong support for DDP, with individuals demonstrating density-dependent immune upregulation in the presence of just one conspecific (Silva*, et al.*, 2013). Much of the underlying immune mechanisms of Lepidoptera, Coleoptera and other insect species, however, appear more complex, with individual immune traits often revealing contradictory results with respect to overall host immunocompetence (Barnes and Siva-Jothy, 2000; Cotter*, et al.*, 2004a).

Crowding experiments using the field cricket (*Gryllus texensis*) have detected no evidence of increased disease resistance in response to increases in population density (Adamo and Parsons, 2006). Variation in immunosupression in response to additional stressors, however, (*e.g.,* exercise, temperature and agonistic behaviour) was found to change across measured traits in the same study. This revealed the importance of considering potential trade-offs between immune function and other physiological mechanisms, particularly within a wider ecological context. Age-dependent immune responses to crowding have also recently been demonstrated in the Mormon cricket (*Anabrus simplex*) suggesting that insect development and age are other critical aspects that may influence DDP investments (Srygley, 2012).

Social insects have also been found to support the DDP hypothesis, despite previous suggestions that this was not the case in permanently social populations (Pie*, et al.*, 2005). In the adult bumblebee (*Bombus terrestris*) significant effects of group size on

immune function were found, indicating an adapted plastic response to density variation (Ruiz-González*, et al.*, 2009). With a higher capacity for behavioural immune defence in social insects, such studies appropriately place a greater emphasis upon behavioural as well as physiological immune defences (Elliot and Hart, 2010).

Altogether, ecological circumstance should be expected to influence the ways in which insects adapt to variation in population density. Whilst the DDP hypothesis proposes an overall increase in immunocompetence at high population densities, results to date also indicate that investments in the maintenance of pathogen resistance and immune function are costly, particularly where group size is highly variable (Barnes and Siva-Jothy, 2000; Wilson*, et al.*, 2002). Trade-offs within the immune system, in response to changes in rearing density, are therefore important considerations when studying the underlying mechanisms of DDP.

DDP and phase polyphenism in locusts

Previous works have suggested that insects exhibiting density-dependent polyphenism might have a higher likelihood of exhibiting DDP, particularly species that frequently occur under variable crowding conditions (Wilson and Reeson, 1998). In locusts, rapid changes in population density are common in the field (Simpson and Sword, 2008). A primary outcome of such density shifts is the occurrence of density-dependent phase polyphenism. This phenomenon, where individuals transition between solitarious and gregarious phases, is typical of all locusts (indeed, it is a defining feature) and ultimately impacts several aspects of their physiology, morphology and behaviour (refer to Chapter 1).

Through a number of approaches, previous studies have found conflicting evidence for and against the DDP hypothesis in locusts. Elevated resistance and antimicrobial activity against the fungus *Metarhizium acridium* (previously named *Metarhizium*

anisopliae var. *acridium*) was found in the gregarious phase of the desert locust, *Schistocerca gregaria*, supporting the DDP hypothesis in these insects (Wilson*, et al.*, 2002). Interestingly, however, this same study also found no difference in temperature preference or behavioural fever response between the two phases.

Molecular studies on the migratory locust (*Locusta migratoria*) have demonstrated expression responses to *M. acridium* infection in nearly twice as many immune-related genes within solitarious individuals compared to their gregarious counterparts (Wang*, et al.*, 2013). In this study, the authors concluded that prophylactic immunity was upregulated in a way that suggested invading pathogens were tolerated rather than eliminated within the host.

Hatchlings of crowd-reared *S. gregaria* parents have been found to exhibit a higher mortality than conspecifics of solitary-reared parents, following infection with *M. acridium* (Miller*, et al.*, 2009a), thereby opposing the findings of Wilson *et al.,* (2002). Fifth instar *Chortoicetes terminifera*, collected from wild marching bands, similarly opposed the DDP hypothesis, showing a negative correlation between total haemocyte counts (THCs) and population density (Miller and Simpson, 2010). Individuals that were removed from marching bands also showed inverse relationships to isolation, with THCs increasing consistently following up to two days of isolation from the group.

Density-dependent phase change in *C. terminifera* has been found to take up to 72 h to achieve complete transition of behavioural traits (Gray*, et al.*, 2009). Analogous to studies in other locusts, physiological changes are not always this rapid (Roessingh and Simpson, 1994; Gray*, et al.*, 2009). How variation in immune function coincides with *C. terminifera* behavioural phase change (following crowding or isolation) remains untested.

Similar to lepidopteran and other orthopteran studies, investigations to date into the prophylactic immune function of locusts indicate that perhaps not all immune traits respond simultaneously to variation in population density (if at all). The resource-intensive nature of immune investment could be one explanation for this (Sheldon and Verhulst, 1996; Zuk and Stoehr, 2002). Whatever the status of the DDP hypothesis, it is clear that changes in population density alter locust behaviour, physiology and morphology. It is therefore of interest to determine whether immune state also differs as part of the suite of characters that accompanies density-dependent phase polyphenism. Whether changes in immune function serve to anticipate an altered risk of pathogen exposure in response to changes in population density is of particular interest.

2.2.3 Aims

An especially important question when considering the complexities of DDP in locusts is how immunological investments are altered during and following the process of density-dependent phase change. How different immune traits respond to locust polymorphism in the presence of infection are of particular importance from the perspective of locust biocontrol.

The following study tests the DDP hypothesis in *C terminifera*, specifically in the context of density-dependent phase polyphenism. Experiments performed investigated both the constitutive and responsive immunity of locusts reared under solitarious and gregarious conditions, as well as locusts that had recently undergone phase polyphenism. The use of the fungal entomopathogen, *M. acridium*, enabled the identification of immune response variation across these phase treatments. By measuring a number of conventional immune traits and locust survival, variation in both constitutive and responsive immune function were compared. Altogether, this

study aimed to: i.) compare immune trait response and survival of *C. terminifera* between fungal-infected and uninfected locusts; ii.) Test the DDP hypothesis in the context of rearing density, behavioural phase and infection, and iii.) understand the interaction between population density and phase change within an ecoimmunological framework.

2.3 Materials and methods

2.3.1 Insect cultures, experimental setup and treatments

Stock cultures of *C. terminifera* were reared under long-term gregarious conditions in facilities at the University of Sydney. Hatchlings were reared in net cages (45x45x45 cm) and fed on fresh wheat grass and wheat germ daily. Cages were established at densities of 45-55 egg pods per cage and maintained as single-age cohorts. Locusts were maintained in temperature controlled rooms of 30-33°C with additional heat lamps on one side of each cage to allow locusts to thermoregulate. Room lighting regimes were set to a photoperiod of 14:10 h (light:dark). Experimental locusts were collected as newly hatched nymphs from the gregarious culture and separated into two initial phase treatments according to previous protocols and outlined below (Gray*, et al.*, 2009). All insects used for experiments were male.

Solitary-reared insects

Solitary-reared locusts (henceforth termed "solitarious") were generated within 2 d after egg hatching by separating nymphs into individual cages (7x8x8 cm). Each locust was fed individually and provided with a clean air supply to avoid transfer of conspecific scent between neighbouring cages. Dividers were used to separate cages to prevent visual stimulation between locusts. Solitarious locusts were fed on

alternate days with fresh wheat grass and wheat germ. Stems of the wheat grass were wrapped in water-soaked cotton wool to retain moisture content. Frass was cleared on alternate days to maintain clean conditions within enclosed cages. Cages were kept in a temperature controlled room of 30-33°C with a photoperiod of 14:10 h (light:dark).

Gregarious culture

Remaining hatchlings were set up in a single cage (45x45x45 cm) as the long-term gregarious treatment and reared at an approximate density of 300 insects/m^2. Gregarious locusts were fed daily with fresh wheat grass and wheat germ and maintained under the same temperature and photoperiod conditions as described for solitary-reared locusts above. Cages were cleared of frass daily. Under these conditions few deaths were observed and thus did not affect the overall rearing density.

2.3.2 Infection treatments with *M. acridium*

Solitarious and gregarious test insect hatchlings were monitored daily until reaching the fifth (final nymph) stadium (this took approximately 12-14 d). At 24 h post-moult, equal numbers of male and female insects were weighed and randomly assigned to fungal infected or uninfected control inoculation treatments.

Immune challenges were performed using the acridid-specific pathogen *M. acridium*, a fungus currently used in the bio-insecticide Green Guard® against locusts swarms and migratory bands in the field. Cultures of *M. acridium* were obtained from subcultures originally isolated from Green Guard® by collaborators at the University of Sydney. The fungi were grown on Potato Dextrose Agar (PDA) at 30˚C until

spores turned from white to dark green (approximately 4 d), an indication of viable sporulation.

Fungal spores were suspended in sterile 0.07% (v/v) Triton-X and enumerated using an Improved Neubauer haemocytometer. Triton-X (0.07% v/v) was also used to dilute the spore solution to four concentrations (25, 250, 500 and 2500 spores/μL). Locusts were infected by inoculating individuals with 2 μL of one fungal dose behind the pronotum. Control insects were inoculated with the same volume of 0.07% (v/v) Triton-X solution only. Since it was logistically difficult to obtain and maintain more individuals, and as the experimental aims sought to investigate the effects of phase and infection only, a third group consisting of uninoculated (unhandled) controls was not included.

2.3.3 Phase treatments

In addition to inoculation treatments, control and infected locusts were assigned to one of four behavioural phase treatments within a fully factorial design: i.) solitarious locusts maintained under solitary-rearing conditions (SS); ii.) solitarious locusts crowded with four gregarious conspecific stimuli (SG); iii.) gregarious locusts maintained under crowded conditions with four gregarious stimuli (GG), and iv.) gregarious locusts isolated to solitary conditions (GS).

All locust treatments (GG, SG, GS and SS) were housed in the same cages as described for rearing solitarious insects (see above), however, individual airlines and visual dividers were not used for gregarious or crowded treatments (*i.e.,* GG and SG). As mentioned above, test locusts within crowded and gregarious treatments were housed with four stimulus conspecifics to achieve adequate crowding conditions. Since *C. terminifera* does not display morphological or colour variation with phase transition, it was not possible to distinguish test insects from stimulus

insects within a crowded treatment. Stimulus locusts used for crowding were therefore manipulated by removing the tibia from each middle leg as a means of differentiation from experimental locusts.

2.3.4 Dose-dependent LD_{50} survival assay

A resistance assay was conducted to determine the lethal dose 50 (LD_{50}) of *C. terminifera* infected with *M. acridium* within each of the four behavioural phase treatments. This is the dose at which 50% of an inoculated population is killed over a specified time. Locusts were inoculated with one of the fungal doses described above and assigned to one of, SS, SG, GS or GG phase treatments. A control treatment group was inoculated with Triton-X (0.07% v/v) only. Locusts were fed wheat grass and wheat germ *ad libitum* and checked for deaths twice daily for two weeks. Survival data obtained enabled approximate LD_{50} calculation.

2.3.5 Density-dependent immune assays and haemolymph collection

Dose-dependent survival assays indicated 500 spores/µL (1000 spores/insect) as the dose closest to the LD_{50}. Using this dose, immune experiments were carried out to identify variation in constitutive and responsive immune function across the different infection and phase treatments. As above, locusts were inoculated with either the LD_{50} dose of *M. acridium* (infected treatment) or 0.07% (v/v) Triton-X (control treatment) before being assigned to SS, SG, GS or GG treatments (n=40). At 72 h post-inoculation, the maximum time determined for the complete behavioural transition between phases in either direction (Gray*, et al.*, 2009), locusts were bled by puncturing a hole in the arthrodial membrane of the hind leg with a sterile pin and drawing up the haemolymph with a micropipette. Insects were bled for the maximum volume of haemolymph possible before being discarded (a minimum of 10µL was

needed for assays). Haemolymph was immediately frozen and stored at -80˚C until use.

2.3.6 Second-generation behavioural phase assays

In an attempt to identify whether impacts on immune function accumulate across generations of behavioural phase treatments, long-term gregarious insects (GG) were also compared to second-generation solitary-reared insects (2SS). These were generated by mating solitary-reared adults, separating their eggs from egg pods prior to hatching and rearing the hatchlings in isolation until their 5th stadium. Eggs were separated from sand and foam within egg pods using distilled water and sterile forceps. Each egg was placed on moist cotton wool in an individual container and incubated at 30-32°C until hatchlings emerged. Upon hatching, nymphs were transferred to individual cages and reared as solitary locusts as described above. Once again, 5th instar solitarious (2SS) and gregarious (GG) locusts (n=40) were inoculated with 2 µL *M. acridium* (500 spores/µL) or 0.07% (v/v) Triton-X and bled for haemolymph at 72 h for immune assays.

2.3.7 Immune and host condition assays

Two conventional immune assays (lysozyme and prophenoloxidase activity) and two host condition assays (total haemocyte counts and haemolymph protein concentration) were conducted to identify variation in immune function or host condition across treatments. These well-established measures were used to gain an overall view of constitutive state, immune response and nutritional reserves.

Total haemocyte counts (THCs)

Thawed haemolymph was diluted 10-fold with 1 M phosphate buffered saline (PBS) and 10 µL loaded onto each side of an Improved Neubauer haemocytometer. Cells

were counted in duplicate in five non-adjacent squares from which the average undiluted haemocyte concentration was calculated. Since previous comparisons of THCs between thawed and fresh haemolymph showed no variation in haemocyte density (data not shown), haemolymph was allowed to remain frozen until enumeration.

Lysozyme activity

Lysozyme activity was measured according to protocols described previously (Cotter*, et al.*, 2004a). Agar plates (10 mL) were prepared containing lypophilised *Microccocus luteus* (5 mg/mL) and the antibiotic Streptomycin (0.1 mg/mL). Twenty holes of 2 mm diameter were aseptically punctured into each agar plate using a glass Pasteur pipette, and 1 µL of 70% ethanol saturated with phenylthiourea (PTU) added to each hole to inhibit haemolymph melanisation. Once the ethanol had evaporated, 1 µL of thawed undiluted haemolymph was pipetted into each hole. Sample triplicates were performed on separate plates to account for plate-to-plate variation. Lysozyme from chicken egg white (Sigma-Aldrich) was serially diluted to 100, 50, 10, 5, 2 and 1 µg/mL for use as a comparative standard.

Plates were left at room temperature for 20 min to allow time for haemolymph to diffuse into the agar before inverting and incubating at 30˚C for 48 h, whereupon all plates were digitally photographed. Lytic zones, produced by antibacterial enzyme activity within the haemolymph, were measured using the program ImageJ (Schneider*, et al.*, 2012). The diameter of each lytic zone is proportional to the natural log of lysozyme activity in the sample; a relationship determined using the standard curve. Sample concentration was calculated using the formula $y = x\ln C - D$, where C and D are enzyme concentration and lytic zone diameter respectively (Wiesner, 1992).

Prophenoloxidase activity

Methods used to measure prophenoloxidase (ProPO) activity followed those described in (Haine*, et al.*, 2008). Briefly, triplicate samples of thawed haemolymph were diluted 10-fold in PBS, spun down and 8 µL of the supernatant added to a 96-well plate containing chymotrypsin (5 mg/mL), L-DOPA (4 mg/mL), PBS and distilled water to a give a final haemolymph dilution factor of 1:100. Since this protocol does not use a comparative standard, equal numbers of samples from each treatment group were run on different plates to control for inter-assay variability. Relative ProPO activity rates were measured as linear increases in colour change at 12 s intervals on a spectrophotometer at 492 nm. The slope of the reaction curve during the linear phase (Vmax) has been previously found to satisfy Michaelis-Menten kinetics at the L-DOPA concentration such that there is a direct correlation between enzyme reaction rate and sample ProPO concentration (Thompson, 2003).

Haemolymph protein assays

Haemolymph protein concentration was measured using the Pierce® BCA protein assay kit (Thermo Scientific) to give an indication of insect protein reserves at the time of bleeding. Thawed haemolymph was diluted 100-fold in PBS and protein measured using a colorimetric assay according to the manufacturer's instructions. Endpoint optical density readings were measured in triplicate on a spectrophotometer at 562 nm and compared to a serial dilution standard (0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/mL) of Bovine Serum Albumin (BSA) set up on each 96-well plate.

2.3.8 Analyses

All analyses were performed using the statistical software SPSS (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). Mantel-Cox tests were applied to dose-dependent survival bioassays to identify variation in locust survivorship across the five inoculation doses tested within each phase treatment. Pairwise comparisons were used to identify specific differences in survivorship between behavioural phase treatments for each fungal dose.

Data for both first- and second-generation experiments were tested for equality of variance using Levene's tests ($p>0.05$) and Wilcoxon tests for normality. For first-generation experiments, two-way ANOVAs were used to analyse treatment variation in lysozyme concentration, ProPO activity and THCs, with phase and infection as the two factors. Data for THCs were log transformed prior to analyses yet are graphically presented as untransformed data. Data for haemolymph protein concentration did not meet the assumptions for an ANOVA. Consequently data were analysed using separate Kruskal-Wallis tests for control and infected treatments. Within this data set, four samples showed negative protein concentration values. These samples were removed as outliers. Differences between treatment groups for all immune assays were identified using Tukey's HSD post-hoc tests.

Variation across phase and inoculation treatments within second-generation experiments were also analysed using two-way ANOVAs. Once again, data for protein concentration did not follow the assumptions for the parametric test. Data was therefore analysed using a Mann-Whitney test for control and infected treatment groups separately and plotted individually.

Where appropriate, all immune parameters in both first- and second-generation experiments were tested for an interaction between phase and inoculation treatments. In instances where no significant interaction was observed (*i.e.,* $p>0.05$) the analysis was repeated with the interaction term removed (Engqvist, 2005).

2.4 Results

2.4.1 Dosage-dependent survival assays: lethal dose 50 (LD_{50})

Overall there was no effect of locust phase on survivorship for each fungal dose tested (Table 2.1). Uninfected controls (0 spores/locust) also showed no significant difference in survivorship between the four phase treatments with 100% survival in all treatments except SG, which suffered a single death.

The effect of dose on survivorship for each phase treatment was analysed to identify the LD_{50} of locusts infected with *M. acridium* over the two-week experimental time-course. Overall comparisons showed significant differences in survivorship between the different fungal doses within all four behavioural phase treatments (Figure 2.1; Mantel-Cox: (a) GG: $\chi^2_{(4)}= 90.891$, $p<0.001$; (b) GS: $\chi^2_{(4)}= 83.483$, $p<0.001$; (c) SG: $\chi^2_{(4)}= 98.373$, $p<0.001$, and (d) SS: $\chi^2_{(4)}= 85.864$, $p<0.001$). Pairwise comparisons revealed that only a few fungal dose treatments were not significantly different from each other (*i.e.,* $p>0.05$) within each behavioural phase (Table 2.2). The closest dose to the LD_{50} was identified to be 1000 spores/insect for all behavioural phase treatments. As mentioned in the methods, this dose was taken as the working spore concentration for all subsequent immune experiments.

Table 2.1 Survival rates of *C. terminifera* inoculated with different doses of *M. acridium*. Locusts were subject to one of four behavioural phase treatments: GG (continuously crowd-reared gregarious insects), GS (isolated gregarious locusts), SG (crowd-reared solitarious insects) or SS (continuously solitary-reared insects). Significance values shown for each dose are overall comparisons between phase treatments.

Dose (spores/locust)	Phase	N	Survival Rate (%)	χ^2	d.f	P-value
50	GG	32	84.4	1.348	3	0.718
	GS	33	90.9			
	SG	34	82.4			
	SS	33	81.8			
500	GG	32	50	3.868	3	0.276
	GS	32	62.5			
	SG	32	68.8			
	SS	33	72.7			
1000	GG	32	37.5	0.728	3	0.867
	GS	32	46.9			
	SG	33	36.4			
	SS	33	45.5			
5000	GG	32	6.3	1.476	3	0.688
	GS	32	12.5			
	SG	33	9.1			
	SS	32	9.4			

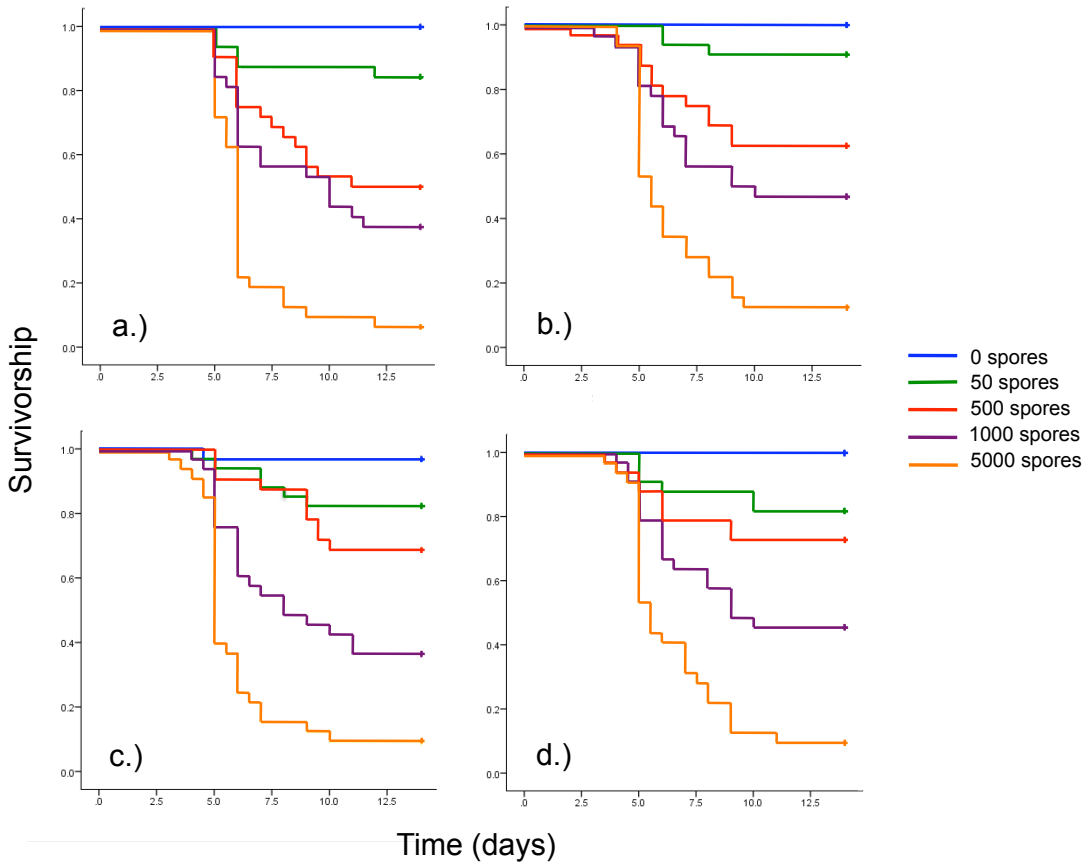

Figure 2.1 Survivorship curves for four behavioural phase groups of *C. terminifera* inoculated with 50, 500, 1000 or 5000 *M. acridium* fungal spores. Phase treatments include: a.) GG (long-term gregarious), b.) GS (gregarious to solitary-reared), c.) SG (solitarious to crowd-reared), and d.) SS (continuously solitary-reared). Uninfected controls (0 spores) were used for comparison. Crosses at the end of survivorship curves indicate data with remaining survivors at the end of the measurement period.

Table 2.2 Pairwise-comparisons of locust survivorship between *M. acridium* doses for each behavioural phase treatment; GG (long-term gregarious), GS (isolated gregarious), SG (crowded solitarious) and SS (long-term solitarious). Data shown are for doses that showed no significant difference in locust survival. All other pairwise comparisons between doses were significantly different (Mantel-Cox, $p<0.05$).

Dose comparison (spores/locust)	Phase	Survival rates (%)	χ^2	p-value
500 *vs.* 1000	GG	50.0 *vs.* 37.5	0.999	0.317
control *vs.* 50	GS	100 *vs.* 90.9	3.002	0.083
500 vs. 1000		62.5 *vs.* 46.9	1.489	0.222
control *vs.* 50	SG	97 *vs.* 82.4	3.664	0.056
50 *vs.* 500		82.4 *vs.* 68.8	1.345	0.246
50 *vs.* 500	SS	81.8 *vs.* 72.7	0.871	0.351

2.4.2 First-generation solitarious versus long-term gregarious locusts

Total haemocyte counts

Total haemocyte counts were significantly affected by both phase and infection treatment (Figure 2.2; two-way ANOVA, phase: $F_{(1,313)} = 13.139$, $p<0.001$, infection: $F_{(1,313)} = 22.074$, $p<0.001$). Here, treatments that experienced phase transitions were significantly different from the long-term phases from which they originated (Tukey's HSD, $p<0.05$), with gregarious locusts that were isolated (GS) showing higher THCs than long-term gregarious treatments (GG). Crowded solitarious locusts (SG), however, had significantly lower THCs than long-term solitarious conspecifics (SS). This pattern was consistent across both Triton-X control and *M. acridium* infected treatment groups. There was no significant interaction between phase and infection treatments.

Lysozyme concentration

As seen in Figure 2.3, a significant difference in lysozyme concentration was observed across phase treatments (two-way ANOVA, $F_{(3,311)} = 6.851$, $p<0.001$). Here, insects from the solitarious phase (SS) were found to have significantly lower lysozyme activity than those originating from long-term gregarious phase treatments (GS and GG) (Tukey's HSD, $p<0.05$). These latter two phase treatments did not differ statistically (Tukey's HSD, $p>0.05$). Solitarious locusts that were crowded (SG) were also not different from long-term solitary (SS) nor long-term gregarious treatments (Tukey's HSD, $p>0.05$).

No statistically significant effect of infection treatment was observed within any of the phase treatments (two-way ANOVA, $F_{(1,311)} = 0.405$, $p = 0.525$), nor was there a significant interaction between phase and infection factors.

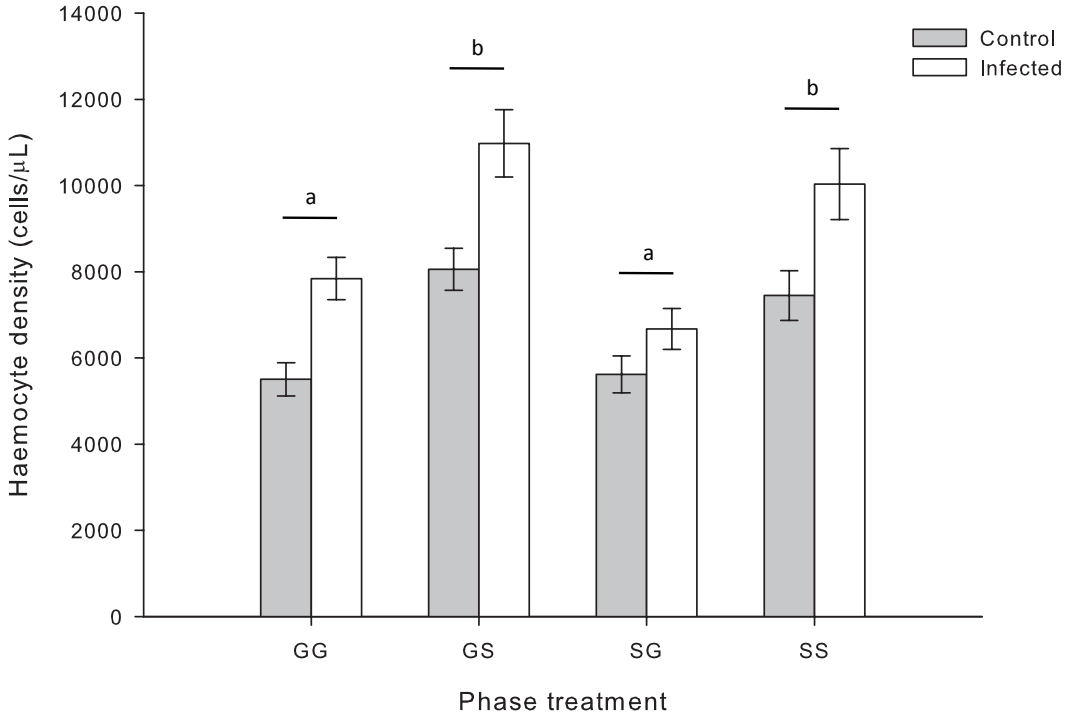

Figure 2.2 Total haemocyte counts of Triton-X inoculated control and *M. acridium* infected locusts across four behavioural phase treatments groups: GG (long-term gregarious), GS (gregarious to solitary-reared), SG (solitarious to crowd-reared), and SS (continuously solitary-reared). Letters denote significant differences between phase treatment groups (Tukey's HSD, $p<0.05$). Error bars = ±SEM.

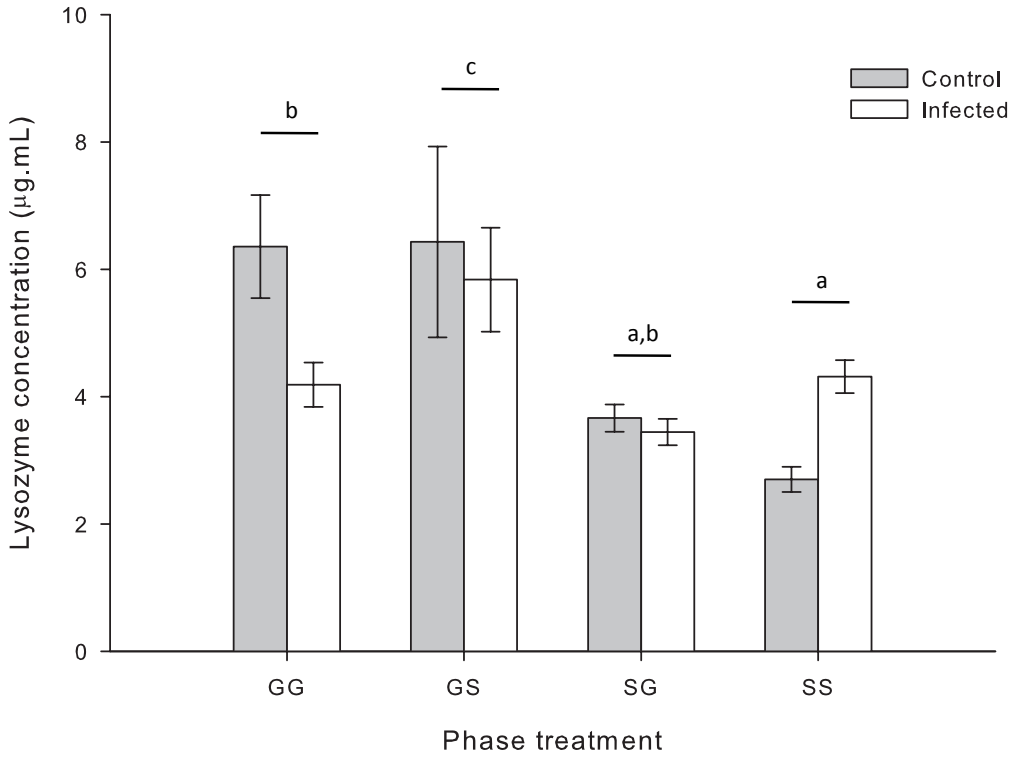

Figure 2.3 Lysozyme concentration of uninfected controls and *M. acridium* infected locusts reared under one of four different behavioural phase regimes; GG (long-term gregarious), GS (gregarious to solitary-reared), SG (solitarious to crowd-reared), and SS (continuously solitary-reared). Different letter indicate significant differences in lysozyme concentrations across phase treatments (Tukey's HSD, $p<0.05$). Error bars = ± SEM.

Prophenoloxidase activity

A significant interaction between phase and infection treatment groups was found for prophenoloxidase activity in first-generation experiments (Figure 2.4; two-way ANOVA, $F_{(3,938)} = 5.906$, $p = 0.001$). Here, statistically significant variation was found across phase treatments (two-way ANOVA, $F_{(3,938)} = 101.028$, $p<0.001$) yet not between infections states (two-way ANOVA, $F_{(1,938)} = 3.435$, $p=0.064$).

Haemolymph protein concentration

Uninfected control locusts showed significant variation in haemolymph protein concentration across behavioural phase treatments (Figure 2.5a; Kruskal-Wallis, $\chi^2_{(3,468)} = 26.618$, $p<0.001$), with long-term gregarious locusts (GG) showing higher protein concentration than insects in the remaining three phases (Tukey's HSD, $p<0.05$).

Fungal-infected insects also showed statistically significant variation in protein concentration (Figure 2.5b; Kruskal-Wallis, $\chi^2_{(3,474)} = 43.008$, $p<0.001$). Here, phase treatments that were long-term gregarious (GG) and those that were crowded (SG) had greater haemolymph protein than isolated (GS) and solitarious (SS) locusts (Tukey's HSD, $p<0.05$).

2.4.3 Second-generation solitarious versus long-term gregarious locusts

Total haemocyte counts

Second-generation solitarious locusts (2SS) showed significantly higher THCs than long-term gregarious conspecifics (GG) (Figure 2.6; two-way ANOVA, $F_{(1,160)}=9.114$, $p=0.003$). In addition, fungal inoculated locusts had significantly higher haemocyte densities than uninfected controls (two-way ANOVA,

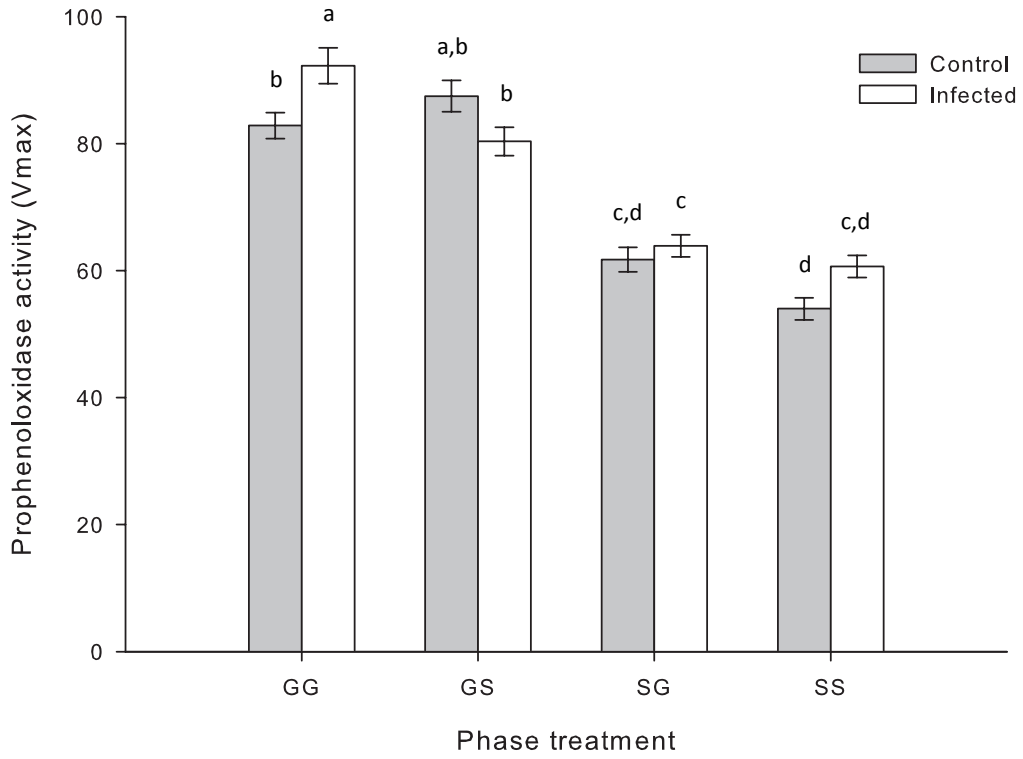

Figure 2.4 Prophenoloxidase activity of GG (long-term gregarious), GS (gregarious to solitary-reared), SG (solitarious to crowd-reared) and SS (continuously solitary-reared) treatments in Triton-X inoculated control and *Metarhizium acridium* infected locusts. Letters show statistical differences between treatments (Tukey's HSD, $p<0.05$). Error bars = ±SEM.

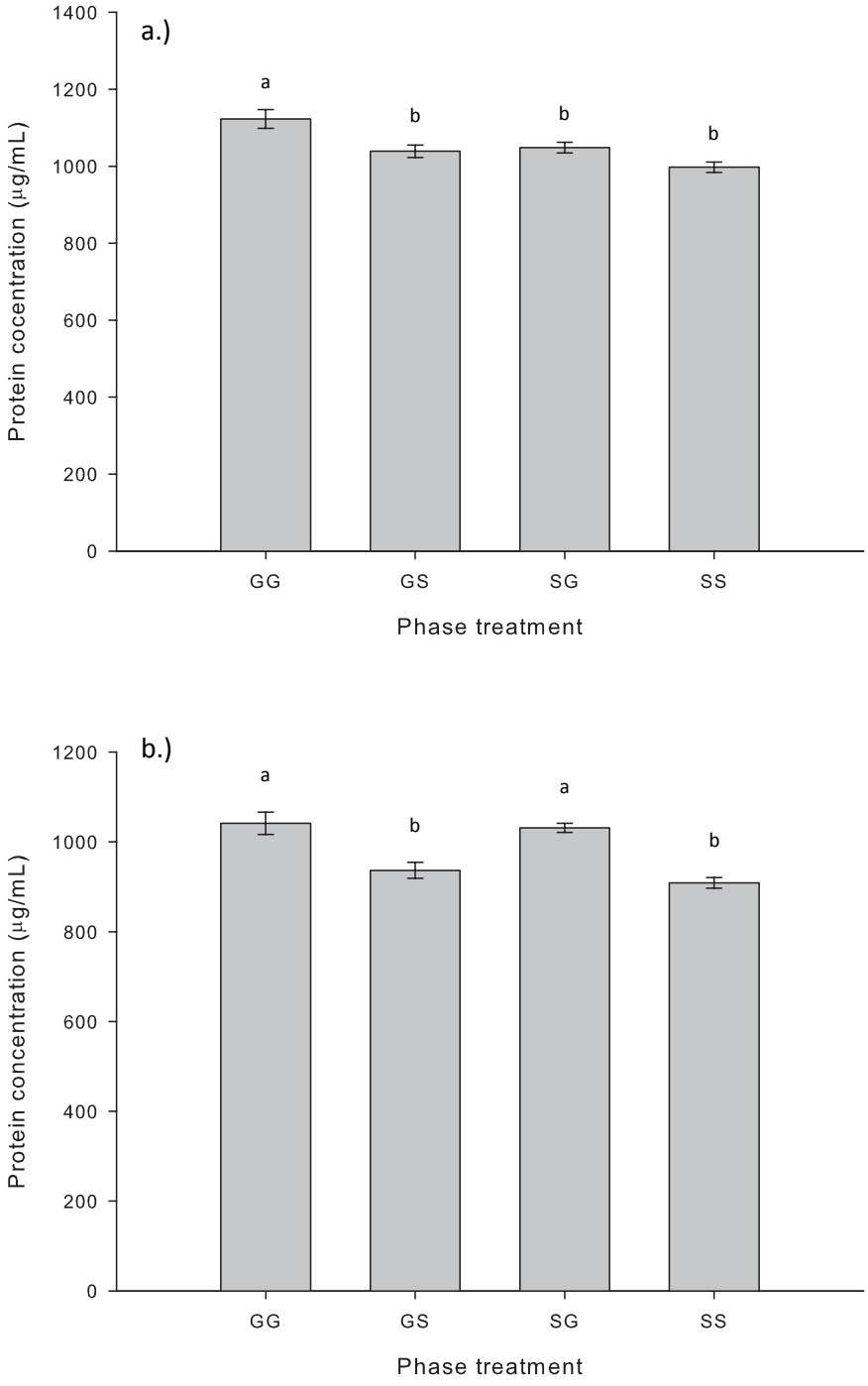

Figure 2.5 Haemolymph protein concentration of a.) uninfected control and b.) *M. acridium* infected locusts from four behavioural phase treatments; GG (long-term gregarious), GS (gregarious to solitary-reared), SG (solitarious to crowd-reared), and SS (continuously solitary-reared). Different letters indicate statistical variation between behavioural phases (Tukey's HSD, $p<0.05$). Error bars = ±SEM.

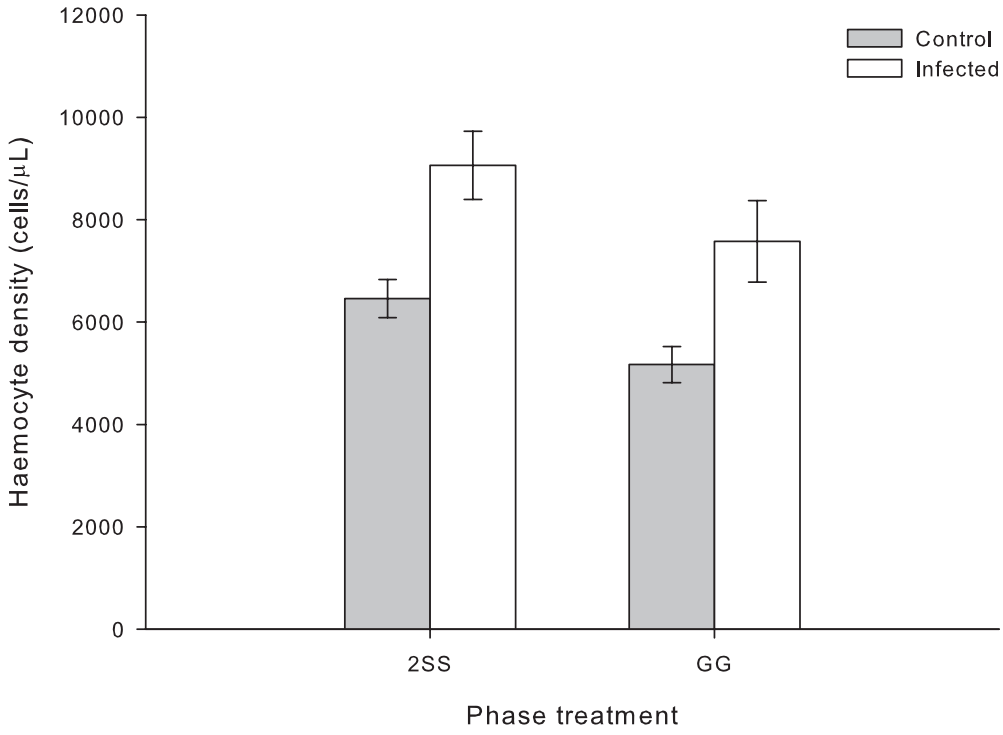

Figure 2.6 Total haemocyte counts for uninfected control and *M. acridium* inoculated *C. terminifera* within second-generation solitarious (2SS) and long-term gregarious (GG) phase treatments. Error bars represent ±SEM.

$F_{(1,160)}$=17.649, p<0.001). There was no significant interaction between behavioural phase and infection treatments.

Lysozyme concentration

Lysozyme concentrations in second-generation solitarious locusts (2SS) were significantly lower compared to those from long-term gregarious (GG) treatments (Figure 2.7; two-way ANOVA, $F_{(1,160)}$=54.286, p<0.001). Infection treatment, however, had no effect on lysozyme concentration, with no significant difference found between control and infected groups within either phase (two-way ANOVA, $F_{(1,160)}$=1.718, p=0.192). The interaction between behavioural phase and infection state treatments was again not significant.

Prophenoloxidase activity

A significant interaction was found between behavioural phase and infection treatment groups (Figure 2.8; two-way ANOVA, $F_{(1,158)}$=4.398, p=0.038). Here, ProPO activity within the solitarious treatment (2SS) was significantly lower in control-inoculated locusts than for fungal-infected (Test of simple effects; Tukey's HSD, p<0.05). In contrast, no significant variation in ProPO activity was observed between infection states within the long-term gregarious treatment (GG) (Test of simple effects; Tukey's HSD, p>0.05).

Haemolymph protein concentration

Long-term gregarious locusts (GG) within control Triton-X inoculated treatments were found to have significantly higher haemolymph protein concentrations than second-generations solitarious (2SS) treatments (Figure 2.9a; Mann-Whitney test, $U_{(1,240)}$ = 3592, p<0.001). In contrast, *M. acridium* infected treatments showed no significant variation in protein concentration between second-generation solitarious

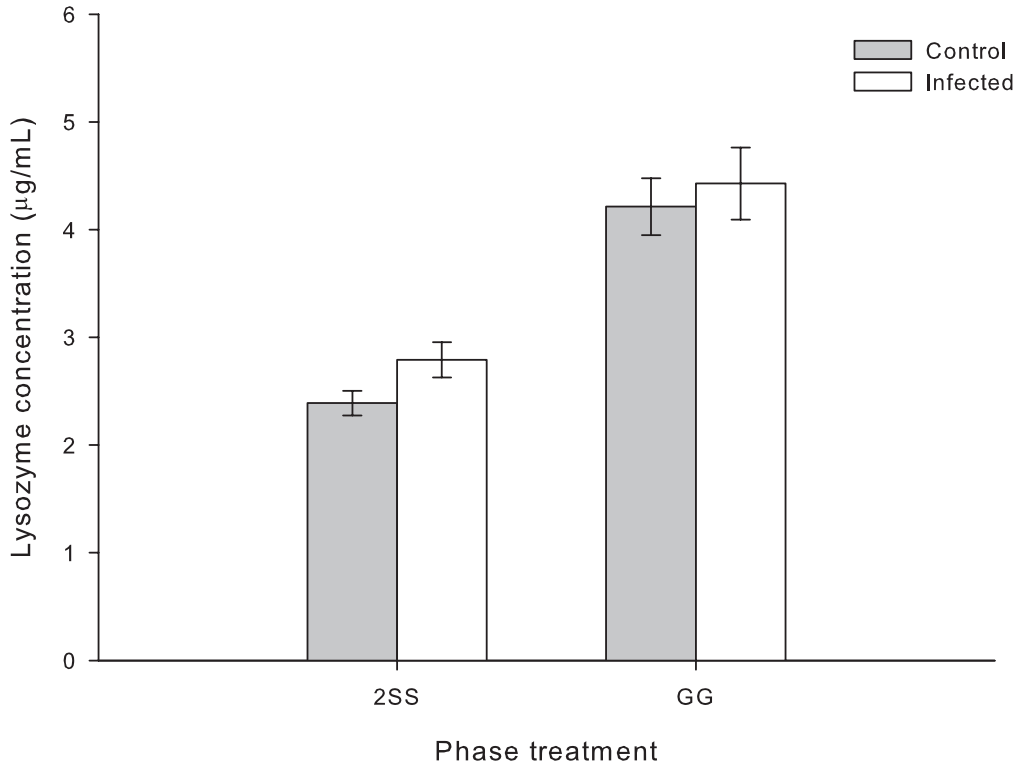

Figure 2.7 Haemolymph lysozyme activity for Triton-X inoculated control and *M. acridium* infected *C. terminifera* within second-generation (2SS) and long-term gregarious (GG) phase treatments. Error bars = ±SEM.

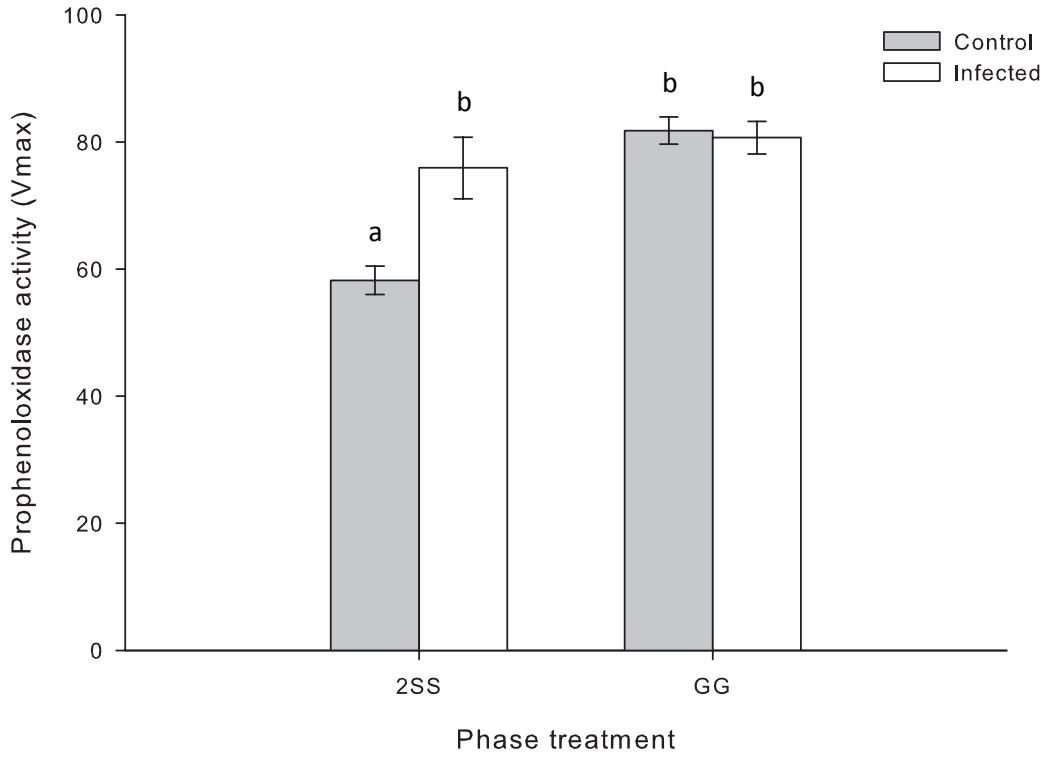

Figure 2.8 Prophenoloxidase activity for control and fungal-infected locusts within second-generation solitarious (2SS) and long-term gregarious (GG) phase treatments. Letters represent significantly different enzyme activities between treatment groups (Tukey's HSD, $p<0.05$). Error bars = ±SEM.

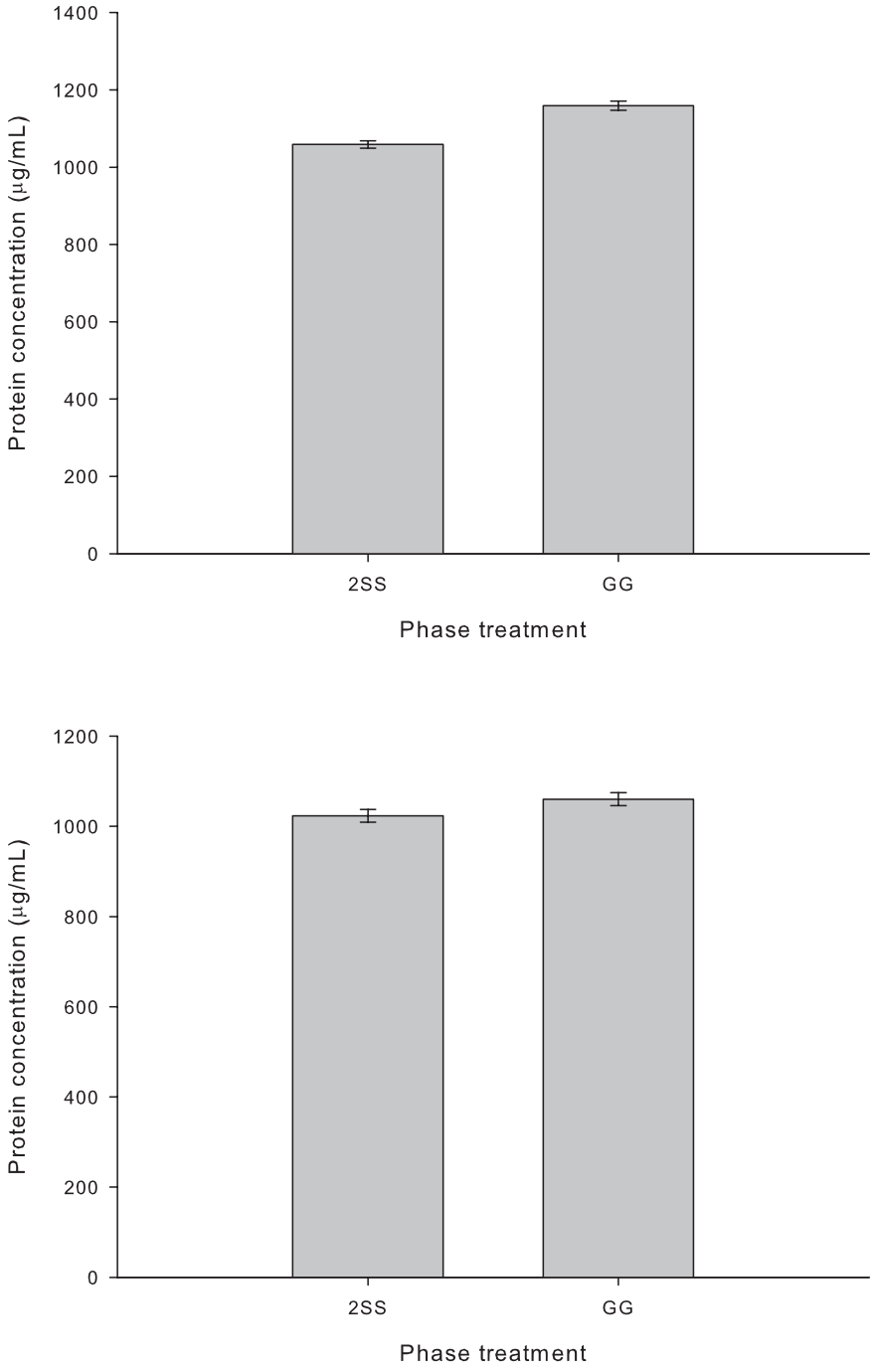

Figure 2.9 Haemolymph protein concentrations of second-generation solitarious (2SS) and long-term gregarious (GG) *C. terminifera* in a.) uninfected control and b.) *M. acridium* inoculated infection treatment groups. Error bars = ±SEM.

(2SS) and long-term gregarious (GG) phase treatments (Figure 2.9b; Mann-Whitney test, $U_{(1,240)} = 6228$, p=0.07).

2.5 Discussion

2.5.1 Density-dependent prophylaxis in *C. terminifera*

In insects, the DDP hypothesis has been largely supported across several orders, including the Lepidoptera, Coleoptera and Orthoptera (Wilson and Reeson, 1998; Barnes and Siva-Jothy, 2000; Wilson*, et al.*, 2002; Wilson and Cotter, 2008). Studies testing DDP across a number of locust species, however, have not been uniformly supportive (Wilson*, et al.*, 2002; Miller*, et al.*, 2009a; Miller and Simpson, 2010). Rather, a more complex interaction between population density and immune function appears to occur.

In these experiments, locust constitutive and responsive immune function was quantified in long-term gregarious, first and second generation solitarious phases, as well as insects that had only recently transitioned between phases over just a few days. Using this setting, this study is the first to investigate the effects of density-rearing duration upon immune function within solitarious and gregarious locust phases.

According to the DDP hypothesis, animals living in groups upregulate their investment in immune function compared to solitarious conspecifics due to a heightened risk of pathogen transmission within a crowd (Wilson and Reeson, 1998). For this hypothesis to be upheld in *C. terminifera*, individuals within the gregarious and crowded treatments (GG and SG) were expected to exhibit elevated expression of constitutive and responsive immune traits, as well as higher protein reserves and longer survivorship than solitarious (SS) and isolated (GS) treatments. Previous

studies looking at survivorship and haemocyte density within locusts, however, have found this not to be the case (Miller*, et al.*, 2009a; Miller and Simpson, 2010). Through the incorporation of GS and SG treatments, in which locusts experienced recent phase transition, the interaction between density-dependent phase polyphenism and locust immunity can now be better defined.

2.5.2 Resistance to fungal infection: locust phase and fungal dose

Unsurprisingly, locust treatments inoculated with increasing doses of *M. acridium* had heightened incidences of mortality across the two-week survival assay. No significant variation in survivorship, however, was identified as a consequence of phase treatment, allowing the identification of a single LD_{50} (and experimental working dose).

Previous survivorship assays on *S. gregaria* have found that hatchlings of solitarious locusts were able to better withstand *M. acridium* infection compared to conspecifics of gregarious parents, suggesting a transgenerational link between phase and immune function, whilst also contradicting the predictions set by the DDP hypothesis (Miller*, et al.*, 2009a). Earlier experiments on *S. gregaria*, however, showed that locusts reared under crowded conditions showed a significantly higher resistance to *M. acridium* than solitary conspecifics (Wilson*, et al.*, 2002), also contradicting the current results from *C. terminifera.*

Several studies have used survival bioassays as a measure of host immunocompetence in response to changes in population density (Roessingh*, et al.*, 1993; Baldal*, et al.*, 2005). Immunocompetence, however, has been defined as an organism's ability to minimise the fitness costs associated with infection (Owens and Wilson, 1999). By this definition, survivorship alone is insufficient in quantifying overall immune function. Therefore, despite many resistance studies showing

support for DDP, a more comprehensive approach is necessary to understand the underlying mechanisms that drive these differences in survivorship (Adamo, 2004a; Cotter*, et al.*, 2004a). Consequently, whilst an effect of rearing density on survivorship was not found in the present experiments, this does not exclude the occurrence of DDP in *C. terminifera* altogether.

2.5.3 Phase duration and immune function

Overall, the results of this study partly support the DDP hypothesis in *C. terminifera*, though not in every immunological trait measured, nor consistently across both infected and uninfected immune states. In first-generation experiments, evidence for DDP was seen in both lysozyme and prophenoloxidase (ProPO) assays and protein concentration, but not in total haemocyte counts, suggesting possible trait prioritisation and differences in transition rates and responses, particularly during phase polyphenism.

Total haemocyte counts

It is generally accepted that hosts challenged by pathogens or other environmental stressors will respond with increases in immune cell proliferation and associated traits (Gillespie*, et al.*, 1997; Lavine and Strand, 2002). In insects, haemocytes are important effectors against infection and known to activate several mechanisms including encapsulation, phagocytosis and nodule formation (Lavine and Strand, 2002). In locusts and most other orthopterans, the haematopoietic tissue is known as the primary source of haemocyte production (Hoffman, *et al.,* 1974). Recent evidence has also shown that circulating haemocytes can replenish themselves through active division during infection and are another key source of immune cell production (Duressa, *et al*., 2015). Variation in haemocyte concentration is therefore

commonly used to indicate changes in the magnitude of such cellular responses (Wilson*, et al.*, 2002).

Contrary to predictions by the DDP hypothesis, THCs in both control and fungal infected groups were lower in long-term gregarious (GG) and crowded (SG) treatments compared to first generation solitarious and isolated gregarious conspecifics (SS and GS). Differences between GG and SS were also statistically significant, revealing a distinct increase in haemocyte density in the solitary treatment. In this case, final rearing density appeared to have strongly influenced THCs in both the infection states tested.

Similarly, THCs in second-generation solitarious locusts were significantly higher compared to long-term gregarious phase conspecifics. Unsurprisingly, fungal infected treatments (both 2SS and GG) in these experiments showed significantly higher THCs compared to uninfected controls regardless of behavioural phase, indicating a possible prioritisation toward an infection response rather than rearing density.

Similar results have been previously found in wild *C. terminifera*, with locusts exhibiting consistent increases in THCs following up to two days of isolation. This suggests a greater risk of exposure to parasitoids and nematodes in the solitarious phase (Miller and Simpson, 2010). Following a three-day isolation period in these experiments, locusts from the GS treatment group (*i.e.,* isolated gregarious insects) were found to further support these findings. In *S. gregaria*, higher (though non-significant) THCs were found in gregarious locusts compared to solitarious conspecifics (Wilson*, et al.*, 2002), a trend that appears to oppose results from all other known locust studies to date. However, since these results were not statistically significant, concrete evidence in favour of DDP cannot be assumed.

In Lepidoptera, similar contrasts between THCs and rearing densities have been observed between species. Wilson *et al.* (2003) identified six solitarious species with higher haemocyte densities than their gregarious counterparts, clearly opposing the DDP hypothesis. Recent works on *Anticarsia gemmatalis*, however, found higher THCs in larvae reared in the presence of conspecifics than those that were reared alone (Silva*, et al.*, 2013). Although *A. gemmatalis* is not known for group living, this response was identified as a direct cause of rearing density, with larval phenotype showing no effect upon immune function.

Lysozyme activity

A decreasing trend in lysozyme concentration, indicating changes in the synthesis of antibacterial enzymes secreted by the fat body (Gillespie*, et al.*, 1997), was observed in both control and infected *C. terminifera* across phase treatments. Here, uninfected treatments that began in the gregarious phase (GG and GS) had significantly higher lysozyme activity than those within the first generation solitarious phase (SS), ultimately supporting the DDP hypothesis. Higher, though not statistically significant, activity was also seen in GG insects compared to crowded solitarious locusts (SG). This suggests that insects experiencing recent transitions in density-dependent phase may still have been undergoing complementary shifts in immune function that may not have been detectable by this assay.

The addition of *M. acridium* infection did not change the relative lysozyme activity between phase treatments. Unlike results for THCs, this suggests that *M. acridium* infected locusts may not be constrained by infection within this immune trait, and that investments toward pathogen response are not prioritised over those to rearing density.

In second-generation treatments, locusts from Triton-X and *M. acridium* inoculated treatments showed no difference in lysozyme activity suggesting no antibacterial response to the presence of infection. Despite this, locusts from the long-term gregarious phase (GG) had significantly higher lysozyme concentrations than second-generation solitarious conspecifics (2SS), supporting the DDP hypothesis as seen within first-generation experiments. Similar results in *S. gregaria* also found significantly higher lysozyme activity in gregarious phase locusts compared to solitarious, a result that strongly supports the occurrence of DDP in locusts (Wilson*, et al.*, 2002).

The current results reveal a constitutive increase in lysozyme activity in response to density-dependent phase polyphenism, regardless of infection state or total rearing duration. Interestingly, no response to infection occurs to alter these density-dependent responses; again suggesting that response to infection does not outweigh immune investments toward rearing density.

In contrast, studies on Mormon crickets have consistently found no short-term plastic density-dependent response of lysozyme activity, despite observations that migratory populations have higher lysozyme concentrations than solitarious individuals (Bailey*, et al.*, 2008; Srygley, 2012). In this case, the lack of plasticity in this trait indicates deep-rooted historical or maternal effects on lysozyme activity, opposing predictions of flexible prophylactic responses to density variation (Bailey*, et al.*, 2008).

In *G. texensis*, lysozyme-like activity, produced by enzymes that have activity against gram-negative (rather than gram-positive) bacteria did not differ between crowded and isolated individuals (Adamo and Parsons, 2006). Other tested stressors such as heat and restraint, however, caused significant decreases in enzyme activity

compared to controls, indicating that investment in this trait in response to crowding may not have been prioritised over other immune traits.

A similar trade-off between antibacterial activity and other immune traits has been suggested to occur in the phase polyphenic species, *Spodoptera littoralis* (Cotter*, et al.*, 2004a). Here, solitary-reared larvae showed significantly higher antibacterial activity than crowded conspecifics, suggesting that such insects may be unable to invest in all types of immune traits simultaneously (Cotter*, et al.*, 2004a).

Prophenoloxidase activity

Prophenoloxidase is a key enzyme implicated in encapsulation, nodule formation and several other immune defences (Hoffmann, 1995; Wilson*, et al.*, 2002). Its main role serves as the inactive precursor to phenoloxidase, a critical enzyme involved in the synthesis of melanin in the cuticle of insects (Hoffmann, 1995; Wilson and Cotter, 2008; Gonzalez-Santoyo and Córdoba‐Aguilar, 2012).

In Triton-X and *M. acridium* inoculated *C. terminifera,* higher ProPO activity was found in treatments that originated in the gregarious phase (*i.e.,* GG and GS) compared to those that were initially solitary-reared (SS and SG). Here, initial phase appeared to strongly influence ProPO activity in locusts in accordance with the DDP hypothesis, regardless of infection treatment. Unlike the results seen in previous traits, this suggests that the distribution of resources toward ProPO activity may be differentially prioritised in favour of rearing density rather than the presence infection. Furthermore, crowded and isolated treatments (SG and GS respectively) varied significantly from each other, yet did not vary significantly from their initial rearing phases. This suggests that ProPO variation in response to rearing density

occurs at a similar rate as the behavioural transitions observed during phase polyphenism in this species.

Support for the DDP hypothesis was also found in second-generation ProPO measurements, though this time in uninfected treatment groups only. Interestingly, effects of *M. acridium* infection were observed only in the solitarious (2SS) phase, with fungal infected individuals exhibiting higher ProPO than uninfected controls as expected. Gregarious locusts showed no apparent response to fungal infection, perhaps due to their higher constitutive immunity compared with solitarious locusts, thereby eliminating the need for an infection response.

Age-dependent variation in ProPO in Mormon crickets has been identified as a response to population density (Srygley, 2012). In young solitary-reared adult crickets, significantly greater ProPO activity was detected compared to group-reared conspecifics. Conversely, older adults reared at high densities showed significantly higher ProPO concentrations than solitary-reared individuals, thereby supporting the DDP hypothesis in older adults only (Srygley, 2012). Such results reveal how host responses to different environmental stressors can vary across an individual's lifetime, a conclusion that has been mirrored in social insects (Ruiz-González*, et al.*, 2009).

Although there are few reports on how rearing density influences ProPO, effects on its product, phenoloxidase (PO), have been investigated in greater depth. High-density phenotypes of *S. littoralis*, for example, have revealed significantly higher PO activity than solitary reared, a result that aligns with this study in favour of the DDP hypothesis (Cotter*, et al.*, 2004a). In contrast, similar studies on other species (including locusts, crickets and beetles) have found no variation in PO with rearing density (Barnes and Siva-Jothy, 2000; Wilson*, et al.*, 2002; Adamo and Parsons,

2006). Since these studies did find positive effects of rearing density in other immune traits, PO regulation appears to be more costly than investment in other traits. In *C. terminifera*, such immune investments may be less costly for its precursor ProPO.

Haemolymph protein concentration

Protein concentration in insect haemolymph is often used as an indication of insect nutrition (*i.e.,* protein reserves) and overall body condition (Cotter*, et al.*, 2004a). Previous authors have proposed that individuals in good condition should be more capable of mounting an immune response and resisting infection than conspecifics in poorer condition (Møller*, et al.*, 1998; Westneat and Birkhead, 1998). In uninfected treatments, *C. terminifera* showed heightened haemolymph protein concentrations within the pong-term gregarious (GG) phase group. indicating a strong effect of long-term crowding on protein reserves. The remaining phase treatments (GS, SG and SS), however, did not differ significantly from each other. These results differ from those seen in *G. texensis*, which saw no evidence to indicate an effect of crowding on haemolymph protein concentration. High variability within groups, however, was suggested to account for this result (Adamo and Parsons, 2006).

Fungal infection in this study caused significant differences in protein concentration across phase treatments, altogether supporting the DDP hypothesis. Here, first-generation solitarious locusts (SS) had significantly lower protein levels than crowded conspecifics (SG) revealing an adaptive change in protein investment when subject to both infection and recent crowding. A similar trend was seen between GG and GS with isolated individuals showing decreased protein levels compared to long-term gregarious counterparts. Comparably, GG locusts were also observed to have slightly greater protein reserves than SS treatments.

Results comparing haemolymph protein concentration of second-generation solitarious and long-term gregarious locusts in uninfected controls were similar to first generation results. Here, second generation solitary locusts (2SS) were found to have significantly lower haemolymph protein levels than gregarious conspecifics (GG). Since both groups received food *ad libitum*, these results suggest that locusts at high density may have allocated more resources toward protein reserves than those reared in isolation, ultimately supporting the DDP hypothesis.

Cotter *et al* (2004a) have suggested that the act of living in groups generally lowers overall host fitness due to fewer available resources. Their study on *S. littoralis* showed a greater body condition (approximated through haemolymph protein and body mass) in solitary-reared larvae compared to crowded conspecifics, despite being offered food *ad libitum*. Such results provide insight into the adaptive responses of larvae to their perceived rearing density, despite the abundance of nutritional resources (Cotter*, et al.*, 2004b).

In the present work, a result opposing DDP was seen in fungal infected phase treatments, with high-density groups exhibiting protein reserves that did not statistically vary from solitarious phases. This difference may suggest that an adaptive response to high rearing density does not occur in the presence of infection, despite increases in the risk of pathogen transmission.

2.5.4 Density-dependent phase polyphenism and locust immunity

Recent works studying DDP in insects have shown that selective resource investment in host immune function is a common observation in response to variation in rearing density (Adamo*, et al.*, 2001; Cotter*, et al.*, 2004b; Adamo and Parsons, 2006; Piesk*, et al.*, 2013). The results found in current experiments appear to support these suggestions, with some traits showing a greater response to locust rearing phase than

others. Rather than oppose the DDP hypothesis in *C. terminifera* altogether, these results provide insight into the complexities between immune function and crowding as an environmental stressor, highlighting the adaptive strategies that locusts have evolved to maximise both their overall fitness and immunocompetence in response to rapid changes in rearing density.

Of the four immune traits tested, ProPO was found to show the strongest response to rearing phase, with clear differences between gregarious and solitarious phase treatments in support of the DDP hypothesis, regardless of infection state or rearing duration. Lysozyme activity was also found to be relatively consistent, changing according to the DDP hypothesis in first- and second-generation uninfected control treatments.

Haemolymph protein concentration appeared to be more labile in response to rearing density. In uninfected controls, protein values appeared to respond slower to phase change following short-term crowding, indicating that this trait may not be the best prophylactic indicator of density-dependent phase polymorphism.

Interestingly, total haemocyte density opposed DDP predictions across all phase and infection treatments, a result that aligns with previous work on *C. terminifera* (Miller and Simpson, 2010). Similarly, no effect of rearing-density was found on locust survival, also contradicting the basic theory of DDP as mirrored in previous works on *S. gregaria* (Miller*, et al.*, 2009a).

It is clear that different immune traits transition and respond to changes in rearing density at different rates within *C. terminifera*. For locusts in general, the effects of small changes in population dynamics can be remarkable. Since other phase-related traits (*e.g.,* morphology, physiology and metabolism) are known to transition asynchronously with behaviour (Roessingh and Simpson, 1994; Gray*, et al.*, 2009), it

is not unexpected to find that the different aspects of immune function also change at different rates. With complex and unpredictable interactions between host physiology, pathogens and other ecological factors, the capacity to flexibly adapt, rather than rely solely on hard-wired responses, is a trait of particular evolutionary benefit.

In the context of density-dependent phase polyphenism, locust immunity appears to function as a multicomponent system where each trait differs in its rate of change. As mentioned above, this may be due to the selective redistribution of finite resources that, during unpredictable and rapid changes in population density, must shift to accommodate traits of greater priority. During periods of infection (an ecological stressor), this appears to again alter resource allocation and investments.

By investigating the effects of rearing density upon single immune traits, many studies risk losing the impact of such trade-offs. Consequently, the interactions between host immunity and population density, implicated by the DDP theory, are often oversimplified. With so many variables influencing host immunity in the field, the ability to tease out one and determine its effects on immune function alone may be somewhat unrealistic.

2.5.5 Conclusions and applied significance

The present work suggests a complex relationship between population density and immune function, one that depends on resource allocation and investment toward individual immune traits. Within the field of ecological immunology, it is becoming increasingly understood individual immune traits measures provide only a limited understanding of the mechanistic responses that occur within the broader suite of integrated host responses (Cotter*, et al.*, 2004a; Cotter*, et al.*, 2004b; Adamo and Parsons, 2006; Lazzaro and Little, 2009). Instead, many authors now recommend a

multifaceted approach, where numerous immune traits are quantified in an attempt to gain a wider insight into overall immune function (Adamo, 2004a; Cotter*, et al.*, 2004a; Schulenburg*, et al.*, 2009). Through the measurement of four well-described host immune and condition traits, as well as dose-dependent survival, this study has followed current advice. By doing so this work has obtained a more detailed understanding into the complex nature of immune investment in *C. terminifera*, in particular how different traits may be prioritised in response to changes in density and rearing duration.

How insects respond to infection on a functional level is of practical importance, particularly when looking at variation in disease resistance of outbreaking pest species. The agricultural devastation caused by *C. terminifera* (an insect that is only destructive to crops when it occurs in plague densities) makes this study one of high priority. The results of such works could be useful to develop and improve the use of infectious agents as biological controls of pest species around the world. With a better understanding of how individual immune traits respond to specific pathogens under different ecological circumstances, there is a greater capacity to identify and target pests at a time where overall immunocompetence may be more vulnerable due to trade-offs with other fitness traits. This is especially important for entomopathogens such as *M. acridium*, where the mechanisms of action as well as other ecological impacts are still poorly understood.

This study has provided evidence in support of the DDP hypothesis in *C. terminifera*. It has also demonstrated that, when stood alone, the results of some immunological assays appear to refute the same theory. The importance of assay selection and sampling time in the study of host immune function has been demonstrated from this work. Indeed, for the selection of measurable traits in future studies, one lesson can be easily conveyed; proceed broadly and with caution.

Chapter 3.

Cannibalism and Immunity

3.1 Summary

Uncovering the links between nutrition, cannibalism and locust migration has been a major step forward in developing a better understanding of the biology and population dynamics of locusts. Limiting resources within crowded migratory bands can lead to cannibalism, where locusts seek to satisfy nutritional deficiencies by consuming their conspecifics. Vulnerable locusts, whether through injury, small size or developmental stage, are often the first to fall victim. Although there are nutritional benefits, there are also costs to cannibalistic encounters. Victims harbouring infection, for instance, may impose a higher risk of pathogen transmission to the cannibal, thereby creating a trade-off between nutritional gain and potential infection. This chapter investigated whether cannibalistic locusts have the capacity to distinguish between fungal-infected and uninfected victims. Experiments used the acridid-specific fungus, *Metarhizium acridium,* to generate freshly killed victims at differing stages of infection. These were offered to potential cannibals in a series of choice, no-choice and survival assays. Results found that cannibals avoided victims where visible indicators of *M. acridium* infection were most obvious. Early-stage infected cadavers, with no apparent symptoms of infection, were not discriminated from uninfected controls and therefore cannibalised with equal likelihood. When presented with victims that were newly-dead of *M. acridium* infection, locusts completely avoided cannibalism, ultimately leading to death by starvation. An analogous result was found for cannibals offered sporulating victims, where infection had progressed even further. With new insights into how locusts are deterred from infection despite the nutritional costs, this chapter facilitates current understandings of locust immune behaviour in the context of cannibalism. The implications for the management of this destructive pest species are discussed.

3.2 Introduction

Cannibalism was once considered a rare event influenced primarily by the physiological stresses of captive animals living in unnatural laboratory environments (Thiele, 1977). However, such behaviours, whether habitual or opportunistic, have been progressively found to be much more common in the natural world than originally thought (Fox, 1975; Polis, 1981; Brunsting and Heessen, 1983; Richardson*, et al.*, 2010). In recent years, observational studies of cannibalism, defined here as intraspecific predation or necrophagy (Fox, 1975; Polis, 1981), have led to a number of interesting findings. The identification of new species that display cannibalism, *e.g.,* grey mouse lemur, red land crabs and crickets (Wolcott and Wolcott, 1984; Simpson*, et al.*, 2006; Hämäläinen, 2012), have led to an anthology of works associating cannibalistic behaviour with its ecological and evolutionary consequences (Polis, 1981; Elgar and Crespi, 1992; Richardson*, et al.*, 2010).

3.2.1 Pathogen transmission and other costs of cannibalism

When carrying out cannibalistic feeding, individuals have often been reported to exhibit behaviours that allow them to avoid the various costs associated with this activity (Pfennig, 1997). During predatory cannibalism (the active killing and consumption of live victims) individuals will attempt to minimise the risk of harm from evenly matched conspecifics by targeting smaller or slower prey (Pfennig*, et al.*, 1998; Rudolf and Antonovics, 2007). Cannibals in several systems have also been documented to avoid consumption of their relatives (Klahn and Gamboa, 1983; Pfennig*, et al.*, 1993; Joseph*, et al.*, 1999; Bilde and Lubin, 2001). One benefit of such cannibalism avoidance as an evolved trait is the capacity for individuals to preserve their indirect inclusive fitness through kin recognition behaviour (Hamilton, 1964a; Hamilton, 1964b).

In a similar way to limited size and mobility, the presence of infection poses an additional level of vulnerability for potential victims. It also presents an additional level of risk to cannibals. The occurrence of pathogen transmission through cannibalism, however, whilst frequently noted throughout the literature, has rarely been studied (Polis, 1981; Williams and Hernández, 2006; Rudolf and Antonovics, 2007). The role of immunity in these processes is also poorly understood. Consequently, how parasites and pathogens influence the adaptive behaviours of potential cannibals is of particular interest.

Studies using theoretical modelling have suggested that the one-on-one transmission of endemic disease from victim to cannibal is a relatively rare occurrence, where animals living and feeding in social groups have a greater chance of acquiring a disease from a shared infected victim (Rudolf and Antonovics, 2007). Several studies across a range of species, however, have reported evidence for the transmission of disease via individual cannibalism, indicating that pathogen transfer could be a critical cost of this behaviour in many species (Prusiner*, et al.*, 1985; Pfennig*, et al.*, 1991; Williams and Hernández, 2006; Pizzatto and Shine, 2011).

3.2.2 Cannibalism and collective movement in Orthoptera

Cannibalistic behaviour is a well-known aspect of orthopteran ecology. Several studies have shown that nutritional imbalance is a primary cause of cannibalism in several species. Experiments using the Mormon cricket, *Anabrus simplex,* have found that protein and (to a lesser degree) salt are two substantially important nutrients in the diets of these insects (Simpson*, et al.*, 2006). Consequently, living in environments where resources are patchy often forces individuals to converge at areas of available food. At high population densities, however, such resources quickly become depleted, whereupon individuals may attempt to satisfy their dietary

requirements by consuming their next closest source of protein; each other (Simpson*, et al.*, 2006).

Just as in *A. simplex*, a locust's appetite for protein drives individuals to migrate, moving forward collectively for two important reasons: (i.) to find new resources, and (ii.) to avoid falling victim to cannibalism themselves (Simpson*, et al.*, 2006). In juvenile flightless locusts, this behaviour results in the formation of densely aggregated marching bands, sometimes stretching several kilometres wide and persisting until alternative resources are found (Bazazi*, et al.*, 2008; Bazazi*, et al.*, 2011). Once a locust's appetite for protein has been satisfied or a new source of protein is encountered, cannibalistic tendencies (and therefore the marching bands that result) diminish.

Uncovering the links between nutrition, cannibalism and migration among several orthopteran species has resulted in important advances in our understanding of many disciplines spanning insect physiology, collective decision-making and even human nutrition (Simpson and Raubenheimer, 2012). It is phenomena such as these that demonstrate the impacts of environmental variability on individual physiology and behaviour, particularly in the context of population dynamics (Buhl*, et al.*, 2011). Consequently, cannibalism has been described as a driving force behind locust migration and potentially a key factor in the evolution of density-dependent phase polyphenism (Bazazi*, et al.*, 2008; Guttal*, et al.*, 2012).

3.2.3 Vulnerability increases the risk of cannibalism in locusts

Where possible, a hungry locust will scavenge upon already dead conspecifics (Lockwood, 1988; Hansen*, et al.*, 2011). Injured, smaller and younger individuals are also among the first to fall victim to cannibalism (Polis, 1981). True for most predator-prey interactions, targeting vulnerable prey allows cannibals to save energy

whilst simultaneously lowering their risk of injury or death from a defensive victim (Barbosa, 2005; Richardson*, et al.*, 2010). Recent studies on the Australian plague locust (*Chortoicetes terminifera*) have shown that mobile victims are less likely to fall prey to cannibalism, where individuals are able to avoid and defend themselves from potential predators (Hansen*, et al.*, 2011). Conversely, immobile and immotile victims are at high risk of being eaten, altogether demonstrating that victim vulnerability has a major influence on the prey choices made by potential cannibals (Simpson*, et al.*, 2006; Hansen*, et al.*, 2011).

In addition to behaviours that minimise injury and avoid consumption of kin, it is of similar interest to determine whether cannibalistic individuals are also deterred by infection (and potential pathogen transmission) when selecting their victims. That locusts will avoid conspecifics that are vulnerable as a result of infection has until recently been supported only by anecdotal evidence. A recent study by Jaronski (2013) has demonstrated cannibalism avoidance behaviour of fungal infected conspecifics in four different orthopteran species. Of the few cannibals that consumed infected conspecifics, fewer still were found to succumb to infection transmission. Nonetheless, fungal infection significantly deterred cannibalistic feeding despite up to 24 h starvation, suggesting a minimal capacity for the horizontal transmission of either *Metarhizium acridium* or *Beauveria bassiana* through cannibalism (Jaronski, 2013). In the present study, similar experiments, including choice, no-choice and survivorship assays were undertaken in the locust species, *C. terminifera.* Whether adapted deterrence to infected conspecifics also occurs in cannibalistic locusts was a main focus of these experiments as was the implications this has to the biological control of locust plagues.

3.2.4 Aims

The primary goal of this study was to determine whether cannibalistic *C. terminifera* have the capacity to distinguish between victims infected with *M. acridium* and uninfected controls. To examine victim choice in greater detail, three victim infection stages were generated in an attempt to associate the degree of risk with cannibal deterrence. Finally, the long-term impact of cannibalistic behaviour was evaluated by performing survival bioassays of locusts offered only uninfected or newly-dead infected victims.

Altogether, the exact aims for this work were to: i.) identify a locust's ability to differentiate between fungal infected and uninfected conspecifics; ii.) determine the stage of victim infection from which locusts will refrain from cannibalism, and iii.) identify the degree of starvation that a locust will experience before consuming an infected conspecific.

3.3 Materials and methods

3.3.1 Insect cultures and treatments

Locusts were cultured according to previously described protocols (refer to Chapter 2). Newly moulted 5^{th} instar males (reared under gregarious conditions) were collected and fed *ad libitum* on fresh wheat grass and wheat germ for 24 h prior to beginning treatments. Cages (45x45x45 cm) were maintained at 30-33˚C. Additional heat lamps were not used to prevent locusts from using thermoregulatory behaviour as a response to infection.

3.3.2 Victim generation

At 24 h post-moult, locusts were inoculated with either a fungal suspension of the *M. acridium* in 0.07% (v/v) Triton-X (Sigma-Aldrich) (infected treatment group) or Triton-X alone (control treatment groups) as outlined below.

Pathogen handling and locust inoculation

Subcultures of *M. acridium* were grown according to methods described in Chapter 2. Spores were then suspended in 0.07% (v/v) Triton-X and enumerated using an Improved Neubauer brand haemocytometer before diluting to a working concentration of 2 x 10^3 spores/µL.

Locusts were infected by pipetting 2µL of the spore solution to the neck membrane underneath the pronotum of each insect (approximately 4000 spores/locust). Control locusts were inoculated with the same volume of Triton-X (0.07% v/v). Following inoculation, locusts were returned to cages and maintained under the rearing conditions described above.

Victim generation: infection stage treatments

Victims of three differing stages of infection were generated from *M. acridium* infected locusts. Three days after inoculation, one third of the infected insects were weighed and killed by freezing at -80°C. As this was before locusts died naturally from *M. acridium*, no visual evidence of infection was observed. These insects were termed the early-infection stage treatment group (Figure 3.1a). Triton-X inoculated control locusts were also weighed and killed at the same time. There were no apparent differences between infected and control insects, which were matched in pairs according to approximate size and mass before storing at -80˚C.

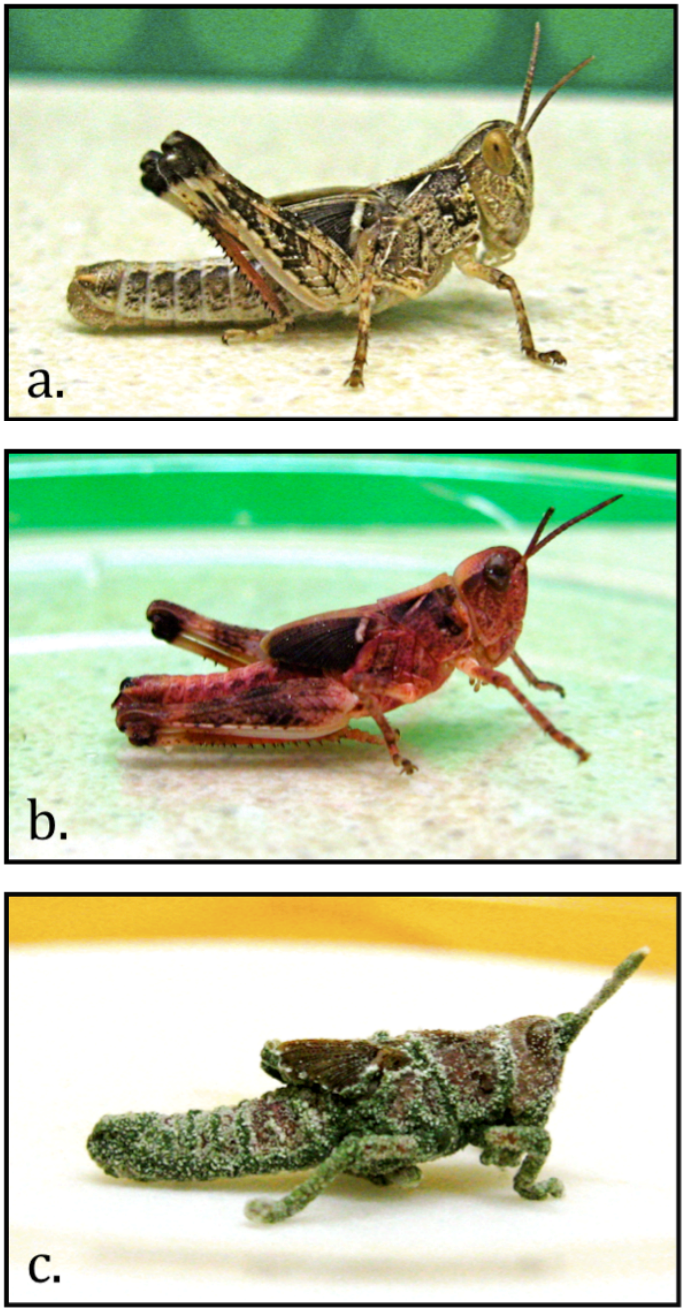

Figure 3.1 Fifth-instar *C. terminifera* were inoculated with *M. acridium* and the infection allowed to progress to three different stages: a.) early-stage locusts, killed at three days post-inoculation before natural death by infection; b.) newly-dead infected locusts that turned bright red in colour upon death, and c.) sporulating infected victims, where newly-dead cadavers were incubated under humid conditions to allow sporulation of fungal mycelia.

Locusts that died naturally of *M. acridium* (but showed no apparent signs of sporulation) were collected as the second stage of infection (Figure 3.1b). At the dosage used, deaths by *M. acridium* generally took 4-6 days following inoculation and were easily identified by their distinctly red cadavers. Newly-dead infected cadavers were collected and weighed as soon as this colour was evident (usually within one hour of death). Control insects of the same age and similar size and mass were collected and paired with infected equivalents as above. Though instar age was constant, individual development was observed to vary slightly between infected and control individuals, with uninfected controls showing faster growth than infected conspecifics (personal observation). Consequently, only locusts that had not yet moulted into adults were used as these controls. Control and newly-dead infected insect pairs were stored at -80°C until use.

Fungal infected cadavers, from which *M. acridium* was allowed to sporulate, were used as the final infection stage treatment (Figure 3.1c). This was achieved by incubating newly-dead cadavers on moistened filter paper in Petri dishes at 30°C for 5 d. The humid conditions generated allowed fungal mycelia to grow and sporulate out of locust cadavers. Although sporulation occurred at different rates and resulted in varied amounts of fungi between insects, this incubation period allowed the majority of all external spores to turn from white to dark green, as described in fungal culturing protocols (Chapter 2). As with the earlier infection-stage treatments, control insects of the same age were weighed to match the mass and size of sporulating cadavers and then killed by freezing at -80°C. Locust cadavers that had undergone sporulation were stored at 4°C and used within three days of generation. At this temperature, *M. acridium* spores remain viable for several months (personal observation).

3.3.3 Cannibal generation and choice behavioural assays

Male and female *C. terminifera* were collected upon moulting to 5th instar and fed *ad libitum* on fresh wheat grass and wheat germ for 48 h. Locusts (*i.e.,* potential cannibals) were then transferred to individual cages and deprived of food for 24 h prior to behavioural assays.

Choice assays

Cannibalism choice assays were conducted in paper circular arenas of 90 mm diameter and 70 mm height in a temperature controlled room of 30-33˚C (Figure 3.2). Potential cannibals (n=52 per infection stage treatment) were placed individually inside arenas and enclosed under blacked out specimen jars for 15 min to allow them to briefly adjust to their surroundings. During this time victim pairs (*i.e.,* early-infection, newly-dead or sporulating infected and control pairs) were thawed and fastened with Blu-tac to the arena paper floors approximately 2 cm apart from each other and 3 cm from the cannibal's starting position. Hind-legs of victims were removed to ensure that cannibal choice was not influenced by position of the femurs, which were often extended asymmetrically as a result of death or freezing. Otherwise, only victims that were completely intact were used for assays. Infected and control victim positions were randomised across arenas.

Arenas were covered with Petri-dish lids to prevent locusts from escaping. Potential cannibals were released into the arenas and left without disturbance for 3 h before terminating the experiment. Locusts that were observed to have cannibalised one or both victim(s) were transferred to individual cages, maintained *ad libitum* on wheat grass and wheat germ and checked twice daily until ecdysis or death.

Figure 3.2 Behavioural arenas used for cannibalism assays. Victim cadavers were fastened with Blue-tac to the top half of arenas and potential cannibals released within the lower half. Control and infected victims were positioned randomly to account for any directional bias by cannibals. Assays were filmed for 3 hours and videos analysed for victim choice and visit duration data.

Video data collection

Locusts were filmed using a JVC camcorder during each 3-h behavioural assay. Video data were used to manually score cannibal behaviour within each infection stage treatment. Parameters scored included first victim choice, total time spent with victims, total number of visits and visit duration with each victim type (*i.e.,* control or infected). As mentioned above, all victims were additionally inspected after each behavioural assay for direct evidence of cannibalism.

3.3.4 Starvation and no-choice assays

No-choice experiments were performed to identify the extent of starvation that *C. terminifera* would endure before consumption of fungal-infected or uninfected cadavers. As a preliminary test, starvation assays were performed to obtain the mean survivorship of starved versus grass fed locusts. These were used to identify the point of starvation at which locusts in no-choice assays would consume *M. acridium* infected conspecifics (if at all).

Starvation curve generation

Newly moulted 5th instar male *C. terminifera* were collected and fed *ad libitum* on fresh wheat grass and wheat germ for 48 h. Insects were separated into fed or starved treatment groups (n=110) and transferred into individual cages (7x8x8 cm). Fed locusts received an excess of fresh wheat grass and wheat germ on alternate days while starved locusts received no food or water of any kind. All cages were maintained under the same photoperiod and temperature-controlled conditions as described above. Locusts were checked twice daily for deaths until reaching 100% mortality within the starved treatment group.

No-choice assays

Potential cannibals in no-choice assays were provided with one victim only (infected or control), thereby forcing individuals to either cannibalise the victim offered or starve. Locusts (n=20) were collected at their 5th instar moult and fed *ad libitum* for 48 h on fresh wheat grass and wheat germ. Insects were then transferred to individual cages (7x8x8 cm) without food or water and allocated to one of two treatment groups; control or infected. Control and newly-dead infection stage victims were generated as described above and stored at -80˚C until required. Potential cannibals were offered a freshly thawed infected or uninfected control victim for 3 h, twice daily. Each victim-offering bout was separated by a 3-h period without victims during the day, and a 15-h period overnight. At the end of each offering period, victims were removed and inspected for signs of cannibalism. Once visual evidence of cannibalism was observed, locusts received one additional victim at the next offering period before being transferred back to wheat grass and wheat germ *ad libitum.* Insect survival was monitored throughout the experiment, regardless of the occurrence of cannibalism. Insects were checked twice daily for deaths until all locusts that were offered infected victims had died.

3.3.5 Analyses

All data were analysed with the statistical software Systat 12 (Systat Software, Inc., San Jose, California, USA). Chi-squared tests were used to compare both the number of first visits to infected or uninfected victims as well as evidence of direct cannibalism of each victim type. These were performed according to the null hypothesis that first visits or direct cannibalism on infected and control victims occurred with equal probability. The total and proportional number of visits or time spent with infected and control victims were all analysed using two-way ANOVAs

(where infection stage and cannibal sex were main factors). Square-root transformations of data were performed prior to analyses for proportion of total number of visits and total visit duration to each victim type. Interactions between cannibal sex and infection stage were also tested. In instances where a significant interaction was not found between factors, the analysis was repeated with the interaction term removed (Engqvist, 2005).

Starvation and no-choice survival assays were analysed using Mantel-Cox tests to identify significant differences in the mortality of starved, grass-fed, and potentially cannibalistic locusts. Survivorship curves were plotted as Kaplan-Meyer curves using the statistical program R (Team, 2013).

3.4 Results

3.4.1 Behavioural choice assays: victim selection

First victims visited

Cannibals offered early-stage infected and uninfected control victims did not differ significantly in their first visit to either victim type (Figure 3.3; $\chi^2_{(1,52)}=0.308$, p=0.5971). First visits by potential cannibals to uninfected control victims, however, were more frequent than visits to cadavers that were newly-dead of infection ($\chi^2_{(1,49)}=12.755$, p=0.0004). The same was observed in the third treatment group with more first visits to uninfected controls than to sporulating infected victims ($\chi^2_{(1,46)}=31.391$, p<0.0001).

Number of visits

Infection stage had no significant effect on the total number of visits to victims made by cannibals (Figure 3.4; two-way ANOVA, $F_{(2,143)}=0.635$, p=0.531). Overall,

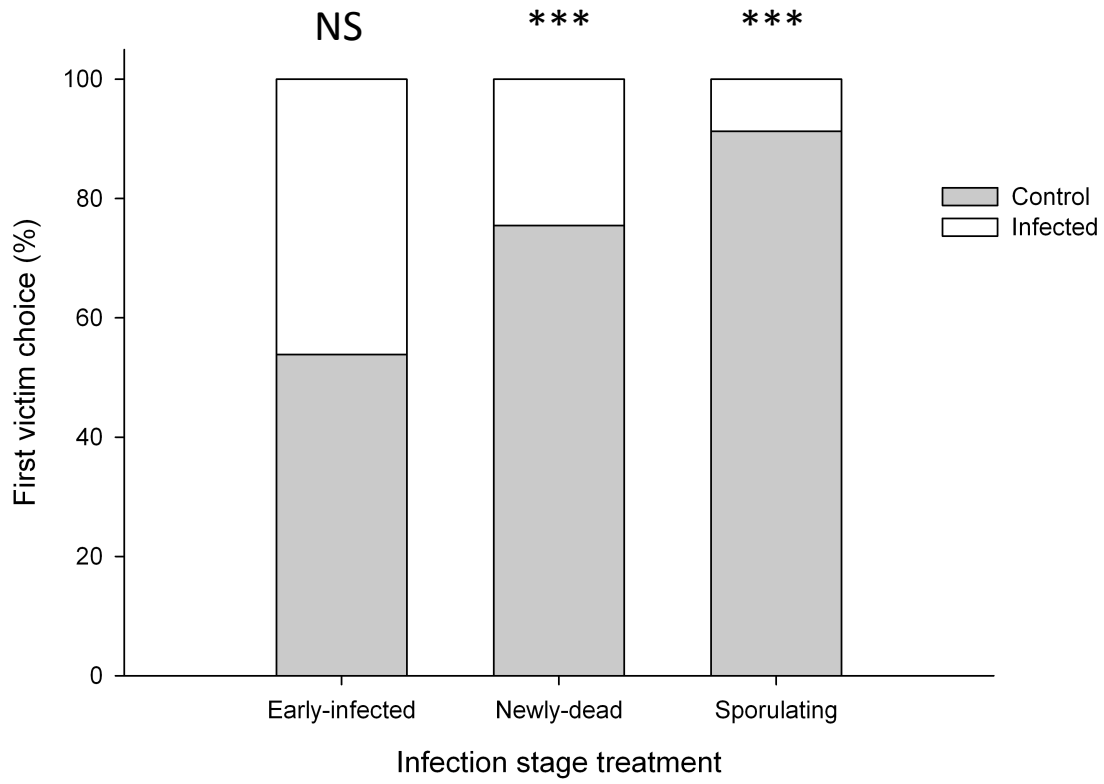

Figure 3.3 First victim visits made by cannibals within early-infected, newly-dead and sporulating infection stage treatments. Significant differences between control (grey) and infected (white) victims within infection stage treatments are indicated by asterisks (χ^2;***$p<0.001$). NS denotes no statistical difference between observed results compared to the expected outcome of an equal number of first visits to each victim type.

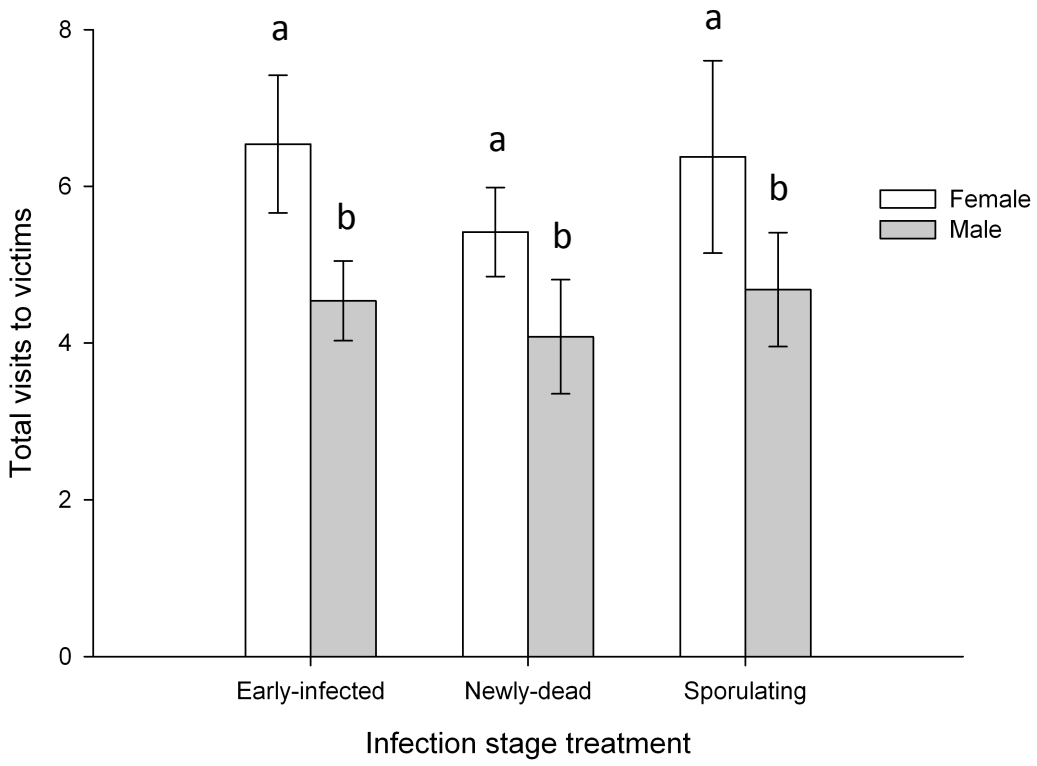

Figure 3.4 Total visits made by male and female cannibals to victims within early-stage, newly-dead and sporulating infection stage treatments. Letters indicate significant differences between the numbers of visits made to victims by each sex (Tukey's HSD, $p<0.05$). Error bars = ±SEM.

however, a significant effect of cannibal sex was identified (two-way ANOVA, $F_{(1,143)}$=6.617, p=0.011) with female cannibals making more visits to their victims compared to males (Tukey's HSD, p<0.05).

The proportion of visits made to control victims by cannibals was significantly different across the infection-stage treatments (Figure 3.5; two-way ANOVA, $F_{(2,143)}$=21.509, p<0.001). Similarly, infection-stage significantly affected the proportion of total visits made by cannibals to infected victims (two-way ANOVA, $F_{(2,143)}$=25.311, p<0.01). Overall, cannibals within newly-dead and sporulating treatment groups made more visits to control over infected victims compared to cannibals in early-infection stage treatments (Tukey's HSD, p<0.05). Cannibals within newly-dead or sporulating treatments were not statistically different in total number of visits made to either control or infected victims (Tukey's HSD, p>0.05). The proportion of visits to control and infected victims did not differ significantly between male and female cannibals (two-way ANOVA, control: $F_{(1,143)}$=0.399, p=0.529, infected: $F_{(1,143)}$=0.214, p=0.645). The interaction between infection stage treatments and cannibal sex was also not significant.

Visit duration

Total visit duration to victims was significantly different across the three infection-stage treatments (Figure 3.6; two-way ANOVA, $F_{(2,143)}$=7.904, p=0.001), with cannibals within the early-stage infection treatment spending significantly more time with their victims than those in the sporulating victim group (Tukey's HSD, p<0.05). In total, males and females did not spend significantly different amounts of time with their victims (two-way ANOVA, $F_{(1,143)}$=0.483, p=0.488). Since female cannibals visited their victims more frequently than males (Figure 3.4), this indicated that female visit duration was shorter. There was no significant interaction between

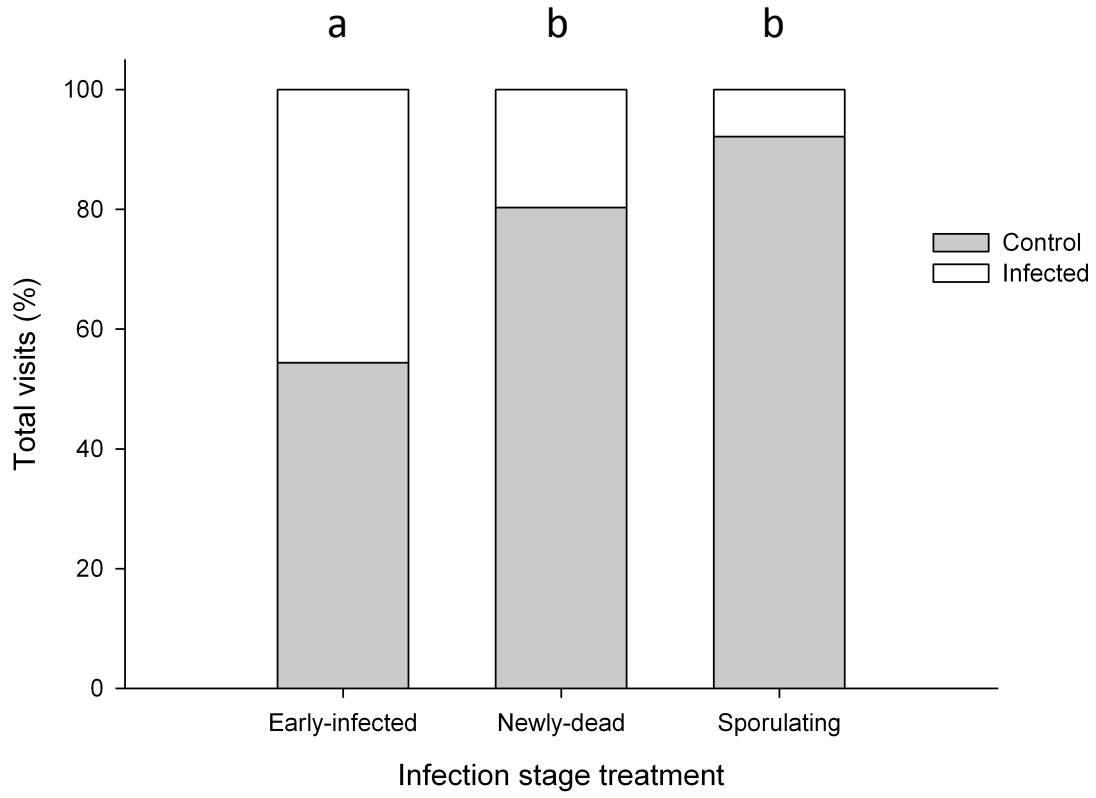

Figure 3.5 Proportion of cannibal visits to control and infected victims across early-stage, newly dead and sporulating infection stage treatments. Different letters indicate differences between the proportions of time cannibals visited control victims (grey) between each of the infection stage treatments. These letters also represent differences across treatments of cannibal visits to infected victims (white) (Tukey's HSD, $p<0.05$).

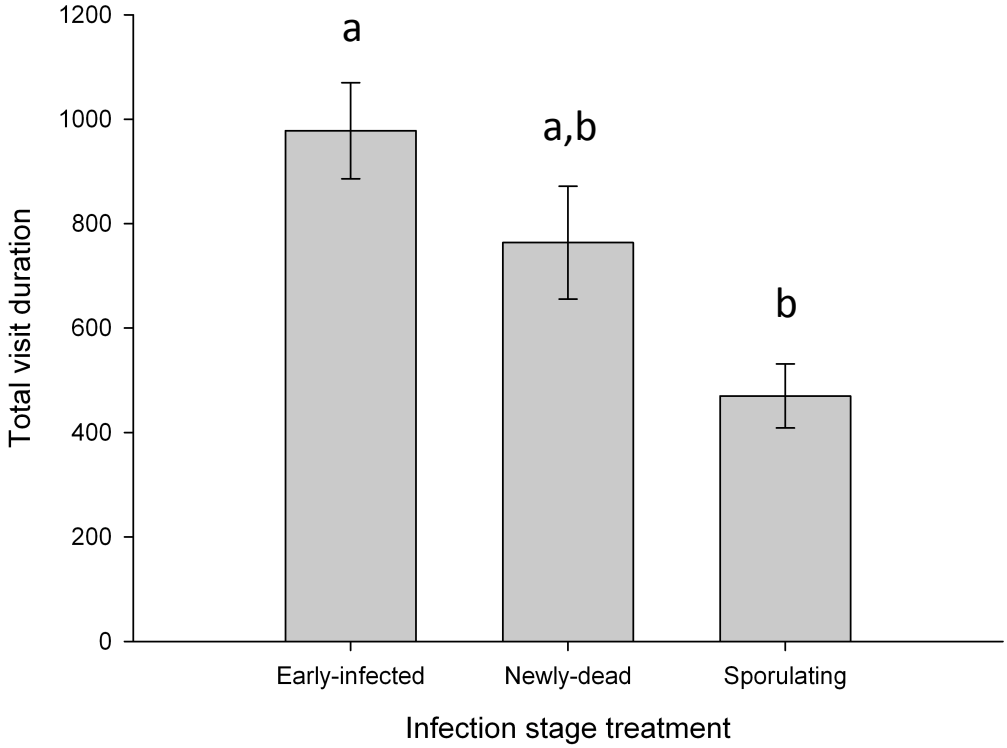

Figure 3.6 Total cannibal visit duration to victims within early-infected, newly-dead or sporulating infection treatments. Letters represent significant differences in the total time cannibals spent with victims (Tukey's HSD, $p<0.05$). Error bars = ±SEM.

cannibal sex and infection-stage treatments for the total visit duration made by cannibals.

The proportion of cannibal visit duration to control and infected victims was significantly different across all the infection-stage treatments (Figure 3.7; two-way ANOVA, control: $F_{(2,143)}=27.015$ $p<0.001$, infected: $F_{(2,143)}=36.979$, $p<0.001$). Cannibals spent more time with control victims than newly-dead or sporulating cadavers compared to locusts within the early-stage infection treatment group (Tukey's HSD, $p<0.05$). Cannibals within these former treatments spent similar time periods with both control and infected victims (Tukey's HSD, $p>0.05$). Cannibal sex had no effect on the time spent with either control (two-way ANOVA, $F_{(1,143)}=0.148$, $p=0.701$) or fungal infected (two-way ANOVA, $F_{(1,143)}= 0.221$, $p=0.639$) victims. There was no significant interaction between infection-stage treatment and cannibal sex for either control or infected victims.

Physical evidence of cannibalism

Since it was sometimes unclear from video footage whether cannibals were feeding on victims or merely resting on or near them, victims were inspected after each behavioural choice assay for physical indications of cannibalism. Overall, no evidence of cannibalism was observed on newly-dead or sporulating infection-stage victims. Within these two-infection stage treatments, 100% of test insects cannibalised uninfected controls (Figure 3.8; newly-dead: $\chi^2_{(1,49)}=49$, $p<0.0001$, sporulating: $\chi^2_{(1,46)}=46$, $p<0.0001$). Cannibals within the early-infected treatment, however, showed no preference to either control or infected victim type ($\chi^2_{(1,52)}=0.103$, $p=0.7477$).

Figure 3.7 Proportion of cannibal time spent with control and infected victims across early-infected, newly-dead and sporulating infection stage treatments. Letters indicate significant differences between the proportion of time cannibals spent with control (grey) and infected (white) victims across the three infection stage treatments (Tukey's HSD, $p<0.05$).

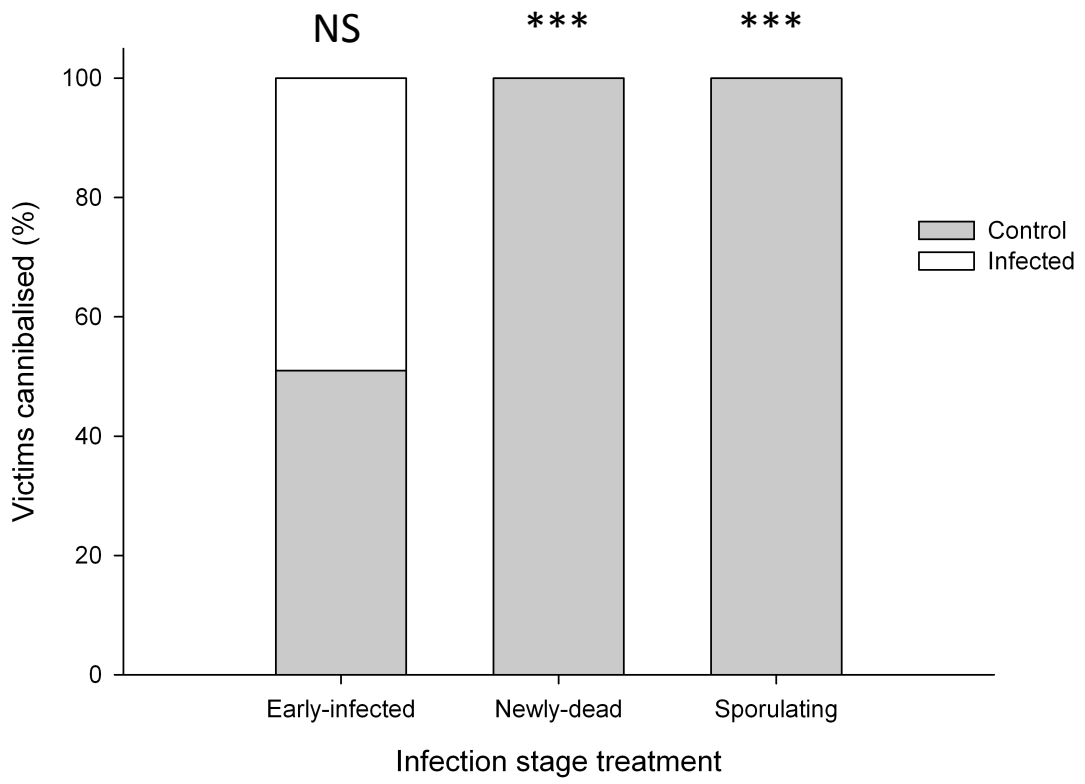

Figure 3.8 Physical evidence of cannibalism of control (grey) and infected (white) victims across the three infection-stage treatments groups: early-infected, newly-dead and sporulating. Asterisks represent a significant difference between actual cannibalism observed and the expected outcome of equal victim consumption (χ^2; ***$p<0.001$). NS denotes non-significance.

Cannibal survival: pathogen transmission

Since physical evidence of cannibalism of infected victims was observed only on early-stage cadavers, only cannibals from this treatment were tested for pathogen transmission through ingestion. Of these locusts, no deaths were observed following cannibalism of early-stage infected cadavers for the entire period tested. Locusts that consumed uninfected controls also had no deaths during this period.

3.4.2 Starvation and no-choice assays

Overall, there was significant variation in survivorship between locusts fed grass, those that were starved and those offered control or newly-dead infected cadavers (Figure 3.9; Mantel-Cox, $\chi^2_{(3)}=206.085$, $p<0.001$). Ninety-percent of locusts that were offered control victims were observed to cannibalise within three days, with a total of 95% cannibalism after seven days. Locusts offered newly-dead infected cadavers did not cannibalise at all. Despite never resorting to cannibalism, these locusts still exhibited a significantly longer survivorship than those that were starved (Mantel-Cox, $\chi^2_{(1)}=10.453$, $p=0.001$). Since the last cannibal in this infection-stage treatment died only one day after the last starved locust, it is possible that the small sample size (*i.e.,* n=20) may account for this observed difference. Locusts fed on grass had the highest survivorship, which was significantly longer than locusts offered uninfected control victims (Mantel-Cox, $\chi^2_{(1)}=8.933$, $p=0.003$).

3.5 Discussion

3.5.1 Locusts avoid ingestion of fungal-infected conspecifics

The capacity of locusts to distinguish between *M. acridium* infected and uninfected conspecifics has now been demonstrated in *C. terminifera*. When presented with two potential victims, food deprived locusts completely avoided the consumption of

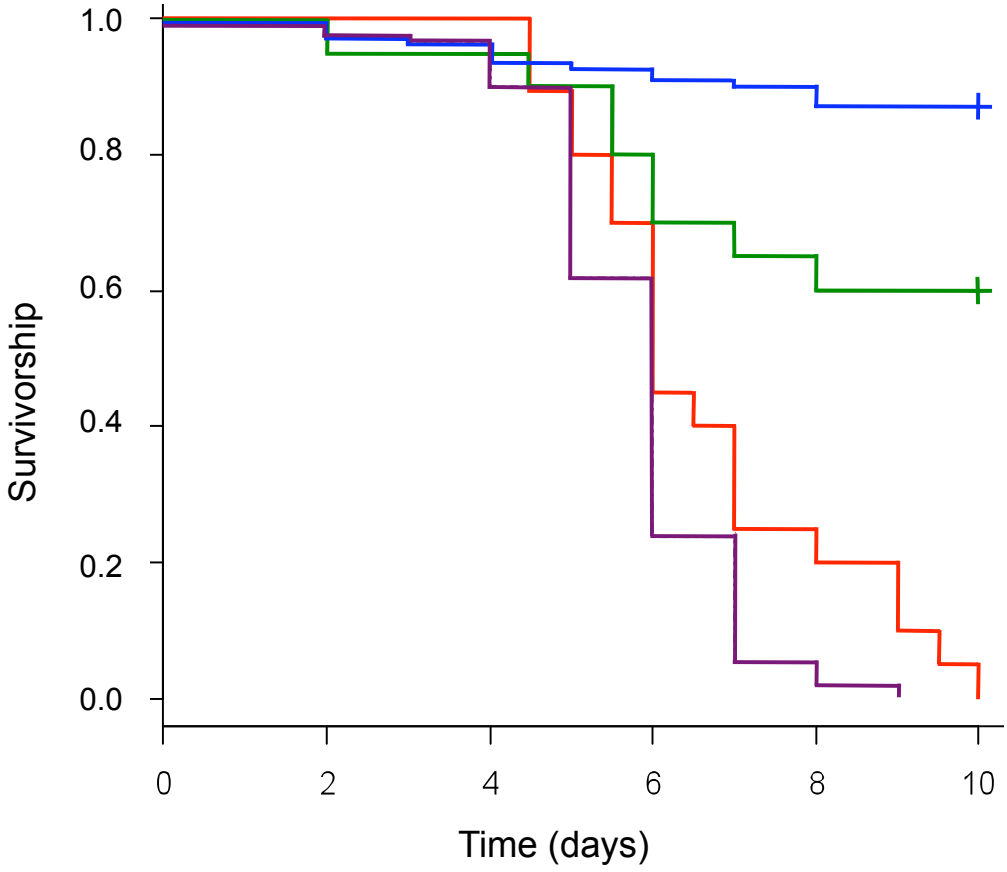

Figure 3.9 Cumulative survival of *C. terminifera* offered grass (blue), uninfected control victims (green), newly-dead infection stage victims (red) or no food at all (purple). Crosses at the end of survivorship curves indicate data with remaining survivors at the end of the 10-d measurement period.

newly-dead and sporulating infection-stage cadavers, instead cannibalising only uninfected control victims. In the absence of an alternative food source, locusts still avoided consumption of newly-dead infected victims, thereby succumbing to death by starvation.

For *M. acridium* infected victims that were yet to show apparent infection symptoms (*i.e.,* early-stage infected victims), no avoidance by cannibals was observed. These early-stage infected victims were just as likely to be cannibalised as uninfected controls, a result suggesting that early-stage infected locusts exhibit neither the physical nor biochemical cues necessary for the detection of *M. acridium* following three days of infection.

The classification of infection-stage treatments (*i.e.,* early-infection, newly-dead and sporulating) in these experiments was primarily based on physical symptoms of the pathogen's progression. Locusts did not eat conspecifics that showed such visible symptoms, indicating that they were repelled by these cadavers, avoided contact with them and did not ingest them if contacted.

Several studies on other insect species have similarly shown a deterrent response to entomopathogenic fungi. A study by Meyling and Pell (2006) on the generalist feeder, *Anthocoris nemorum*, showed that leaf surfaces inoculated with *B. bassiana* substantially repelled insects from contact with host plants during foraging. Similarly, the termite *Macrotermes michaelsenii* showed a significant propensity to move towards the fungus-free (control) arm of an olfactory-based y-maze choice experiment. Here, *M anisopliae* and *B. bassiana* isolates were found to repel termites in a positive dose-dependent manner (Mburu*, et al.*, 2009). This suggests that these insects can detect and avoid fungal contact based on olfactory emissions. Feeding behaviour of a different termite, *Coptotermes formosanus*, similarly showed that

individuals were repelled by *Metarhizium anisopliae*, leading them to forage in spaces furthest from fungal sources (Hussain*, et al.*, 2010). Such behaviour in insects could be an evolved response to avoid infection or may simply reflect that the deterrent chemical cues from fungal infected conspecifics are also found in other unpalatable substrates.

In such instances fungal volatile organic compounds (VOCs) may be responsible for this deterrence, with several studies currently investigating the use of VOCs as potential biocontrol agents (Morath*, et al.*, 2012). The adaptive nature of fungal detection and avoidance in insects demonstrates the intricate ecology shared between insect and pathogen (Vega*, et al.*, 2009). These experiments have shown that the interaction between cannibalistic *C. terminifera* and *M. acridium* infected conspecifics is similarly complex.

It is well known that chemical profiles, such as epicuticular hydrocarbons, are used for chemosensory communication between insects (Chapman*, et al.*, 2013). As well as serving a primary role in water-proofing (Singer, 1998), many species use hydrocarbon signals for a number of social interactions. This includes the recognition and differentiation of nest-mates, and evaluation of immunocompatability during mate selection (Fan*, et al.*, 2004; Richard*, et al.*, 2008; Ali and Tallamy, 2010). The fungal spores of *M. acridium* take several days to penetrate and germinate within the host. It is therefore possible that the immune stimulated biochemical cues in early-stage infected victims were still indistinguishable from that of uninfected controls.

Several studies have shown that entomopathogenic fungi, such as *M. acridium* and *B. bassiana*, utilise a variety of hydrocarbon structures as metabolic fuel for both germination and biosynthesis of cellular components (Napolitano and Juárez, 1997; Jarrold*, et al.*, 2007). These include compounds that are both similar and identical to

those found on insect hosts, particularly in the stages prior to fungal penetration through the host cuticle. A study by Jarrold *et al.,* (2007) showed that hydrocarbons and other extracts from the wings of the desert locust (*Schistocerca gregaria*) were strong promoters of *M. acridium* growth and that without these extracts fungal germination was relatively poor. Consequently, during the early stages following *M. acridium* inoculation in these experiments, host hydrocarbons produced in response to infection may have been utilised and degraded by the fungus itself. Any varied response in the cuticular chemical profile may have therefore remained undetectable to cannibals.

Although locusts showed no avoidance of early-stage infected victims, they may still have been capable of distinguishing these victims from uninfected controls. If so, early-infection stage victims may simply not have deterred cannibals, perhaps reflecting a low level of risk since the infection was in its early stages. That the threat was indeed low was indicated by the absence of a measurable increase in mortality rate following ingestion of early-stage infected victims. Since Orthoptera rely heavily upon olfactory cues for both food location and conspecific interactions, these results support this rationale (Hopkins and Young, 1990; Bomar and Lockwood, 1994a; Bomar and Lockwood, 1994b; Chapman*, et al.*, 2013).

3.5.2 Infection avoidance eliminates pathogen transmission through cannibalism

Pathogen transmission is often reported as an important limitation to cannibalism, a behaviour that might otherwise be exploited far more frequently as a means of gaining nutrition in many species (Pfennig*, et al.*, 1991; Williams and Hernández, 2006; Richardson*, et al.*, 2010). Behavioural avoidance of infected conspecifics is not uncommon in the animal kingdom, particularly in species classified as being gregarious or eusocial (Kramm*, et al.*, 1982; Behringer*, et al.*, 2006). Selective

cannibalism can also occur in reverse, where the spread of infection is controlled through favourable consumption of hosts bearing parasites (Reed*, et al.*, 1996). This, however, relies upon a low risk of transmission via ingestion.

The most common mode of horizontal spread of *M. acridium* in locusts is through tactile interaction with infected conspecifics. Since *C. terminifera* avoid ingestion of newly-dead and sporulating infected conspecifics, pathogen transmission through cannibalism is an unlikely mode of secondary infection. What's more, locusts that cannibalised early-stage infected victims showed no sign of increased mortality as a result of *M. acridium* ingestion, indicating a low risk of fungal transmission at this stage of infection. However, since freezing at -80˚C was used to kill these early-infection stage victims, it is possible that this procedure also killed the fungus itself. An increase in cannibal mortality following infected-victim consumption may therefore be unlikely in this instance.

These experiments were unable to demonstrate whether a late-stage infected locust would have the capacity to transmit a fungal pathogen to a cannibalistic conspecific (a question that can only be answered when such cannibalism takes place). However, when subject to no-choice behavioural assays, locusts still clearly avoided consumption of infected cadavers, suggesting that transmission of *M. acridium* by this means may have been a threat. Through their avoidance behaviour, locusts conferred the benefits of infection evasion. This was done, however, at the cost of nutritional deficiency and inevitably death.

Previous experiments within other orthopteran species have found a significant "reluctance" by individuals to cannibalise conspecifics infected with either *M. acridium* or *B. bassiana* (Jaronski, 2013). Of those that did, a maximum of just 18.9% exhibited fungal transmission through cannibalism (Jaronski, 2013).

Similarly, horizontal transmission of the nuclear polyhedrosis virus (SfNPV) in *Spodoptera frugiperda* resulted in a total mortality of 32%, again suggesting that cannibalism is a less efficient mode of SfNPV transmission (Chapman*, et al.*, 1999).

3.5.3 Cannibalism, infection and plague control

Secondary cycling of *Metarhizium flavoviride* has been proposed as an important mode of natural biocontrol within locust and grasshopper populations (Thomas*, et al.*, 1995). Here, modelling studies were used to suggest that cadavers serve as important infective units (due to their long infection profile), resulting in the initiation of new infections as a persistent mode of biocontrol. For migratory populations, where infected cadavers may be rapidly left behind, this may be less effective. The potential for later fungal cycles to infect subsequent migratory bands, however, is still possible by this means.

Currently, crop spraying of the bio-insecticide Green Guard® (which contains *M. acridium* as its major agent) is the primary mode of locust control using this pathogen. Whether *M. acridium* has the potential to infect locusts through ingestion of spores is still unclear. However, the adaptive behaviour of infection avoidance demonstrated in locusts, and previously in grasshoppers and *A. simplex* (Jaronski, 2013), makes the exploitation of cannibalism as an additional means of *M. acridium* recycling in locusts less promising.

As mentioned in the introduction, locust cannibalism (a behaviour motivated by the search for nutrients) is considered an important aspect of nymphal migration (Simpson*, et al.*, 2006; Bazazi*, et al.*, 2008). Locusts that are vulnerable (immotile or immobile) have been shown to be easy targets for cannibalism (Hansen*, et al.*, 2011). The use of dead victims in the present experiments meant that cannibals had no risk of injury or death from defensive victims, suggesting that the observed avoidance

was indeed influenced by the presence of infection. In a marching band context, the avoidance of *M. acridium* infected cadavers would enable locusts to migrate beyond areas of infected conspecifics, ultimately reducing periods of physical contact with fungal infected locusts and lowering the likelihood of tactile horizontal transmission.

The exploitation of cannibalism as a secondary approach to pest control, however, should not be disregarded altogether. Studies on rangeland grasshoppers have demonstrated the successful transmission of *Nosema locustae,* a microsporidian protozoan, through the cannibalism of infected cadavers (Lockwood, 1988). The successful biocontrol of such an approach in *C. terminifera* using *N. locustae* or a similar pathogen may therefore still be conceivable.

3.5.4 Conclusions and future research

This work has demonstrated that *C. terminifera* are able to detect, distinguish and avoid ingestion of newly-dead and sporulating conspecifics infected with the fungus *M. acridium*. These results indicate that locusts are deterred from infectious cadavers; an adaptation that enables effective avoidance responses yet results in nutritional loss and (in the absence of alternative resources) ultimately starvation. Whilst the mechanisms behind locust infection avoidance behaviour remain unclear, the complexity of interactions between locusts and their pathogens suggest that individuals must frequently adopt such strategies to benefit both individual and group outcomes.

Future identification of the processes involved in infection deterrence during cannibalism will provide greater insight into the circumstances by which *M. acridium* infection becomes: i.) detectable by individuals; ii.) infectious to conspecifics, and iii.) repellent to other locusts. In addition, studies looking at alternative pathogens as a means to exploit secondary infection cycling through

cannibalism will be important in identifying new benefits in the use of biological agents for plague control. The ability of hosts to anticipate and respond to infection (and potential secondary transmission risk) in this way is also of particular interest. Such experiments, looking at immune anticipation in locusts exposed to both dead infected cadavers and live conspecifics with developing infections, are presented in Chapter 4.

Chapter 4.

Predictive Immune Function

4.1 Summary

The Australian plague locust, *Chortoicetes terminifera*, detects and avoids conspecific cadavers infected with the entomopathogenic fungus, *Metarhizium acridium*. Previous studies have found that food deprived locusts are deterred by cadavers that are newly dead or sporulating from *M. acridium* infection, leading to avoidance behaviour and ultimately death by starvation. Interestingly, locusts still cannibalise victims at an early infection stage, where apparent symptoms of infection are absent. How locusts respond to such infection exposures on an underlying immune level, however, is still unclear. The extent (if any) of anticipatory immune activation in response to *M. acridium* exposure is of particular interest. This chapter investigated whether *C. terminifera* demonstrates evidence of predictive immune activation following exposure to fungal-infected conspecifics. The experiments presented quantify the variation in the constitutive immunity of locusts exposed by direct contact or proximity to infected and uninfected conspecifics across a progressive time-course. Since such scenarios of infection exposure are commonly encountered in the field, upregulation of locust immune traits in response to potential infection transmission was expected. Despite this, no clear evidence for plastic immune anticipation was found. Whilst locusts have the capacity to detect and avoid the ingestion of fungal-infected conspecifics, physiological immune reactions of locusts exposed to infected conspecifics were undetectable. Immune anticipation of a different sort, however, was identified. Here, locusts showed significant upregulation of several immune traits across the experimental time-course. This observed immune activation is suggested to occur as part of the physiological preparation locusts experience leading up to ecdysis. Anticipation of increased risk of infection during this vulnerable process may be one adaptation where hard-wired (rather than plastic) predictive immune investment is worthwhile.

4.2 Introduction

4.2.1 Infection prediction and detection

A host's ability to protect itself from infection is paramount for reproduction and survival. Host immune defences span several levels; from the outer integument that separates self from non-self, to behavioural strategies and physiological responses (Schmid-Hempel, 2011). When looking at host immunity and infection response, an important consideration is also the way in which organisms are able to anticipate potential disease onset (Schulenburg*, et al.*, 2009; Schmid-Hempel, 2011). Tell tale signs of increased risk of infection include environmental cues, *a priori* experience and social interactions (Schulenburg*, et al.*, 2009).

The cues and mechanisms that animals use for anticipatory defence are varied and often complex, particularly for hosts living in unpredictable environments. Despite the costs of resource investment toward heightened immune function, the ability of hosts to predict and defend against an increased risk of infection is also beneficial.

Immune anticipation in response to ecological factors

A host's constitutive and responsive immune function is greatly influenced by its ecology. Environmental variables can therefore be reliable indicators for variation in pathogen diversity and host susceptibility (Nelson, 2004; Altizer*, et al.*, 2006; Córdoba-Aguilar, *et al.*, 2009). Seasonal variation, for instance, can greatly affect pathogen dynamics, thereby impacting the hosts they invade. In general, warm temperatures are optimal for microbe growth and reproduction, often elevating pathogen abundance and virulence (Harvell*, et al.*, 2002). However, colder seasons, whilst potentially poor for pathogen replication, can also be energetically demanding for hosts, particularly where resources are limiting (Nelson and Demas, 1996). With

a huge capacity to adapt to new environments, pathogens are often able to opportunistically exploit vulnerable hosts in variable conditions, revealing the costs of a delayed immune response (Raffel*, et al.*, 2006).

The ability to anticipate changes in pathogen dynamics, for instance through the detection of seasonal shifts, is frequently exploited by hosts. Environmental alerts including variation in climate, nutritional resources and daily photoperiod enable hosts to adapt their energetic investment toward immune function using infection risk as a gauge for predictive response. The regulation of melatonin as a seasonal clock, for example, is one adapted strategy that allows such immune anticipation (Nelson and Demas, 1996; Nelson and Drazen, 2000).

Population density can also influence immune function in animals. The density-dependent prophylaxis (DDP) theory (see Chapter 2) describes how animals living in groups are more likely to encounter a greater number and variety of pathogens than conspecifics that live at low density or in solitary (Wilson and Cotter, 2008). As a result, animals occurring at higher densities are predicted to invest more in their constitutive immune function than those at low density as a strategy to reduce the risk of infection transmission. Many species, especially social or gregarious groups, have been found to support the DDP hypothesis, demonstrating how plastic physiological responses can aid to anticipate increased risks of pathogen transmission in response to variable (and often unpredictable) external cues (Wilson, *et al.,* 2002; Altizer, *et al.,* 2011).

Response, protection and immune priming

An adaptive and rapid response to infection is critical for both individual and group fitness. Host infection response greatly depends on pathogen type and likelihood of transmission (Cremer*, et al.*, 2007). Physiological responses may include induced

fever, lymphocyte or haemocyte proliferation, melanisation, phagocytosis and production of antimicrobial compounds or antibodies (see Chapter 1) (Schmid-Hempel, 2011). However, host investment toward preventative immunity is equally important and often less resource-intensive than a direct and specific infection response. Behavioural adaptations are commonly employed as preventative measures against the spread of infection across populations. Avoidance, triggered by the initial response of 'disgust' or deterrence, is often the first to take place (Oaten*, et al.*, 2009). Preparing for potential infection can increase overall inclusive fitness of individuals, ultimately favouring the evolutionary success of a species (Hamilton, 1964a; Hamilton, 1964b). Many organisms use previous experience of parasitism as a way to protect themselves from pathogens that are likely to be re-encountered in the future (Moret, 2006; Sadd and Schmid-Hempel, 2009b).

The production of antigen-specific antibodies in vertebrates (and the lesser known humoral aspects of invertebrates) allows the protection of individuals and their offspring in response to repeated encounters with the same pathogen (see Chapter 1) (Schmid-Hempel, 2005). Transgenerational immune priming and parental care is often linked to investment in offspring immunity. The transfer of antibodies and antimicrobial peptides have been shown to provide elevated immune protection to offspring based on a parent's own parasitic and environmental history (Schmid-Hempel, 2011; Roth *et al.,* 2010). Microbial ingestion through coprophagy and transfer of antimicrobial peptides during early development has also been frequently reported throughout the animal kingdom (Moret, 2006; Freitak*, et al.*, 2009).

Selective mate choice by females is another form of parental immune anticipation. In *Diabrotica undecimpunctata howardi*, females are known to reject males with cuticular hydrocarbon profiles similar to their own (Ali and Tallamy, 2010). Selection toward mates with greater variation in their hydrocarbon signatures has

been shown to produce offspring with higher immunocompetence compared to offspring of parents with similar immune signatures. Anticipation of offspring encounters with parasitism is suggested to be driving such parental reproductive strategies (Ali and Tallamy, 2010).

Group and social immunity in insects

As indicated above, surface cuticle hydrocarbon signatures are a crucial component of communication between insect conspecifics (also see Chapter 3). Such chemical profiles play a vital role in allowing insects to identify the immune status of their conspecifics, thereby enhancing reproductive and social fitness through selective interactions (Chapman*, et al.*, 2013). Healthy individuals may actively avoid contact with infected conspecifics based on hydrocarbon signals (Ugelvig and Cremer, 2007). In some cases these chemical cues serve as reliable indicators for the removal of infected individuals from a population (Heinze and Walter, 2010; Bos*, et al.*, 2012). Allogrooming, based on chemosensory interactions, is also commonly used within large populations to eliminate pathogens following infection, as well as a form of self-vaccination (Reber*, et al.*, 2011; Konrad*, et al.*, 2012; Sun and Zhou, 2013; Zhukovskaya*, et al.*, 2013).

Konrad *et al.,* (2012) have recently identified active upregulation in the immune function of ants following exposure to fungal-infected nest mates. Increases in survivorship, antifungal defence, and elevated immune gene expression, indicated that such immune upregulation was the result of conspecific allogrooming behaviour where transfer of fungal conidia served as a mode of self-vaccination and protection. Interestingly, these responses were pathogen-specific, with no detectable upregulation of antibacterial or antiviral immune traits. Such behavioural response

strategies, to defend against acquired infections, are fast becoming a new topic of interest within the field of insect ecological immunology.

4.2.2 Pathogen recognition demonstrated in locusts

The capacity of the Australian plague locust, *Chortoicetes terminifera,* to discriminate between conspecifics infected with the fungus *Metarhizium acridium* and uninfected controls has been demonstrated in Chapter 3. When subject to choice assays, locusts only cannibalised uninfected control victims, or those at an early stage of fungal infection. Cannibals were clearly deterred, however, from cadavers harbouring more developed symptomatic fungal infections, selecting to ingest uninfected victims only. Locusts offered a single victim that was newly-dead of *M. acridium* infection again explicitly avoided cannibalism, leading to death by starvation. Comparatively, cannibals offered only uninfected victims consumed their prey well before the risk of death from starvation was apparent.

With the new knowledge that cannibalistic locusts have the capacity to detect and avoid the ingestion of infected conspecifics, this work aimed to identify the extent of predictive immune protection that individuals may also confer in similar conditions. In this study, three well-established immune traits, lysozyme concentration, prophenoloxidase (ProPO) activity and haemolymph protein concentration were measured in an attempt to identify any detectable differences in locust immune state following various exposure treatments to infected and uninfected conspecifics.

4.2.3 Aims

The goal of this chapter was to determine whether proximity or direct contact with *M. acridium* infected conspecifics induced detectable upregulation of immune traits within *C. terminifera*. With infection recognition and avoidance behaviour already

well demonstrated, these experiments investigated whether an elevated threat of pathogen transmission might also induce physiological immune anticipation. Specifically, this chapter aimed to identify and compare variation in the constitutive and responsive immune function of locusts: i.) exposed by proximity to fungal infected or uninfected conspecifics; ii.) infected directly with *M. acridium* compared to uninfected controls; iii.) exposed by direct contact to live or dead uninfected conspecifics, and iv.) exposed by direct contact to uninfected or fungal-infected cadavers.

4.3 Materials and methods

4.3.1 Test and stimulus insects: control, infected, alive and dead

All experiments were conducted in a temperature-controlled room at 30-33˚C using *C. terminifera* obtained from a gregarious-reared laboratory culture as described previously (Chapter 2). Test insects used were newly moulted 5th instar males fed *ad libitum* on fresh wheat grass and wheat germ for 24 h prior to beginning exposure treatments.

Stimulus insects were inoculated with *M. acridium*, 24 h after moulting to their 5th stadium. As described previously (Chapter 3), 2 µL of *M. acridium* solution (approximately 2500 spores/µL) was applied to the pronotum of locusts such that individuals died naturally of the fungal infection 4-6 d later. Newly-dead cadavers (distinctly red in colour) were stored at -80˚C until use. Uninfected dead stimuli were similarly inoculated with an equal volume of Triton-X (0.07% v/v) and killed 3 d later by freezing at -80°C. Live stimuli (control or infected) were used immediately following inoculation. All stimulus locusts were maintained on fresh wheat grass and wheat germ until either killed or used for live exposure treatments.

4.3.2 Proximity exposure: live infected and uninfected stimuli

Proximity experiments tested for the occurrence of immune anticipation within test locusts in the presence of live stimulus conspecifics that were either uninfected (controls) or harbouring developing fungal infections (infected). These exposure treatments excluded direct tactile interaction between test and stimulus insects while still allowing the exchange of visual and olfactory cues. Test locusts (n=10) were individually transferred to one side of cages containing two equally sized compartments (Figure 4.1a). These were separated by two layers of fly screen, 1cm apart, to prevent physical contact between test and stimulus locusts in neighbouring compartments (Figure 4.1b). Four live stimulus insects, inoculated with either *M. acridium* or Triton-X (0.07% v/v), were transferred to the second compartment of each cage. Control and infected stimuli were inoculated immediately before starting exposure treatments. A third treatment group contained test locusts but no stimulus group. Test locusts were exposed to stimuli for 6, 30 or 78 h and immediately bled for haemolymph for use in immune assays. In addition, individual insects were randomly selected from each stimulus group and also sampled for haemolymph at each of the three sampling time points. This was performed to quantify and track the immune function and response of stimulus insects infected with *M. acridium* compared to uninfected controls.

4.3.3 Direct contact exposure: live and dead uninfected stimuli

The effects of direct exposure to live versus dead conspecifics, in the absence of infection, were tested in these experiments. Test locusts (n=10) were exposed to one of three stimulus treatments and one of three sampling-time treatments. Stimulus treatments included direct exposure within individual cages (7x8x8 cm) to a single live conspecific, a single dead conspecific or no stimulus at all (Figure 4.1c). All

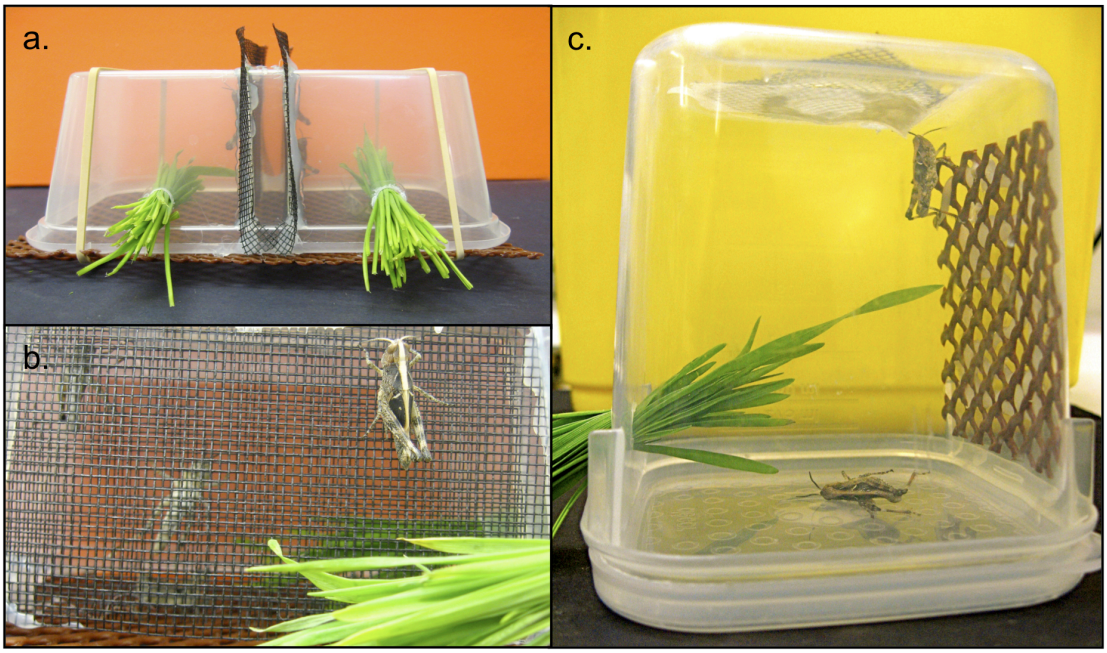

Figure 4.1 Experimental cages for immune anticipation experiments. a.) Proximity exposure cages housed individual test locusts on one side of a divided cage. Stimulus groups of four live conspecifics, inoculated with *M. acridium* (infected) or Triton-X (control), were placed in the opposite side. b.) Test locusts in proximity experiments were separated from stimulus groups using two walls of fly screen to allow visual and olfactory, but not tactile interactions between conspecifics. c.) Contact exposure assays placed test locusts (top right) in the same cage as stimuli (bottom centre). Live or dead controls and infected cadavers were used as individual stimuli.

stimulus insects (live and dead) were Triton-X inoculated only (*i.e.,* uninfected). Exposure treatments and the sampling time course are illustrated in Figure 4.2. Briefly, test locusts were exposed to stimulus treatments for 3-h exposure bouts, separated by 3- and 15-h periods for a total treatment time of 6, 30 or 78 h. Three-hour exposure blocks were considered a reasonable duration, following which no apparent signs of cadaver decay were observed.

Locusts sampled at 6 h received one 3-h exposure period followed by 3 h without stimuli before haemolymph collection. Test locusts sampled at 30 h were subject to three exposure periods in total, separated by 3- and 15-h intervals without stimuli. Test locusts sampled at 78 h received seven 3-h exposure periods, again separated by 3- or 15-h intervals without stimuli (see Figure 4.2).

New stimulus insects (live or freshly thawed) were used for each exposure period. All live locusts (test and stimuli) were provided with fresh wheat grass and wheat germ throughout experiments to prevent cannibalism. All haemolymph sampling was performed 3 h after the final exposure period for each treatment.

4.3.4 Direct contact exposure: infected and uninfected dead stimuli

Immune effects of direct contact exposure to fungal infected conspecifics were tested across three sampling time treatments using dead stimulus insects only. Test locusts in individual cages (Figure 4.1c) were directly exposed to one of two stimulus treatment groups: locusts newly dead of *M. acridium* infection or Triton-X inoculated cadavers. As described above, test locusts within 6-h sampling treatments received one 3-h stimulus exposure period and were sampled for haemolymph 3 h later. Locusts sampled at 30 and 78 h received three and seven exposure periods respectively with 3- and 15-h intervals between exposure periods. Once again,

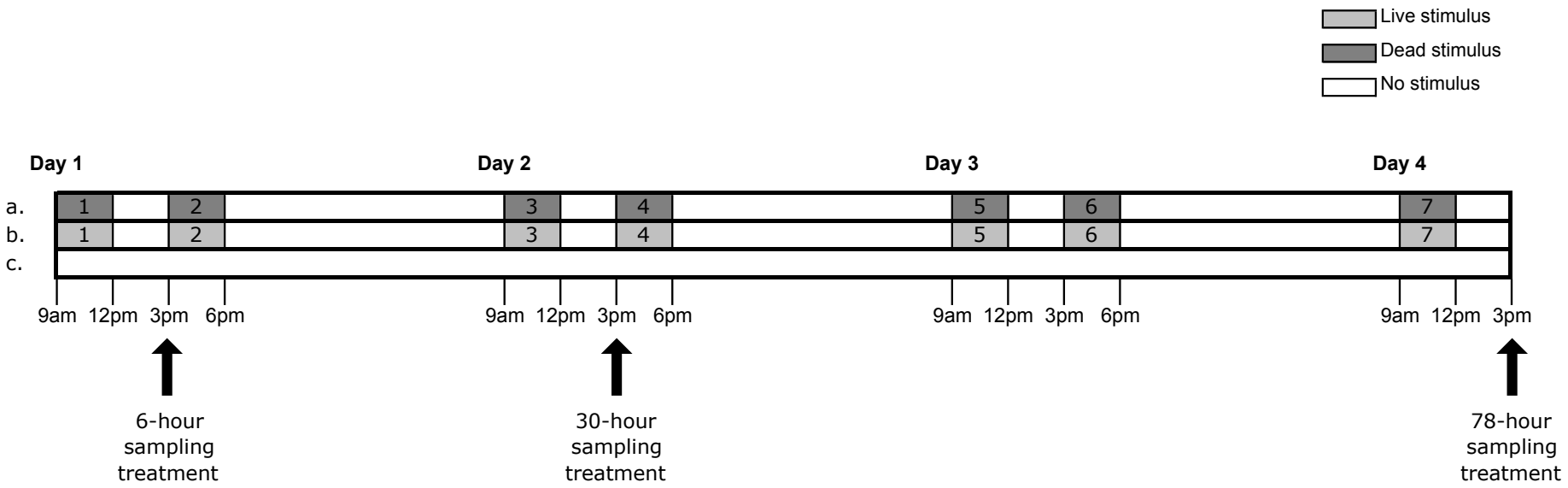

Figure 4.2 Exposure treatments and haemolymph sampling time-course for contact exposure experiments. Test locusts were exposed to; a.) live (light grey), or b.) dead (dark grey) conspecific stimuli for one, three or seven 3-h exposure periods (indicated by numbers). Exposure periods were separated by 3- or 15-hour intervals. Control groups without stimuli (c.) were used for comparison. Periods when test locusts were not exposed to stimuli are shown in white. Haemolymph was collected from the different exposure treatment groups at 6, 30 or 78 hours following the start of the first exposure (indicated by arrows). These collection times were performed three hours after the last exposure period for each treatment group.

haemolymph was sampled 3 h following final exposure periods for all stimulus treatments.

4.3.5 Haemolymph collection and assays

Using a sterile pin, locusts were sampled for haemolymph by puncturing the left hind leg membrane between the coxa and the metathorax. The maximum volume of haemolymph possible was collected from each insect using a micropipette before being transferred to a microcentrifuge tube and immediately frozen at -80°C until use.

Thawed haemolymph was assayed to quantify protein and lysozyme concentrations as well as the prophenoloxidase (ProPO) activity of each sample. These latter two traits are well-established measures in invertebrate immunity, used frequently throughout the literature, while protein concentration is used as an indication of protein reserves and host condition (see Chapter 2). When measured and reported collectively, such traits provide a greater insight into the underlying workings of host immunity. Assay protocols are described in detail in Chapter 2.

4.3.6 Analyses

Trait variation across exposure and sampling time treatments were analysed for all immune assays with two-way ANOVAs using the statistical software SPSS (Version 21.0. Armonk, NY: IBM Corp.). All non-significant interactions terms between these two variables were removed from the model and the analysis repeated (Engqvist, 2005). Specific differences between treatment-groups were identified using Tukey's HSD post-hoc tests ($p<0.05$). Since variation in immune parameters might be linked to nutritional reserves, and due to the comparative nature of these experiments, data

were not corrected for protein concentration. Therefore values of immune and condition traits within each treatment are not reported as units/mg protein.

4.4 Results

4.4.1 Proximity exposure to live infected, live control or no stimulus groups

Effects of proximity exposure (*i.e.,* without direct contact) of test locusts to groups of live stimulus insects were investigated to identify whether developing infections in neighbouring conspecifics may elicit an anticipatory immune response.

Haemolymph protein concentrations in test locusts were not significantly differently between infected, uninfected and no stimulus exposure treatments (Figure 4.3; two-way ANOVA, $F_{(2,85)}$=0.066, p=0.937). Protein concentrations, however, did vary significantly across sampling times (two-way ANOVA, $F_{(2,85)}$=80.886, p<0.001), with an increasing trend between time and protein concentration within each exposure treatment. There was no significant interaction between sample time and stimulus type.

Neither stimulus type nor sampling time treatments had a significant effect on ProPO activity (Figure 4.4; two-way ANOVA, stimulus type: $F_{(2,85)}$=0.323, p=0.725, sampling time: $F_{(2,85)}$=0.245, p=0.784). The interaction between sample time and stimulus type was also not significant.

No difference in lysozyme activity was detected between test locusts exposed to uninfected stimulus groups, fungal infected stimulus groups or no stimulus group treatments (Figure 4.5; two-way ANOVA, $F_{(2,84)}$=0.285, p=0.753). A significant effect of sampling time was, however, detected with an increasing trend between lysozyme concentration and exposure time for all treatment groups (two-way

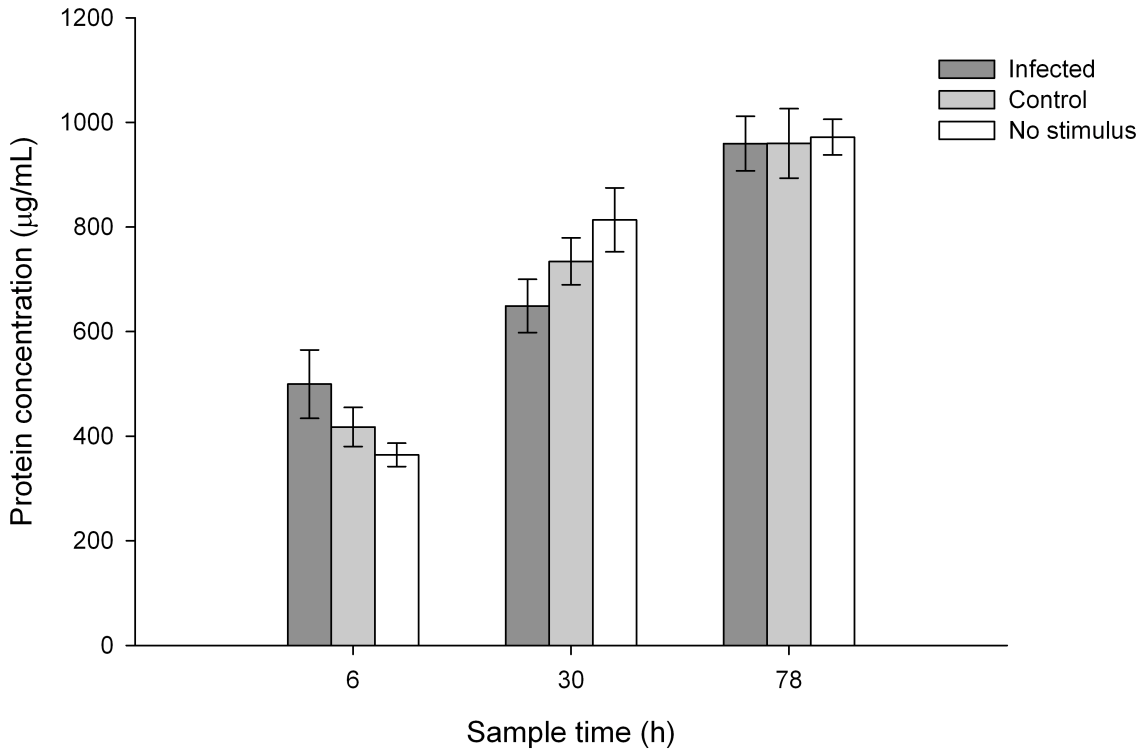

Figure 4.3 Haemolymph protein concentrations of test locusts housed in proximity to live stimulus groups. Stimulus conspecifics were either infected with *M. acridium* (dark grey) or inoculated as uninfected controls with Triton-X (light grey). A third group of individuals exposed to no stimulus group was also included (white). Protein was sampled from locusts after 6, 30 or 78 h exposure to stimuli. Error bars = ±SEM.

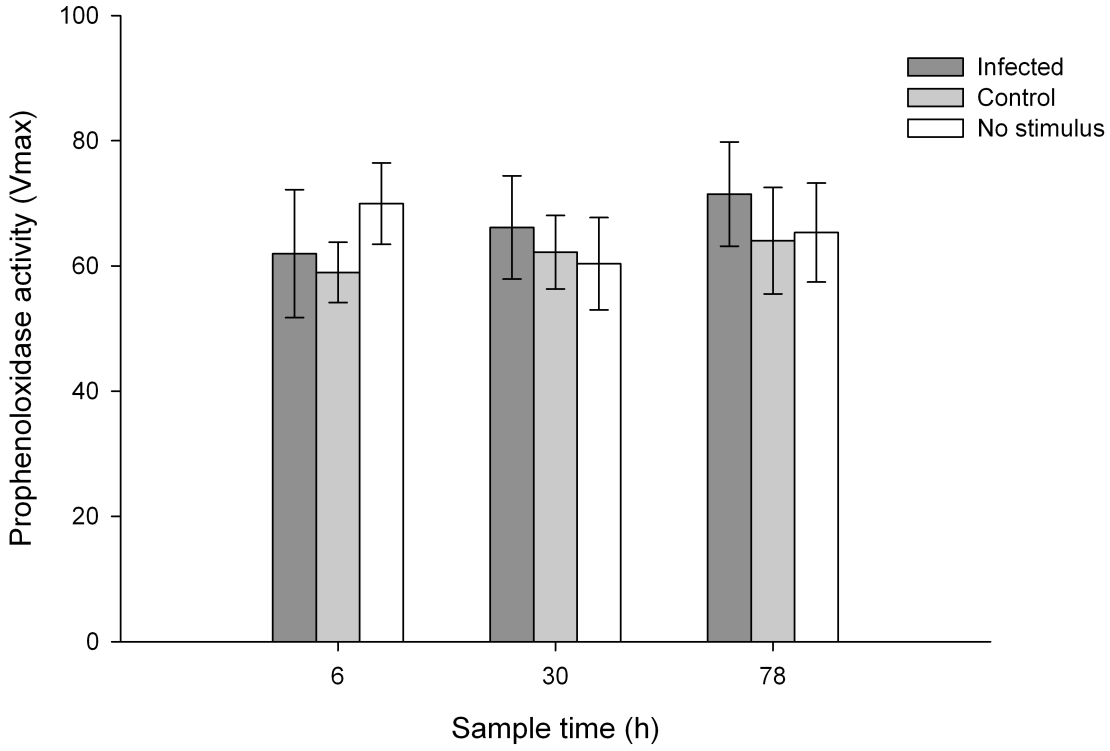

Figure 4.4 Prophenoloxidase activity of test locusts exposed by proximity to live stimulus groups with developing *M. acridium* infections (dark grey), live uninfected control stimuli (light grey), or no stimulus group at all (white). Haemolymph ProPO was measured at 6, 30 or 78 h from initial exposure. Error bars = ±SEM.

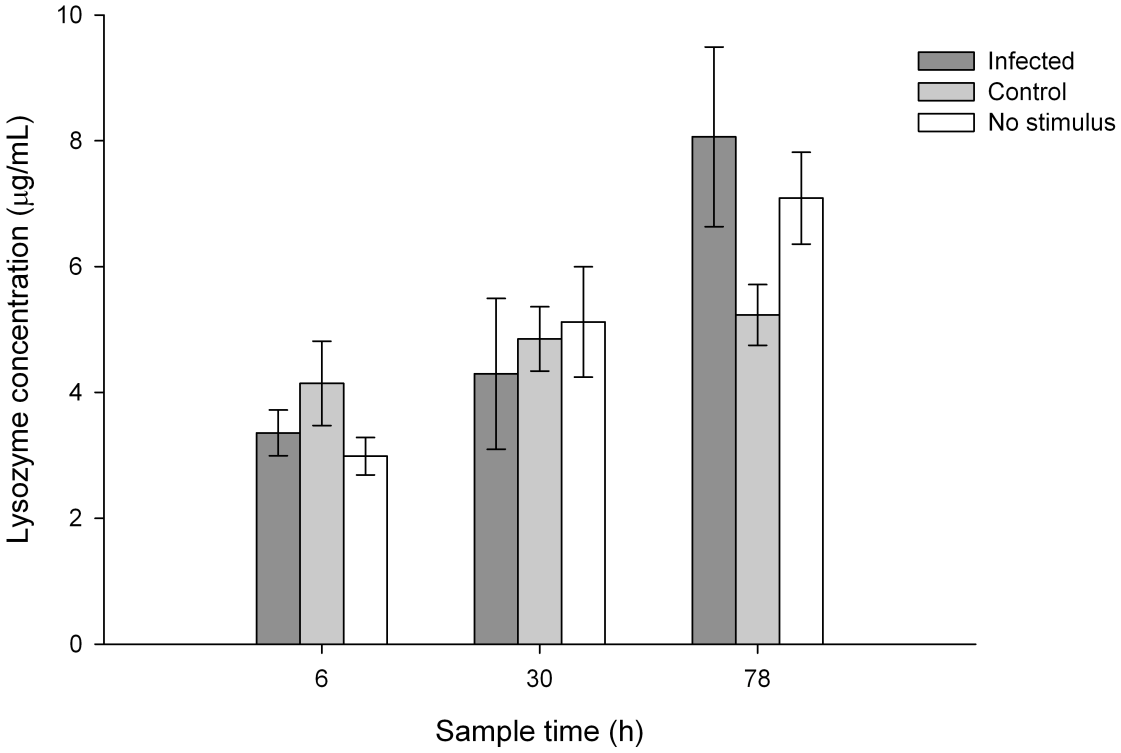

Figure 4.5 Test locust lysozyme concentration following proximity exposure to fungal infected stimulus groups (dark grey), uninfected control stimulus groups (light grey) or no stimuli at all (white). Haemolymph was sampled following 6, 30 or 78 h of proximity to exposure treatments. Error bars = ±SEM.

ANOVA, $F_{(2,84)}$=11.773, p<0.001). There was no significant interaction between stimulus type and sample time.

4.4.2 Stimulus insects: fungal infected versus uninfected controls

As outlined above, individuals selected from each stimulus group were sampled to quantify immune variation in infected and uninfected stimuli, across the sampling time course.

Haemolymph protein concentration was significantly influenced by both infection treatment and sampling time (Figure 4.6; two-way ANOVA, infection: $F_{(1,55)}$=6.124, p=0.016, sampling time: $F_{(2,55)}$=88.870, p<0.001). Post-hoc tests showed that differences between infection treatments occurred at the 78-h sampling time with uninfected controls showing higher concentrations of protein than infected conspecifics (Tukey's HSD, p<0.05). In addition, protein concentration increased significantly across sampling time (Tukey's HSD, p<0.05). The interaction between sample time and stimulus type was not significant.

Contrary to protein results, there was neither a significant difference in ProPO activity observed between *M. acridium* infected and control stimulus insects, nor across sampling times (Figure 4.7; two-way ANOVA, infection: $F_{(1,55)}$=0.448, p=0.506, sampling time: $F_{(2,55)}$=3.000, p=0.58). There was also no significant interaction between infection and sample time as main factors.

Lysozyme activity was significantly affected by infection treatment (Figure 4.8; two-way ANOVA, $F_{(1,52)}$=4.160, p=0.046). In addition, lysozyme concentration increased significantly with sampling time (two-way ANOVA, $F_{(2,52)}$=8.895, p<0.001). Overall, fungal infected stimuli showed a faster increase in lysozyme activity than

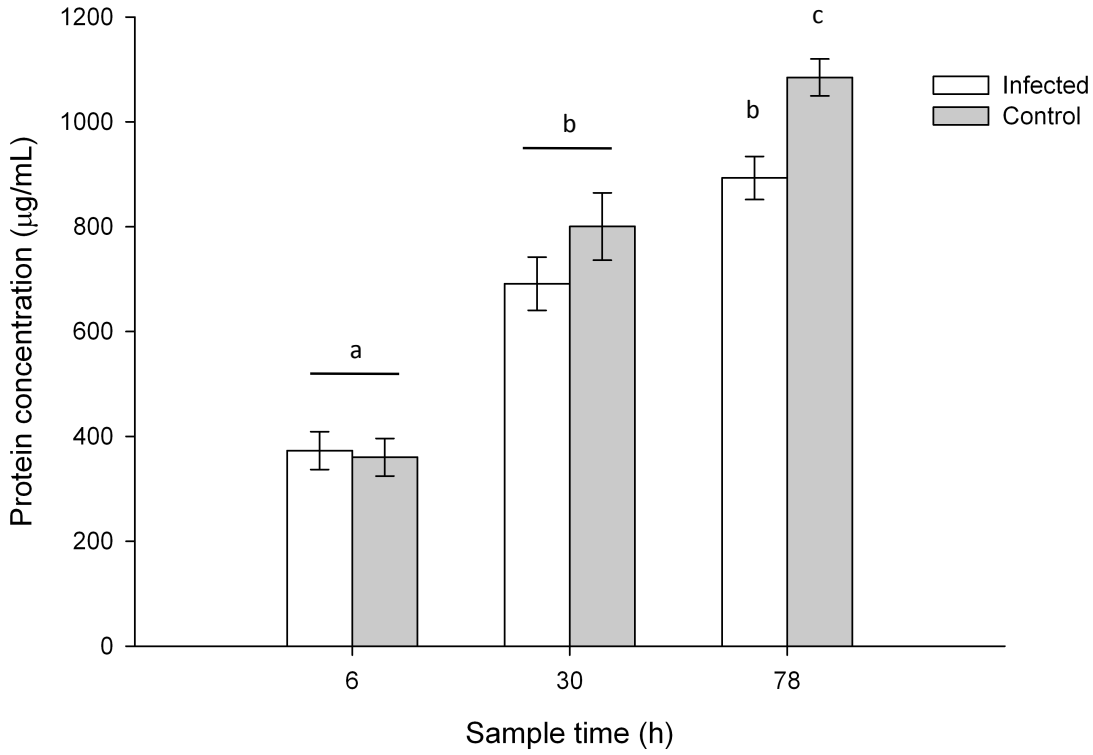

Figure 4.6 Haemolymph protein concentrations of stimulus locusts inoculated with *M. acridium* (white) or Triton-X (grey). Individuals were randomly selected from stimulus groups and sampled at 6, 30 or 78 h post-inoculation. Letters indicate significant differences in protein concentration across different sample times (Tukey's HSD, $p<0.05$). Error bars = ±SEM.

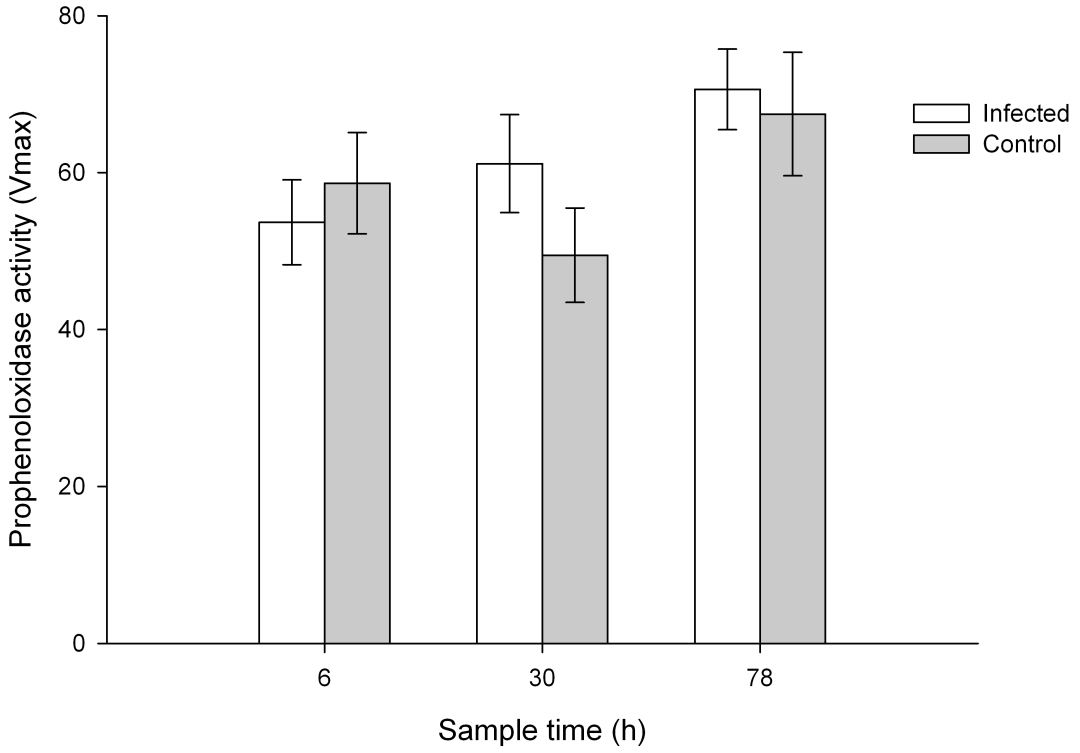

Figure 4.7 Prophenoloxidase activity (Vmax) measured in fungal-infected (white) and uninfected control (grey) stimulus insects at 6, 30 or 78 h post inoculation. Error bars = ±SEM.

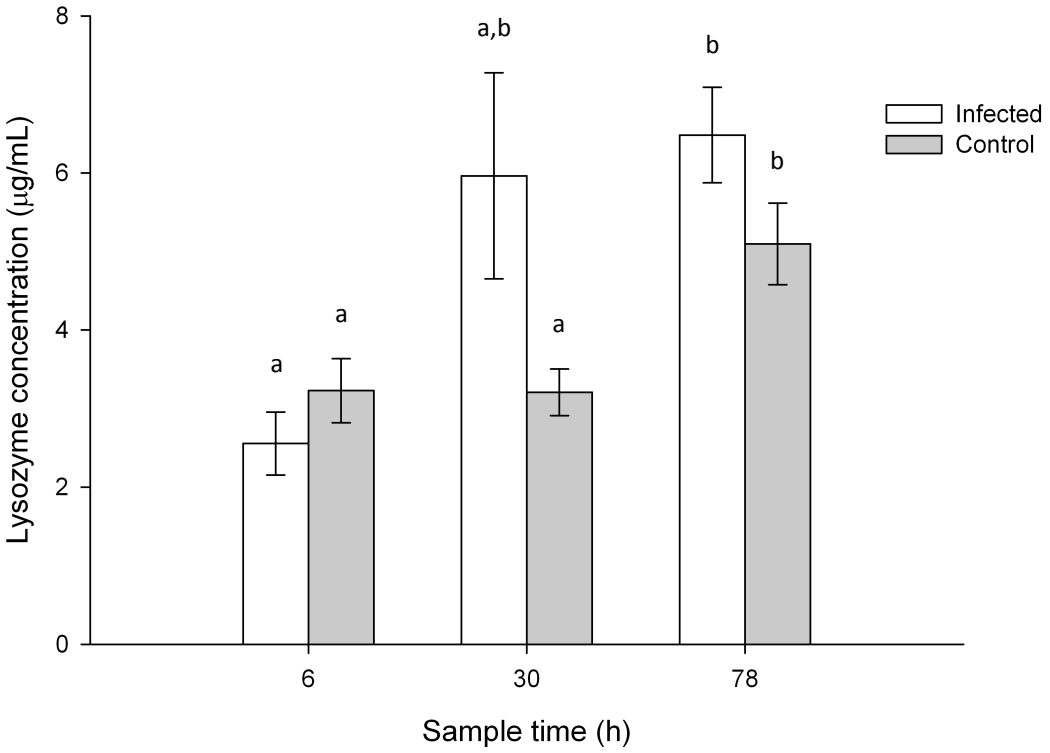

Figure 4.8 Lysozyme concentration of *M. acridium* infected stimulus locusts (white) or uninfected control stimuli (grey). Haemolymph was sampled at 6, 30 or 78 h post-inoculation. Letters indicate differences in lysozyme concentration between infection treatments across time (Tukey's HSD, $p<0.05$). Error bars = ±SEM.

uninfected controls (Tukey's HSD, $p<0.05$). No significant interaction was found between sample time and stimulus type treatments.

4.4.3 Direct contact exposure: effects of live and dead uninfected stimuli

Effects on the constitutive immunity of test locusts exposed by direct contact to live and dead control conspecifics, or no stimulus were quantified in these experiments.

Haemolymph protein concentration was not significantly affected by exposure treatment (Figure 4.9; two-way ANOVA, $F_{(2,85)}=2.254$, $p=0.111$), with no differences in protein concentration found between locusts exposed to dead controls, live controls or no stimulus. Sampling time however, did have a significant effect on protein concentration levels (two-way ANOVA, $F_{(2,85)}=148.378$, $p<0.001$). Here, locusts in all exposure treatments demonstrated the same positive relationship between protein concentration and sampling time (Tukey's HSD, $p<0.05$). There was no significant interaction between exposure treatment and sampling time.

A significant effect of exposure treatment was found on ProPO activity for test locusts exposed to uninfected live controls, uninfected dead controls or no stimuli (Figure 4.10; two-way ANOVA, $F_{(2,84)}=10.911$, $p<0.001$). Test locusts exposed to uninfected cadavers showed lower ProPO activity than those exposed either to live controls or no stimulus at all. Although this was only statistically different for locusts sampled at 30 h (Tukey's HSD, $p<0.05$), a similar trend was observed for locusts sampled at 6 and 78 h. No significant effect of sample time was found on ProPO activity (two-way ANOVA, $F_{(2,84)}=0.905$, $p=0.408$). The interaction between sample time and stimulus type was also not significant.

No significant differences between exposure treatments were detected for lysozyme concentration measurements (Figure 4.11; two-way ANOVA, $F_{(2,85)}=0.632$, $p=0.53$).

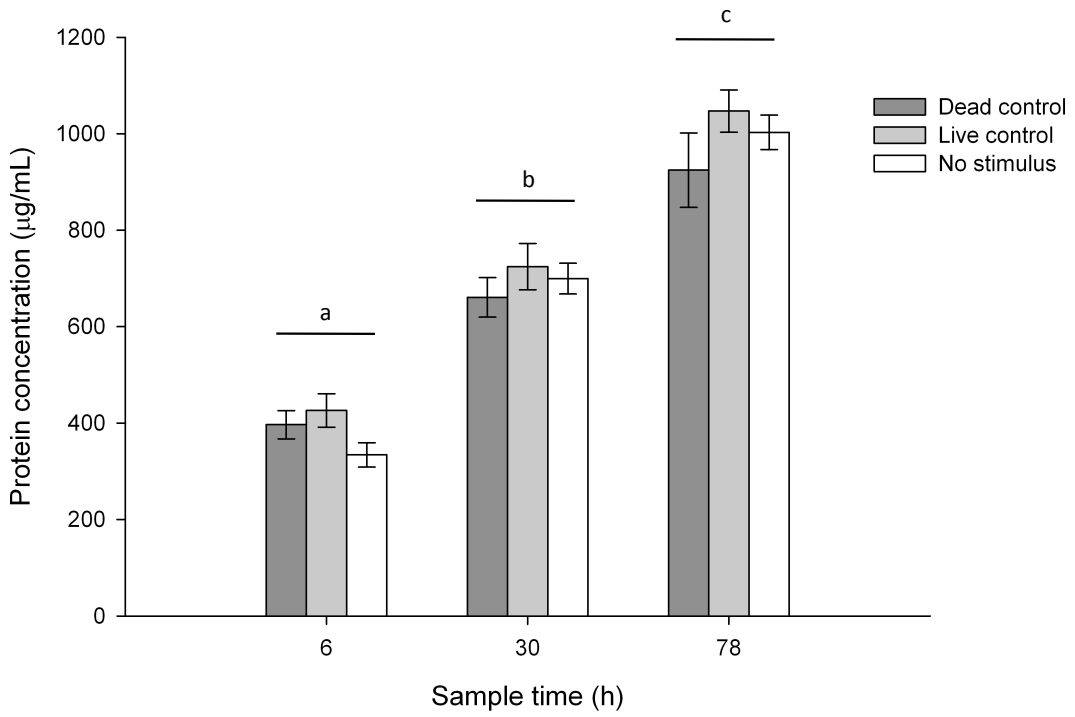

Figure 4.9 Haemolymph protein concentration of test locusts exposed to dead uninfected (dark grey), live uninfected (light grey) or no stimulus (white) conspecifics. Haemolymph samples were taken from test locusts at 6, 30 or 78 h post initial exposure. Letters represent significant differences between treatment groups (Tukey's HSD, $p<0.05$). Error bars = ±SEM.

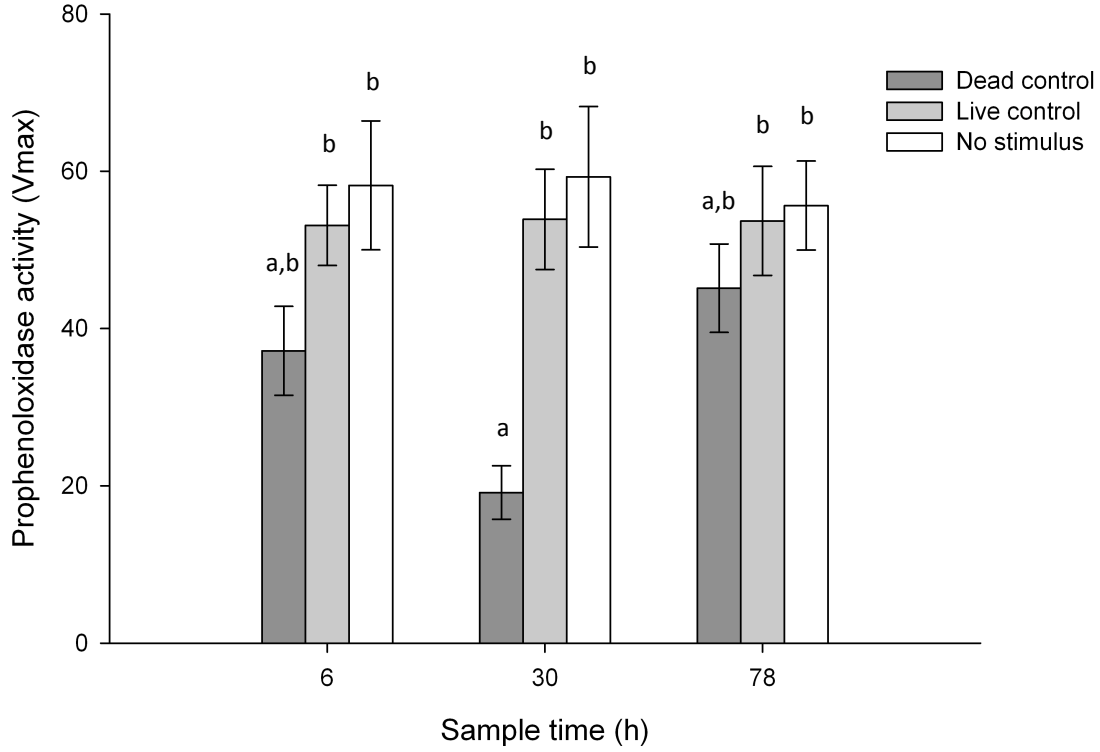

Figure 4.10 Prophenoloxidase activity (measured as Vmax rates) of test locusts exposed to dead uninfected controls (dark grey), live uninfected controls (light grey) or no stimulus at all (white). Haemolymph was sampled at various times following initial exposure (6, 30 or 78 h). Letters indicate significant changes in ProPO activity between stimulus exposure treatments (Tukey's HSD, $p<0.05$). Error bars = ±SEM.

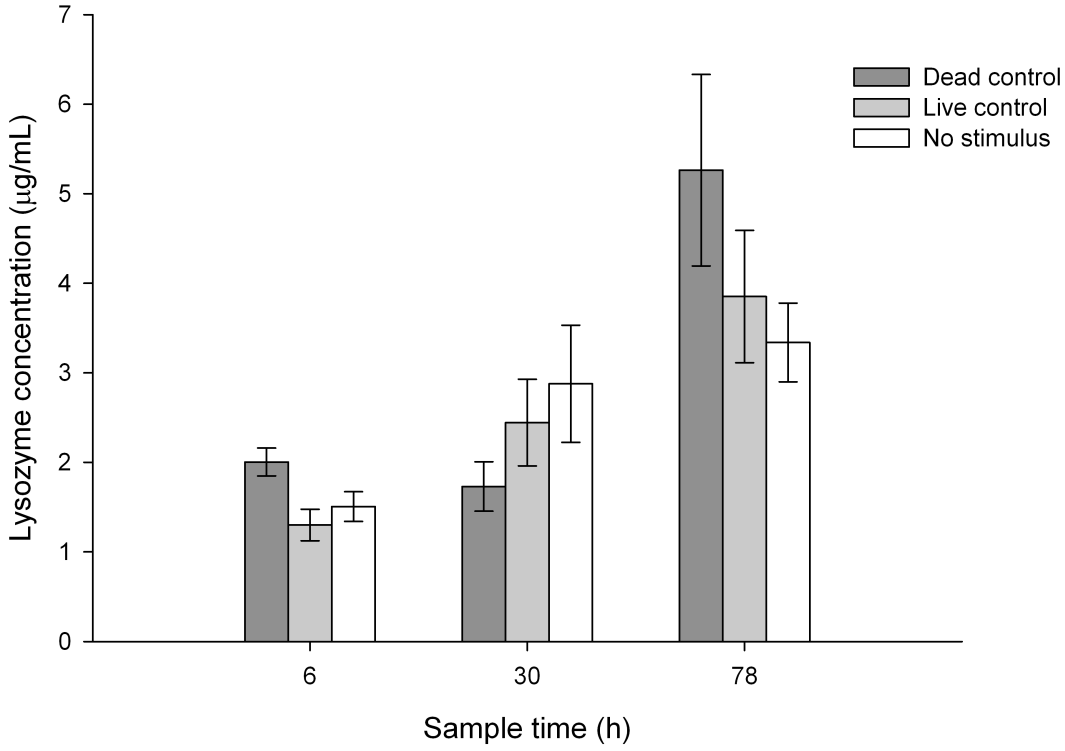

Figure 4.11 Lysozyme concentration of test locusts exposed to dead uninfected controls (dark grey), live uninfected controls (light grey) or no stimulus at all (white). Haemolymph was sampled at 6, 30 or 78 h following initial exposure. Error bars = ±SEM.

Significant differences in lysozyme activity were found across sampling times, again showing the same increasing trend with time (two-way ANOVA, $F_{(2,85)}$=16.283, p<0.001). Exposure treatment and sampling time revealed no significant interaction.

4.4.4 Direct contact exposure with infected and uninfected dead stimuli

Contact exposure to Triton-X inoculated or newly-dead infected cadavers examined the effects of infection exposure of dead conspecifics on the anticipatory immune function of test locusts.

Haemolymph protein concentration was not significantly affected by direct exposure to either control or infected stimuli (Figure 4.12; two-way ANOVA, $F_{(1,56)}$=0.949, p=0.334). Sampling time for both exposure treatments, however, did have a significant effect on protein concentration (two-way ANOVA, $F_{(2,56)}$=72.432, p<0.001). Once again, a significantly increasing trend between protein concentration and sampling time was observed (Tukey's HSD, p<0.05). Since no difference was observed between live and dead stimulus exposure treatment groups (Figure 4.9), this change in protein concentration over time was unlikely to be a result of test locust exposure to dead conspecifics. No significant interaction was found between exposure treatment and sampling time.

Prophenoloxidase activity was not significantly different between test locusts exposed to control and fungal infected cadavers (Figure 4.13; two-way ANOVA, $F_{(1,55)}$=0.071, p=0.790). Significant effects of sampling time, however, were demonstrated (two-way ANOVA, $F_{(2,55)}$=12.567, p<0.01), with locusts at 78 h showing significantly higher ProPO activity than those sampled at 30 h (Tukey's HSD, p<0.05). Similar to test locusts exposed to uninfected control cadavers (Figure 4.10), locusts sampled at 30 h appeared to be driving this decrease in ProPO activity, though no statistical difference between 6 and 30 h sampling times was detected.

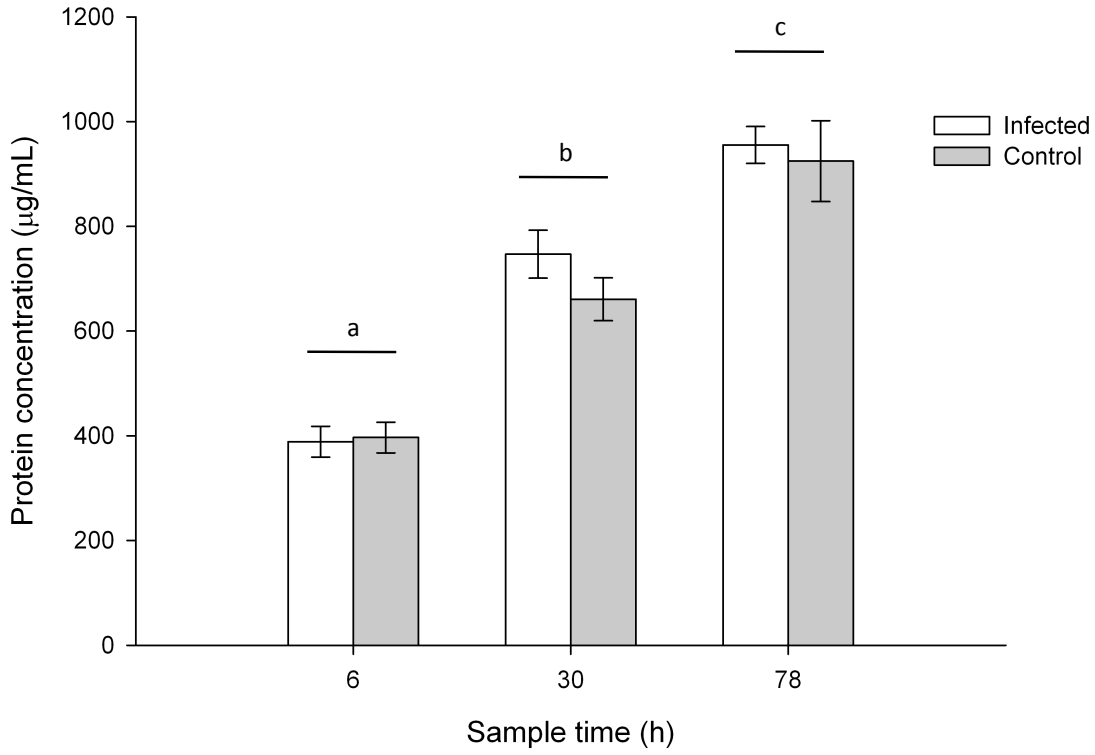

Figure 4.12 Protein concentration of test locust haemolymph exposed to uninfected control cadavers (grey) or newly-dead *M. acridium* infected cadavers (white). Haemolymph was sampled at 6, 30 or 78 h after initial exposure. Different letters represent significant variation across the sample time treatment groups. Error bars = ±SEM.

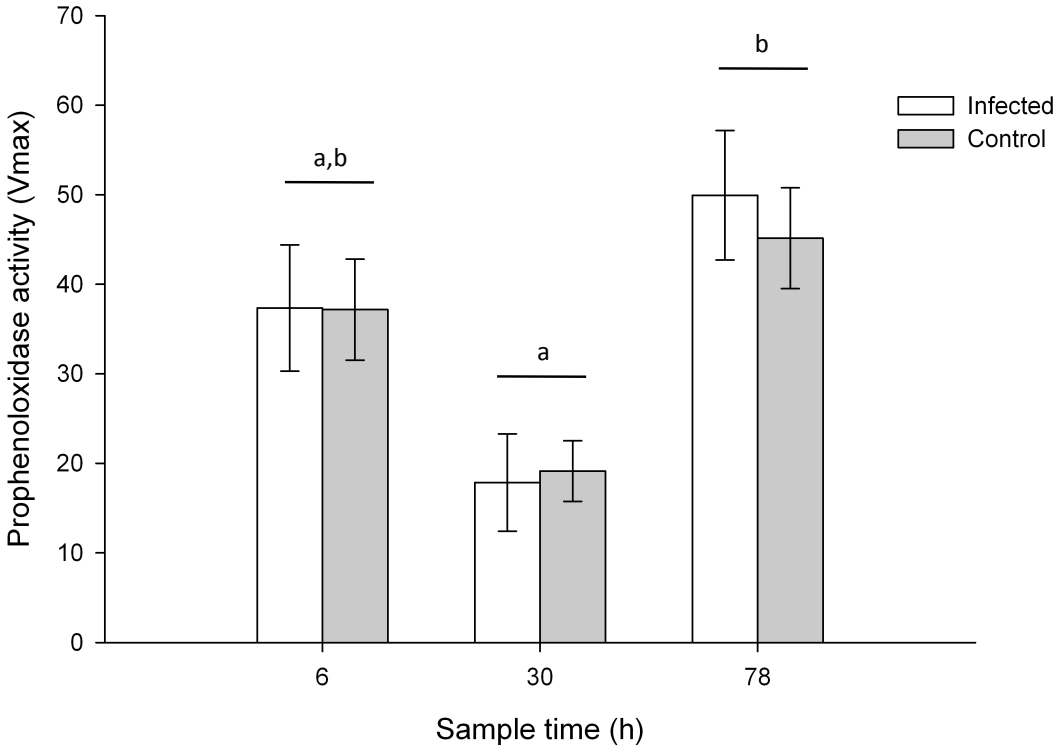

Figure 4.13 Prophenoloxidase activity of test locusts exposed to uninfected control (grey) or newly-dead fungal infected (white) dead conspecifics. Haemolymph samples were taken at 6, 30 or 78 h following initial exposure to stimulus insects. Letters indicate significant differences in enzyme activity across the different sample times (Tukey's HSD, $p<0.05$). Error bars = ±SEM.

There was no significant interaction between sample time and stimulus type treatments.

Test locusts in contact with Triton-X control or *M. acridium* infected stimulus cadavers showed no significant difference in lysozyme activity (Figure 4.14; two-way ANOVA, $F_{(1,56)}$=0.006, p=0.937). Sample time, however, was again found to have a significant effect on lysozyme concentration (two-way ANOVA, $F_{(2,56)}$=22.851, p<0.001). Here, individuals at 78 h showed a significant increase in lysozyme activity compared to the other time points (Tukey's HSD, p<0.05). Since there was no difference in lysozyme activity between test locusts exposed to uninfected live, uninfected dead or no stimulus (Figure 4.11), this effect was not likely to be a response of exposure to dead conspecifics. The interaction between sample time and stimulus type was not significant.

4.5 Discussion

In nature, locusts are in frequent contact with pathogens, often through direct interaction with their conspecifics. As mentioned above, previous experiments have demonstrated that *C. terminifera* are capable of distinguishing and avoiding fungal-infected from uninfected victims during cannibalistic encounters (Chapter 3). This deterrence of infected cadavers clearly demonstrates pathogen recognition in locusts, where avoidance behaviour prevents infection transmission through ingestion. Other studies presented on this species have found evidence of immune upregulation in response to increases in population density (Chapter 2). These results support the theory of density-dependent prophylaxis, a hypothesis suggesting that animals in groups have increased constitutive immune investment in anticipation of an elevated risk of pathogen transmission between conspecifics.

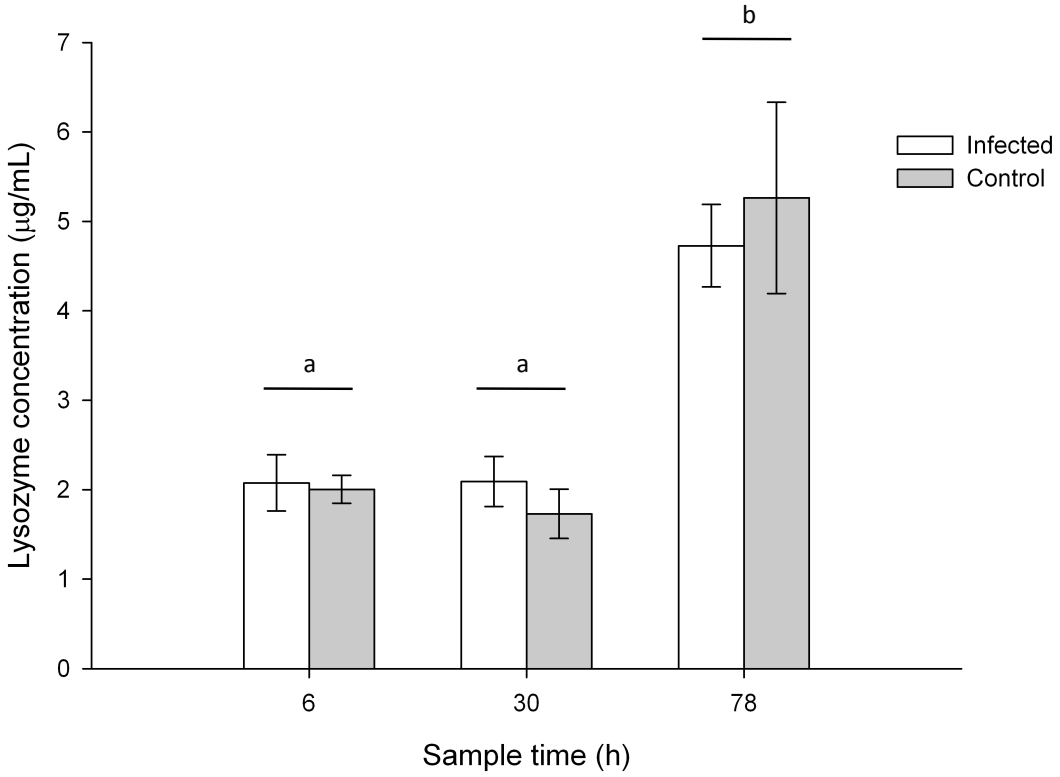

Figure 4.14 Lysozyme concentration of test locusts exposed to uninfected control (grey) or *M. acridium* infected (white) cadavers. Haemolymph was bled from test locusts after 6, 30 or 78 h following exposure to stimulus treatments. Different letters represent statistical variation in lysozyme concentration across sample time (Tukey's HSD, $p<0.05$). Error bars = ±SEM.

With the knowledge that *C. terminifera* exhibit adaptive behavioural and physiological responses to infection, the capacity of locusts to plastically anticipate and respond to changes in pathogen exposure was similarly expected in this species. Two approaches of infection exposure (proximity and direct contact) were therefore employed to identify how *C. terminifera* respond to heightened infection risk on a mechanistic level. Overall, however, *C. terminifera* showed no evidence of immune upregulation following up to 78 h exposure to fungal-infected conspecifics. This result was consistent across test locusts exposed both by contact and proximity to live and dead infected stimuli, suggesting that these insects do not show plastic anticipatory responses of this sort.

Studies on the ant species, *Lasius neglectus*, have recently found fungal-specific immune upregulation in individuals that engaged in social contact with *Metarhizium anisopliae* infected nest-mates (Konrad*, et al.*, 2012). Here, allogrooming behaviour allowed the transfer of *M. anisopliae* between conspecifics before the cuticular attachment of fungal conidia. This was found to significantly inhibit fungal growth and increase antifungal immune gene expression. Such adaptive immunisation has been found to be highly beneficial in social communities, particularly in long-lived species that commonly re-encounter such pathogens (Konrad*, et al.*, 2012). Contrary to predictions, however, similar indications of immune anticipation in *C. terminifera* were not detected.

4.5.1 Fungal infection and detection

Infection by *M. acridium* starts with the adhesion of conidia to the outer cuticle of insects. Infection structures and cuticle degrading enzymes then aid penetration of spores through the host's integument (Leger*, et al.*, 1987; Ortiz-Urquiza and Keyhani, 2013). Once inside the haemocoel, spores grow and multiply rapidly by

exploiting nutrients in the haemolymph, ultimately killing the insect from within (Clarkson and Charnley, 1996). Several days, however, are generally needed to facilitate the mechanisms leading up to initial cuticlular penetration. It was during this early infection-stage that test locusts in proximity experiments were exposed to fungal-inoculated stimulus groups. It is therefore possible that insects did not yet present cues or stimuli sufficient to induce an anticipatory response.

In order to identify whether *M. acridium* infected stimulus insects were in fact experiencing detectable immune responses during proximity experiments, locusts from control and infected stimulus groups were randomly sampled at each of the three time points. In conjunction with increasing effects of time, uninfected stimuli sampled at 78 h showed a higher protein concentration compared to fungal infected treatments indicating greater protein reserves in these insects. Lysozyme concentration in infected stimuli increased faster than in uninfected controls, at 30- and 78-h sampling points respectively. Prophenoloxidase, however, showed no detectable response to either infection or sampling time treatments. These results suggest that while *M. acridium* did affect trait investment in infected stimuli, progression of the infection (and therefore infection response) was perhaps still only in its early stages. This is consistent with this pathogen's mechanism of action and may explain why no induction of immune response was detected in test locusts following proximity exposure to infected stimuli.

To verify this, experiments also measured the immune function of test locusts with direct contact exposure to newly-dead cadavers killed as a result of *M. acridium* infection. Once again, no evidence of immune anticipation was found in any of the immune traits measured when compared to exposure controls, further indicating that plastic immune anticipation following exposure to fungal-infected conspecifics does not occur in these insects.

4.5.2 Time-dependent increases in immune function

Plastic adaptive immune responses were not detected in test locusts following infection exposure treatments. Significant increases in measured traits were observed, however, across the sampling time-course in nearly all the immune assays conducted. This result may be the outcome of a hard-wired constitutive upregulation of immune function in preparation for locust ecdysis. This resource-intensive developmental process is known to heighten host vulnerability, thereby potentially impacting the capacity for immune protection (Beckage, 2011). Numerous studies have previously demonstrated immune upregulation during insect development, an adaptation to protect against opportunistic pathogens (Wago and Ichikawa, 1979; Gillespie *et al.*, 1997). The similar patterns observed in *C. terminifera* may therefore be therefore intuitive.

Changes in hydration levels might explain the trait upregulation seen across the experimental time-course, however, the known behavioural changes of locusts in the days leading up to ecdysis suggest that this is unlikely. Previous studies have shown that locusts generally decrease their food intake prior to moulting, with the highest food intake usually occurring in the middle of the stadium (Hill and Goldsworthy, 1968; Simpson, 1982). Although food was provided *ad libitum*, no observations of increased intake were made in these experiments. The overall process of ecdysis, including the need to compensate for overall energy usage and prepare for increased vulnerability to infection, is therefore still a more probable cause of the observed trait upregulation.

Upregulation of protein and lysozyme concentrations across stadium duration was evident for both test and stimulus insects in all exposure treatments. Prophenoloxidase, however, did not always show a clear increase in response to

sampling time. Contact experiments showed a significant decrease in ProPO levels at the 30-h sampling point. Unexpectedly, this dip was observed only in test locusts exposed to dead stimuli, regardless of their infection state. Protein and lysozyme concentrations did not show this response.

Phenoloxidase (for which ProPO is the inactive zymogen) has been suggested to serve a fundamental role within insect developmental physiology, in particular exoskeleton repair (Terwilliger, 1999; Cerenius and Söderhäll, 2004). The observed decrease (rather than increase) of ProPO activity in test locusts exposed to dead conspecifics was unexpected but may be related to the insect developmental process. In this case, longer sampling periods might give a clearer picture of how investments in ProPO regulation are prioritised, in the context of both infection exposure and hard-wired developmental responses.

Although these experiments found no evidence of anticipatory immune upregulation in response to infection exposure, the observed variation between immune assays across the experimental time-course is still an important result, particularly for studies that may fail to control for age. Here, the variation seen between measured time points could have lead to considerable differences in interpretation if examined individually. Such results therefore serve to caution future studies in immune experiments, revealing the importance of considering ontogenetic immune variation.

4.5.3 Immune assay sensitivity

It is possible that the immune assays used in these experiments were unable to detect the subtle changes in immune state that may have occurred as a result of exposure to conspecific infection. Low (but still detectable) lysozyme activity, for instance, has been reported in insects where constitutive immunity is unchallenged by infection (Chapman*, et al.*, 2013). Although the immune assays used in this study have been

tried and tested throughout previous studies, it is possible that acute and early immune reactions by hosts may have been missed as a consequence of sampling time, response strength or assay sensitivity. For this reason, comprehensive approaches that incorporate the measurement of several traits (as was done here) are highly desirable when studying the innate immunity of animals (Adamo, 2004a; Bradley and Jackson, 2008).

Recent shifts toward molecular approaches have broadened the sensitivity and precision through which insect immune responses can be detected and quantified (Pedersen and Babayan, 2011). In line with these advances, Chapters 5 and 6 present and apply the development of reverse transcription quantitative PCR as an additional tool to quantify locust immune response alongside conventional approaches. This further cements suggestions that a broader use of available techniques can in turn provide a wider view of immunological study systems.

4.5.4 Conclusions and further research

In the field, migrating locust bands will typically leave behind infected individuals and unpalatable cadavers. Selective avoidance of infected conspecifics during cannibalism has also been clearly demonstrated (Chapter 3). The present study, however, found no evidence for boosted immune function in response to exposure to fungal-infected conspecifics. Since avoidance behaviour and migration collectively lower infection exposure times and therefore pathogen transmission risk, the additional upregulation of immune function, within the scenarios explored in this chapter, might represent a greater resource cost for individuals that do not warrant the benefits of increased resistance to infection.

Test locusts in contact exposure treatments had been expected to show the most prominent evidence of predictive immune upregulation. However, for test locusts

exposed to either control or fungal- infected cadavers, no evidence of immune anticipation was detected. It is possible that the degree of stimulus infection used in these experiments represented a low risk of pathogen transmission or was otherwise undetectable to test locusts altogether. Exposure to more developed infections (*e.g.,* sporulating cadavers) for longer periods may serve to either confirm current findings or trigger a detectable plastic immune response in test locusts. Conflicting results and false positives brought on by changes in insect developmental physiology, however, must be considered.

Immune upregulation in locusts, as preparation for ecdysis, was demonstrated through the time course of these experiments. Ecdysis is an essential developmental process repeated several times throughout the life cycle of a locust. Physiological and morphological changes that occur during moulting may therefore act as reliable indicators of latent changes in immune vulnerability. Upregulation of immune traits in preparation for ecdysis is likely to be a foreseeable part of an individual's life history and therefore a 'hard-wired' component of the developmental process. No evidence, however, was found for an additional plastic anticipatory response to exposure to fungal infected conspecifics.

Chapter 5.

Tissue-specific Immune Gene Expression

5.1 Summary

A host's ability to recognise non-self and respond to infection involves several complex immune recognition pathways. Broadly conserved pathogen-associated molecular patterns (PAMPs) allow individuals to target and react to a range of invading microbes. However, several studies on the innate immunity of insects have provided evidence that different species show activation of alternative immune pathways in response to the same pathogen. Response variation in different tissue types has also been observed, making the question of assay and tissue selection increasingly complex. This chapter looks at the immune gene expression of a range of tissue-types within *Locusta migratoria* in response to an immune challenge using lipopolysaccharides (LPS) from *Escherichia coli,* a gram-negative bacterium. By examining expression variation in three immune-associated genes encoding peptidoglycan-recognition protein SA (PGRP-SA), gram-negative binding protein 1 (GNBP1) and prophenoloxidase (ProPO), this work demonstrates how different tissues express varying levels of constitutive immunity. Variation in immune response to LPS injection was also found across tissue-types. Overall, results showed an increase in PGRP-SA expression in response to LPS across all seven of the tissues assayed. Relative PGRP-SA expression levels between tissues, however, differed greatly, suggesting tissue specificity in response to this immune challenge. Constitutive expression of GNBP1 also differed between tissue types, yet showed an unexpected decrease in the fat body in response to LPS injection. Increases in ProPO expression in response to LPS injection could only be detected in the three gut sections (foregut, midgut and hindgut), again suggesting that immune response pathways are specific to the tissue being examined. Such effects are expected to also demonstrate pathogen dependence. The following study demonstrates the importance of tissue and gene selection when performing immune gene expression assays.

5.2 Introduction

5.2.1 Immune specificity in insects

Insect immunology, a field that interfaces with many areas of insect biology and ecology, is critical in understanding many disciplines spanning from insect physiology to parasitology and agriculture. Understanding the mechanisms and pathways responsible for immune function in insects is of great interest, not only with respect to the ecology and economics that surround invertebrates systems, but also as a model to study the innate immunity of human and other vertebrate systems.

To fight infection, insects rely on innate immune defences to rapidly induce responses against invasive microbes (see Chapter 1). Microbial non-self recognition enables hosts to recognise conserved, stereotypical (rather than unique) microbial structures that are common to a wide range of microorganisms. The best-known examples of such structures are called pathogen-associated molecular patterns (PAMPs). These include the lipopolysaccharides (LPS) of gram-negative bacteria and the peptidoglycans of gram-positive bacteria (Medzhitov and Janeway, 2002). These, along with other PAMPs, are commonly recognised by pattern-recognition receptors (Janeway Jr and Medzhitov, 2002).

Two molecules involved in PAMP detection are the peptidoglycan recognition protein SA (PGRP-SA) and the gram-negative bacteria binding protein 1 (GNBP1). PGRPs are highly conserved between insects and mammals (Royet and Dziarski, 2007), yet have been shown to activate different immune pathways to GNBP1 depending on the species under study. In *Drosophila*, PGRP-SA and GNBP1 form a protein complex that recognises gram-positive peptidoglycans and activates the Toll pathway, a series of protein receptors that play a critical role in insect immune defence (Michel*, et al.*, 2001; Gobert*, et al.*, 2003; Wang*, et al.*, 2006; Lemaitre and

Hoffmann, 2007). In beetles, it has been shown that cell wall components of both gram-positive and gram-negative bacteria are recognised by PGRP-SA and GNBP1 respectively, which induces Toll and prophenoloxidase (ProPO) activation (Yu*, et al.*, 2010). This suggests that Coleoptera and Diptera may have different antimicrobial peptide gene induction systems, revealing the importance of further investigations into PGRP-SA and GNBP1 in other insect species, particularly from a comparative physiology viewpoint.

5.2.2 Tissue-specific immune responses

Innate immune activation in insects has been classically measured in targeted tissues such as the haemolymph and fat body, two tissues that are known to play important roles in cell production and immune response (Hoffmann, 1995). Haemocyte density is frequently used to measure cell proliferation, where processes such as encapsulation and phagocytosis give indication of foreign particle response (Lavine and Strand, 2002). Fat body plays a large role in the production of antimicrobial peptides and is one of the first tissues to respond to immune challenges through the secretion of synthesised proteins and lipids into the haemolymph (Boman and Hultmark, 1987; Hoffmann, 1995). The gastrointestinal tract of insects also produces antimicrobial peptides (as well as reactive oxygen species) in response to invading pathogens (Vallet-Gely*, et al.*, 2008; Chapman*, et al.*, 2013). With limited information on comparative immune activation across different tissues, the suitability of many of these tissue types, when assessing insect immune function, remains largely unknown.

With an array of organs and tissues interacting and responding to different pathogens, studies that incorporate multiple immune assays and tissue types to

quantify insect immunity are now becoming more frequent, and are now considered necessary when attempting to describe insect immune function (Adamo, 2004a).

With the associated difficulties of tissue acquisition, storage and analysis it is clear that finding accurate and sensitive approaches to describe and quantify invertebrate immunity and resistance is vital for a better understanding of innate immune function. Despite the diversity of immune assays available, results obtained from such measurements are often contradictory, making it difficult to establish a precise relationship between induced changes in individual trait investments and overall host response. Conflicting results between four conventional immune measures in Chapter 2, for example, found that trait response to infection and density treatments in *Chortoicetes terminifera* did not support the same hypothesis. The use of multiple immune traits in this way reveals the complexities of innate immune function and highlights the importance of a broad approach when attempting to describe how ecology influences immune response and physiology.

The ability to detect and measure host immune state on a molecular level is also challenging, with a high likelihood that different immune pathways will be activated in a way that depends on environment, pathogen and tissue type. In an attempt to identify newly informative tissues to describe insect immunity, this study used reverse transcription quantitative polymerase chain reaction (RT-qPCR) to follow the immune gene expression in several tissue-types within the African migratory locust, *Locusta migratoria* (Orthoptera: Acrididae).

5.2.3 Study species and applied importance

Locusta migratoria is a species of great biological, agricultural and global economic importance that is estimated to impact millions of people around the world. In April 2012, a colossal locust plague in Madagascar affected the livelihoods of an estimated

13 million people (more than half the total population). Following the predictions of the Food and Agriculture Organisation of the United Nations (F.A.O), at least 1.5 million hectares of land would be infested, representing two-thirds of the country's size (F.A.O, 2013). With agricultural devastation of this magnitude, obtaining a better understanding of locust immunity and therefore population biocontrol is essential, particularly since the major approach to locust biocontrol is through the application of infectious disease agents. The comparison of tissue-specific immune function in this study will therefore provide a greater insight into how insects respond to infection at the level of three important immune genes. In addition, this work will enable the identification of tissue-types that might be more informative when studying insect immunity using similar molecular approaches.

5.2.4 Aims

The following experiments focused on the development of gene expression assays to identify variation in the constitutive and responsive immune function across seven tissues within *L. migratoria*. Genes coding for PGRP-SA and GNBP1 were investigated in their response to an immune challenge with LPS (extracted from *E. coli*). The gene coding ProPO, an enzyme involved in insect melanisation and also triggered by microbes derived by molecules such as LPS (Jiravanichpaisal*, et al.*, 2006), was also analysed for expression variation.

Specifically this study aimed to: i.) optimise RT-qPCR as a technique for the identification of changes in three immune associated genes; ii.) identify differences in immune gene expression and response across seven tissue types to enable better selection of informative tissues, and iii.) compare *L. migratoria* immune gene expression to results from other studies to reveal variation and similarities across species.

5.3 Materials and methods

5.3.1 Insect cultures and injection treatments

Adult *L. migratoria* were reared to 14 d maturity under gregarious culture conditions at the University of Sydney as described previously (Clissold*, et al.*, 2010). These rearing conditions mirrored those outlined in Chapter 2. Insects were fed daily with fresh wheat grass and wheat germ and provided with water *ad libitum*. Two treatment groups (n=10) were generated from the same stock cage. Locusts were injected with either 100 µg LPS from *E. coli* (purified by phenol extraction; Sigma Aldrich) that was resuspended in 20 µL insect saline (Goldsworthy*, et al.*, 2003), or 20 µL of saline only as sham controls. Injections were made into the thoracic haemocoel via the intersegmental membrane between the third and fourth tergum using a 50-µL volume Hamilton micro-syringe with a 26-gauge bevelled needle. Following injections, locusts were returned to gregarious culture conditions for 7 h before being bled and dissected.

5.3.2 Insect bleeding, dissections and tissue collection

Haemolymph was obtained by piercing the arthrodial membrane at the base of the hind leg using a sterile needle and the expelled fluid collected using a micropipette. Each insect was bled to obtain the maximum volume of haemolymph possible per individual, with a minimum of 10 µL used for RNA extractions. Individuals from which less than 10 µL haemolymph was obtained, were discarded as this had previously been demonstrated to be an insufficient amount for obtaining a high quality RNA yield (personal observations). These insects were not included in the final sample size of n=10. All haemolymph samples were frozen directly at -80˚C without fixation. Bled locusts were then immediately dissected to obtain the head, hind femur (leg), fat body, and gastrointestinal tract (Figure 5.1). The latter was

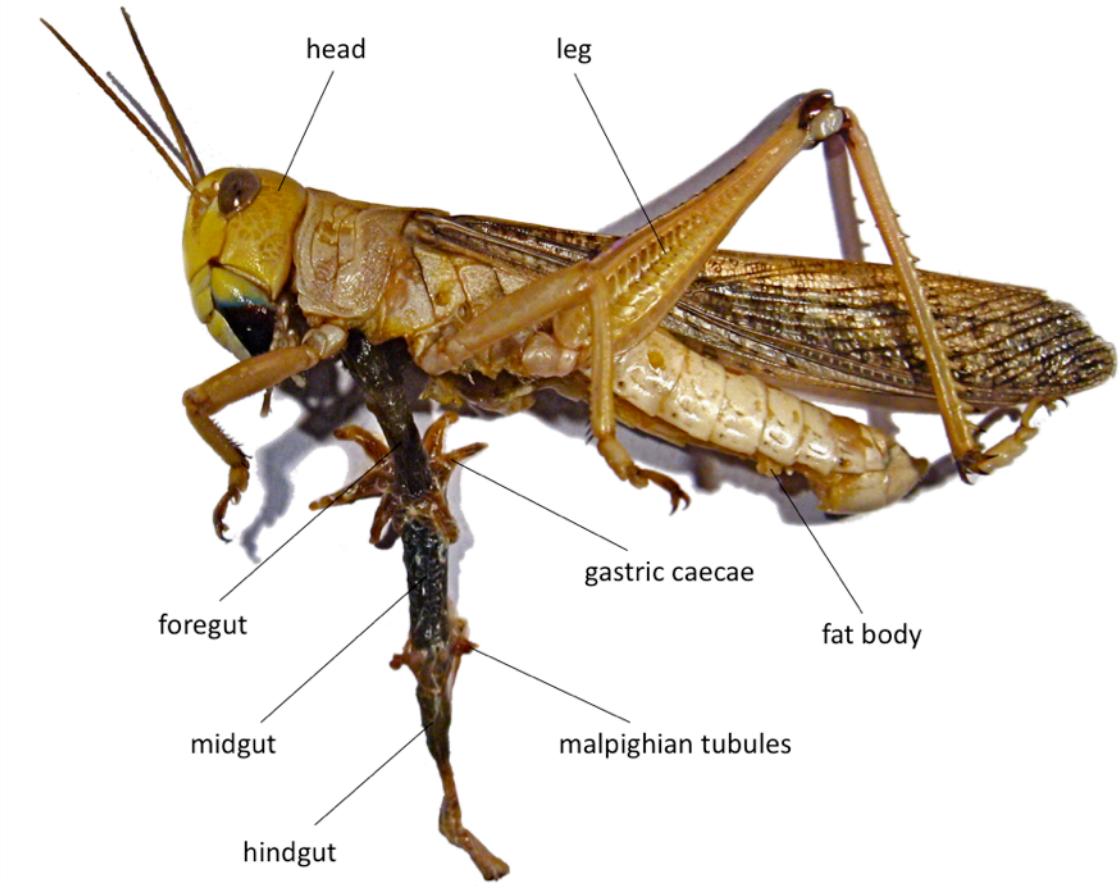

Figure 5.1 Dissection of *L. migratoria* showing the various tissue types analysed for gene expression assays including head, leg (hind femur), fat body, foregut, midgut and hindgut. Gut sections were separated at gastric caecae and malpighian tubules. Haemolymph was collected prior to dissections and is not shown.

separated at the gastric caecae and malpighian tubules to obtain the three gut sections: foregut, midgut and hindgut. Each gut section was dissected open and washed in insect saline to remove the gut contents. All tissues were immediately placed in *RNAlater* (Invitrogen) and held at 4˚C overnight before long-term storage at -80˚C.

5.3.3 Gene expression assays

All protocols used conformed to the MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments (Bustin*, et al.*, 2009). This section outlines the essential information, *sensu* (Bustin*, et al.*, 2009), required to allow reliable interpretation of the corresponding RT-qPCR results.

RNA isolation

RNA extractions were performed using a combination of Trizol-chloroform extraction procedures and the Qiagen *RNEasy* Plus Mini kit protocol for animal tissues as previously described (Chapuis*, et al.*, 2011; Ponton*, et al.*, 2011b). Briefly, tissues were placed in 1 mL Trizol reagent (Ambion) and homogenised using 5 mm stainless steel beads in a tissue lyser (Qiagen) for 40 s at 25 Hz. Following a 15 min incubation at room temperature, samples were centrifuged for 10 min at 4˚C (12000 g). From this the supernatant was retained and washed in ¼ volume chloroform for phase separation. An additional step was added where the RNA-containing supernatant was aspirated three times using a 26-gauge syringe (Livingstone). This was performed to shear genomic DNA (gDNA) prior to the addition of chloroform. After a 3-min incubation period at room temperature, samples were centrifuged again for 20 min at 4˚C (12000 g) and the upper phase transferred to gDNA eliminator columns (*RNEasy* Plus Mini Kit, Qiagen). RNA extraction procedures then followed the kit protocol until RNA elution. Samples

were eluted twice; once with RNAse free water, and the second time with the eluate obtained from the first elution to increase RNA concentration. To eliminate residual gDNA, the purified RNA was treated with a DNAse DNA-free kit (Ambion) according to the manufacturer's protocol. Total RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies) and the samples stored at -80˚C.

Reverse transcription and cDNA synthesis

Complementary DNA was synthesised following the manufacturers protocol from the SuperScript® VILO kit (Invitrogen). Reactions used 250 ng of total RNA added to DEPC treated water, 5x VILO™ reaction mix and 10x SuperScript® enzyme mix to a final reaction volume of 20 µL. RNA extractions containing less than 250 ng template in the maximum allowable volume (14 µL) were not used. No template controls (NTC) and no reverse transcription enzyme (no-RT) controls were also run for each sample. Reverse transcription reaction conditions consisted of an initial incubation period of 10 min at 25˚C, followed by 60 min at 42˚C. The reaction was terminated at 85˚C for 5 min. All cDNA samples and controls were stored at -20˚C.

Primers

Primers (Sigma Aldrich) were designed for immune genes based on sequences obtained from the Migratory Locust EST Database (LocustDB) (Ma*, et al.*, 2006). Immune genes selected were from sequences coding for peptidoglycan recognition protein SA (PGRP-SA; LMC_003396), gram-negative bacteria binding protein 1 (GNBP1; LMC_001357) and a prophenoloxidase encoding gene (ProPO; LMC_001102). Reference genes used were Actin and Elongation factor 1a (EF1a), two genes that have previously been shown to be stably expressed in locusts (Chapuis*, et al.*, 2011). Primers were designed to 8-22 bp in length, 59-61°C Tm, 40-

60% GC content, a maximum self-complementarity of 5 bp and a maximum 3' self-complementarity of 2 bp using the software Primer3 (v.0.4.0) (Untergasser*, et al.*, 2012). Product sizes ranged between 80-150 bp. All other parameters were kept as default. Primer sequences, melting temperatures (Tm), accession numbers and amplicon lengths are shown in Table 5.1.

Reverse transcription quantitative polymerase chain reactions (RT-qPCR)

Triplicate first strand cDNA aliquots for each sample served as templates for RT-qPCR using SYBR Green PCR Master Mix (Applied Biosystems) and a LightCycler480 instrument (Roche Diagnostics). Amplification reactions were performed manually in 384-well optical plates (Perkin Elmer/Applied Biosystems Divisions) to a total volume of 5µL. This included 1 µl of cDNA template (diluted 1:20), 2.5 µL SYBR Green PCR Master mix (Applied Biosystems), 100 nM of each primer and ultra-pure water (Invitrogen).

Quantitative PCR was carried out using the following incubation conditions: 50ºC for 2 min, 95ºC for 10 min, and 45 cycles of 95ºC for 15 s and 60ºC for 1 min. Equal amounts of stock cDNA from each sample across all treatments were pooled and serially diluted (*i.e.,* 1/3, 1/9, 1/27, 1/81, 1/243, 1/486 and 1/972) to generate relative standard curves for the gene transcripts. Reverse transcription qPCR efficiency (E) was determined for each gene and treatment with the slope of a linear regression model (Pfaffl, 2001). These were calculated according to the equation: E = (10[-1/slope]-1) x100 (Radonić*, et al.*, 2004). Dissociation-curve analysis after 45 cycles of amplification revealed that all primer pairs amplified a single PCR product. All PCRs displayed a coefficient of correlation superior to 0.99. Efficiencies ranged between 98 and 120% and were shown to be reproducible across the different

Table 5.1 Primer sequences for immune genes encoding peptidoglycan recognition protein SA (PGRP-SA), gram-negative bacteria binding protein 1 (GNBP1) and prophenoloxidase (ProPO) used in RT-qPCR gene expression assays. Reference genes (Actin and EF1a) were used to normalise relative expression. Amplicon length, melting temperature and accession numbers are shown.

Accession number	Gene name	Primer F/R	Primer sequence	Amplicon length (bp)	Tm (˚C)
JF915527	PGRP-SA	F	AGGAGTTCATGGAGGTGCAG	87	64.3
		R	GCCAAGACGGTGGAGTACAT		63.9
JF915523	GNBP1	F	GGGAAGAGTTCAACCACCAA	83	63.9
		R	GCAAGCGTAGATTTCCAAGG		63.5
FJ771024.1	ProPO	F	TGTGCCTCATTGTCGTTGTT	137	64.3
		R	TACCTGGACGTGTGCTGAAG		64.0
KC118986	Actin	F	CTTTTCCCTGTTTGCCTTTG	104	63.4
		R	AAATCTGGCACCACACCTTC		63.9
AB583233	EF1a	F	CAGCCTGTGACGTTCCTGTA	112	64.0
		R	ATTGACATTGCGTTGTGGAA		64.0

plates. Samples were verified as free of DNA contamination through NTC and no-RT controls.

5.3.4 Data mining and statistical analyses

Expression levels were determined by the number of amplification cycles needed to reach a fixed threshold in the exponential phase of the PCR reaction (Walker, 2002). This number is referred to as the quantification cycle (Cq) value (Lefever*, et al.*, 2009). Cq values were transformed into quantities via the standard curve using PCR efficiencies as described previously (Vandesompele*, et al.*, 2002). Target gene expression was normalised using the geometric mean of the reference genes (Vandesompele*, et al.*, 2002). Sample outliers were removed from treatment groups prior to statistical analyses, thereby accounting for final sample size variation across tissue-types.

Differences in expression levels between saline- and LPS-injected insects were analysed using t-tests. Since the same individuals were used to measure expression levels across the different tissue types, variation in gene expression between tissues were tested using Friedman tests with individual number as a blocking variable. All statistical analyses were performed using the software package Systat 12 (Systat Software, Inc., San Jose, California, USA).

5.4 Results

Gene expression levels of inoculated locusts were compared across LPS- and saline-injected treatments as well as between the seven tested body parts for PGRP-SA, GNBP1 and ProPO.

5.4.1 Immune gene expression after LPS injection

A significant increase in PGRP-SA expression was found in LPS-injected insects compared to saline controls in all seven body-parts analysed (Table 5.2, Figure 5.2). This increase differed depending on the body part being examined. The greatest variation in PGRP-SA was observed in the head and leg of LPS-injected individuals with 7.0- and 6.8-fold expression increases respectively.

GNBP1 gene expression was not significantly upregulated in LPS-challenged locusts in any of the tissue types tested. Unexpectedly, fat body showed significantly lower GNPB1 expression in the LPS-injected treatment compared to saline injected controls (Table 5.3, Figure 5.3).

Expression levels of the gene coding for ProPO were significantly different between immune challenged treatments within each of the three gut sections (*i.e.,* foregut, midgut and hindgut) (Table 5.4). Here, increases in ProPO expression were observed in LPS-injected insects compared to saline-injected controls (Figure 5.4). No effects of LPS-injection were detected in any other body part.

5.4.2 Immune gene expression across tissue types

Immune gene expression levels were also compared between tissue-types within LPS and control treatments. Significantly different PGRP-SA expression levels were found across the seven body parts for both treatments (Figure 5.2; Friedman test, saline controls: $\chi^2_{(6)}=42.429$, $p<0.001$; LPS-injected: $\chi^2_{(6)}=47.810$, $p<0.001$). Highest expression levels were observed in the fat body and haemolymph for both control saline and LPS-injected locusts. Expression levels of PGRP-SA in all gut segments were the lowest of all tissue-types for both inoculation treatments.

Table 5.2 T-test comparisons of mean relative expression levels of the gene encoding peptide recognition protein SA (PGRP-SA) between LPS-injected and saline control treatments in seven tissues of *L. migratoria*. All differences shown are statistically significant ($p<0.05$). Values in parentheses indicate treatment sample sizes (n).

Tissue type	Relative expression		t	d.f	P-value
	Saline control	LPS injected			
Head	18.54 (10)	129.58 (9)	5.45	17	< 0.0001
Leg	13.04 (10)	88.94 (10)	5.98	18	< 0.0001
Fat body	57.10 (10)	259.33 (10)	4.49	18	0.0003
Haemolymph	40.02 (8)	251.26 (10)	2.26	16	0.0383
Foregut	1.17 (10)	13.34 (10)	5.63	18	< 0.0001
Midgut	0.27 (7)	0.67 (9)	2.98	14	0.0099
Hindgut	1.50 (8)	14.77 (10)	5.30	16	< 0.0001

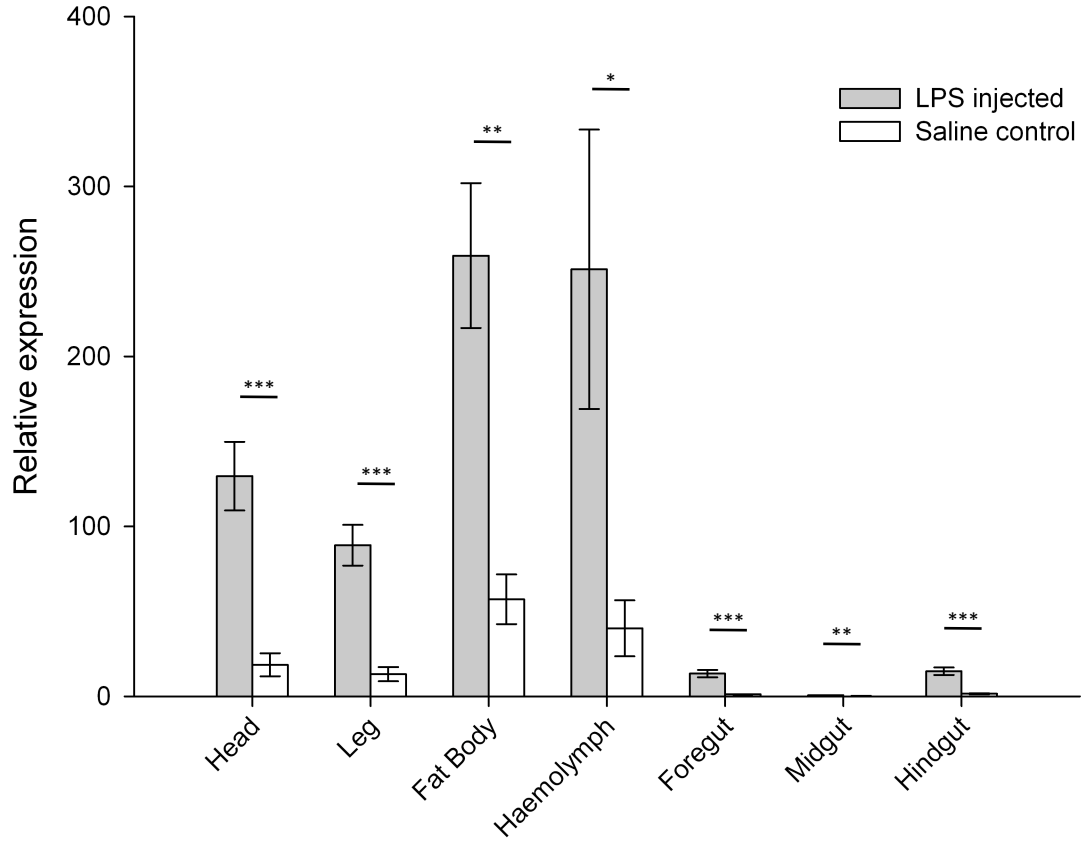

Figure 5.2 Relative expression levels of saline control and LPS-injected *L. migratoria* for the immune gene encoding peptidoglycan recognition protein SA (PGRP-SA) in seven tissue types. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Error bars = ±SEM.

Table 5.3 Relative expression level comparisons of the gene encoding gram-negative binding protein 1 (GNBP1) between LPS-injected and saline control *L. migratoria* in seven tissue types. Statistically significant differences between treatments (determined by t-tests) are shown in bold ($p<0.05$). Values in parentheses indicate treatment sample sizes (n).

Tissue type	Relative expression		t	d.f	P-value
	Saline control	LPS injected			
Head	55.82 (10)	24.53 (9)	1.68	17	0.1104
Leg	97.54 (10)	74.66 (10)	0.34	18	0.7402
Fat body	10.31 (10)	5.12 (9)	2.32	17	**0.0331**
Haemolymph	293.34 (6)	376.15 (8)	0.45	14	0.6597
Foregut	0.90 (10)	7.20 (10)	1.04	18	0.3128
Midgut	1.15 (8)	0.78 (10)	1.10	16	0.3254
Hindgut	8.97 (8)	20.91 (10)	0.93	16	0.3684

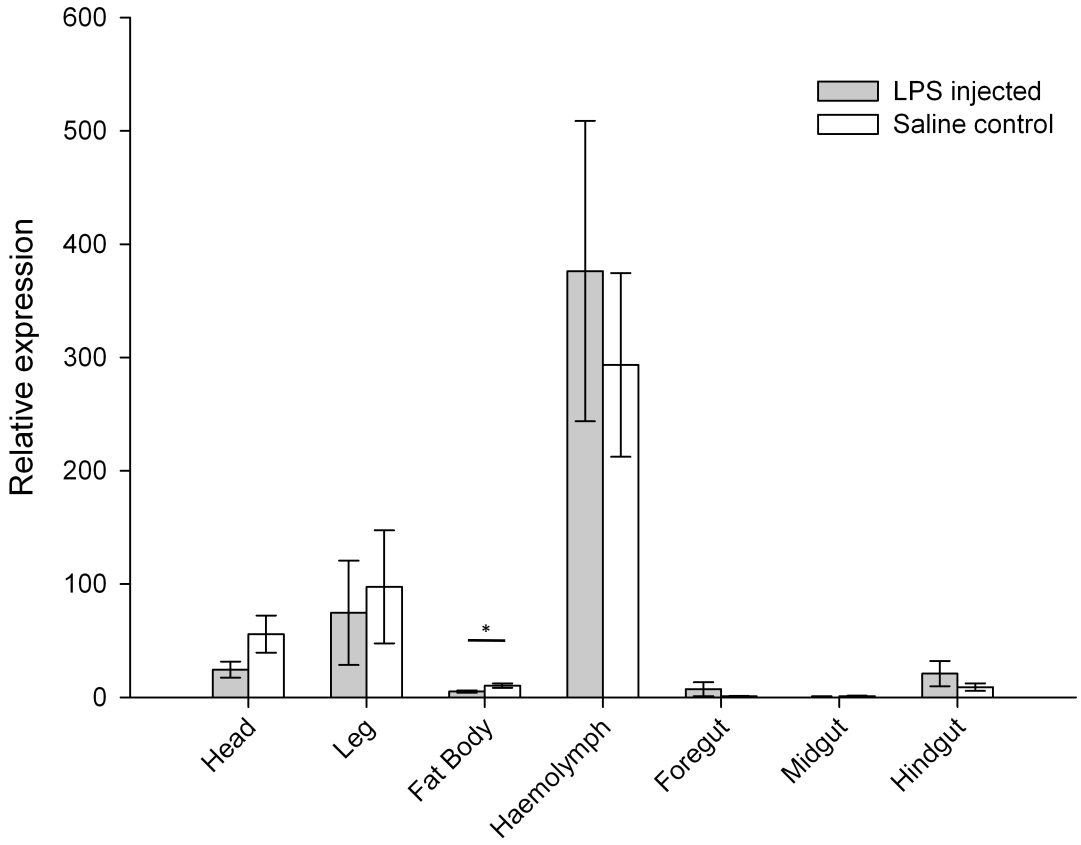

Figure 5.3 Relative expression levels of the gene coding for gram-negative binding protein 1 (GNBP1) across seven tissue types in LPS-injected and saline control *L. migratoria.* Error bars = ±SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Table 5.4 T-tests of mean relative expression levels of the prophenoloxidase gene (ProPO) between LPS-injected and saline control treatments in seven tissues of *L. migratoria*. Statistically significant differences between treatments are shown in bold ($p<0.05$). Values in parentheses indicate treatment sample sizes (n).

Tissue type	Relative expression		t	d.f	P-value
	Saline control	LPS injected			
Head	72.10 (10)	68.27 (9)	0.15	17	0.8811
Leg	90.86 (10)	85.05 (10)	0.23	18	0.8197
Fat body	15.32 (10)	22.04 (10)	1.29	18	0.2126
Haemolymph	76.17 (8)	89.45 (8)	0.45	14	0.6608
Foregut	44.30 (10)	133.71 (10)	3.48	18	**0.0027**
Midgut	1.26 (7)	2.89 (9)	2.75	14	**0.0157**
Hindgut	18.90 (8)	36.79 (10)	2.95	16	**0.0095**

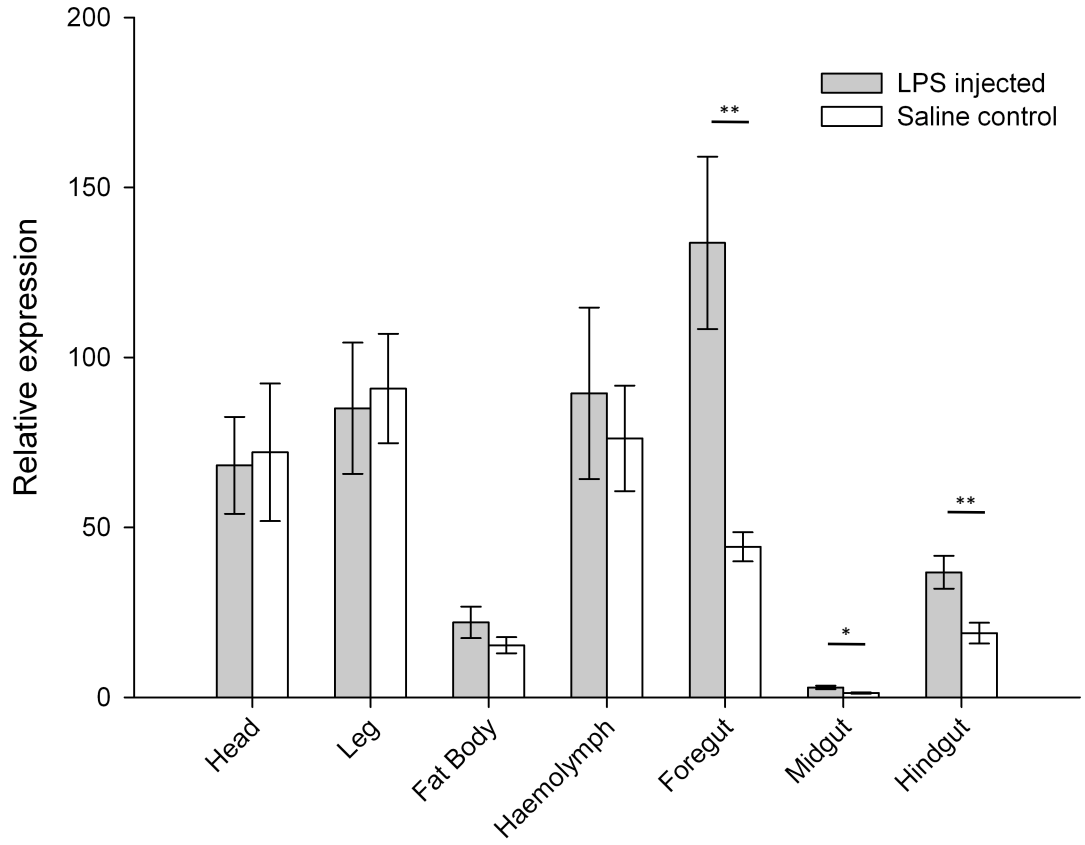

Figure 5.4 Prophenoloxidase (ProPO) gene expression levels for saline control and LPS-injected *L. migratoria* for seven tissue types. Error bars = ±SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Expression levels of GNBP1 were significantly different between tissue types for both saline- and LPS-injected insects (Figure 5.3; Friedman test, saline control: $\chi^2_{(6)}=42.696$, $p<0.001$, LPS-injected: $\chi^2_{(6)}=42.381$, $p<0.001$). A similar pattern of variation was found for both treatments with greater expression levels in the haemolymph, head and leg than observed for gut sections and fat body.

Prophenoloxidase expression levels were significantly different between tissue-types for locusts within both inoculation treatments (Figure 5.4; Friedman test, saline control: $\chi^2_{(6)}=40.500$, $p<0.001$, LPS-injected: $\chi^2_{(6)}=43.048$, $p<0.001$). Here, ProPO expression in fat body and midgut body parts was lower than in any of the other tissues.

5.5 Discussion

5.5.1 Tissue-specific immune gene expression

This study has found evidence of tissue-specific immune gene expression in *L. migratoria.* Variation in gene expression between body parts appears to be dependent both on the gene being analysed and the infection status of the host. Relative expression patterns between tissue-types were generally consistent in both LPS- and saline-injected treatments for each gene tested. Haemolymph consistently showed high relative expression levels compared to the other tissues. Gut sections showed moderately low expression of PGRP-SA and GNBP1, particularly within the midgut. Foregut and hindgut however, showed comparatively high ProPO expression relative to the other tissue-types tested.

Interestingly, fat body in both LPS- and saline-injected treatments showed the least consistency in relative immune gene expression across the different tissue-types. Here, PGRP-SA expression was among the highest of all the tissues for both LPS

and control treatment groups. Fat body ProPO expression fell in the middle of the range compared to other tissue types. Expression of GNBP1 in fat body, however, was comparatively low in contrast. Fat body also showed significantly lower GNBP1 expression in LPS injected locusts than saline controls, a counter intuitive response to an immune challenge with a gram-negative pathogen. Haemolymph and head, whilst not statistically significantly, showed a similar trend. Altogether, these results suggest that immune response in fat body may play a more specific role in immune function than perhaps other tissue types might. This is supported by evidence showing that fat body functions as a major source of antimicrobial peptide synthesis within hosts, and shares an important role in energy storage and metabolic function (Boman and Hultmark, 1987; Arrese and Soulages, 2010).

Immune response specificity in this tissue might therefore also be particularly sensitive. In this case, the analysis of just one tissue-type or gene represents an immune reaction to a narrow set of conditions, providing little information of overall host response. The importance of informative tissue and gene selection when performing immune studies is therefore strongly demonstrated in these results, supporting current suggestions of an integrated approach in ecoimmunological studies (Adamo, 2004a).

5.5.2 Immune gene significance

Peptidoglycan recognition protein SA (PGRP-SA)

First reported in *Drosophila*, PGRP-SA has been described as a non-catalytic peptidoglycan recognition protein that activates the Toll pathway in response to the presence of most gram-positive bacteria (Royet and Dziarski, 2007). However, investigations in other model insects (*e.g.,* beetles) have shown that PGRP-SA can also recognise gram-negative peptidoglycans, thereby activating both Toll and Imd

pathways (Yokoi*, et al.*, 2012). Results from this study found that injections of LPS, from the gram-negative bacteria *E. coli,* upregulated PGRP-SA expression in *L. migratoria,* suggesting that locust immune function may share similarities with the coleopteran system.

Wang *et al.,* (2013) have recently measured PGRP-SA expression in the haemocytes, fat body and midgut of solitarious and gregarious *L. migratoria* in both fungal-infected and uninfected treatments. In gregarious locusts, a higher relative expression of PGRP-SA was found in fat body compared to haemocytes, with even lower (nearly undetectable) expression levels in the midgut (Wang*, et al.*, 2013). Results from the current study show a similar trend, with some expression still detectable in midgut tissues despite being comparatively low relative to the other tissues. What's more, the expression level of PGRP-SA in the midgut was much lower than that quantified in the other gut parts (*i.e.,* foregut and hindgut). Previous studies on transcriptome variation in *Drosophila* gut regions have also shown PGRP-SA expression to differ between the different gut compartments (Buchon*, et al.*, 2013). Such results may indicate tissue-specific expression of PGRP-SA within the digestive tract itself.

Gram-negative binding protein 1 (GNBP1)

Gram-negative bacteria binding proteins (a second class of immune recognition protein) were initially purified from the haemolymph of the silkworm *Bombyx mori* (Lee, *et al*., 1996). As their name suggests, these proteins have been found to have a strong affinity for gram-negative bacteria surface molecules. In *Drosophila*, GNBP1 is implicated in responses to gram-positive as well as gram-negative bacterial infection (Pili-Floury*, et al.*, 2004). Here, physical interactions with PGRP-SA and gram-positive peptidoglycans are necessary for downstream Toll signalling (Wang*,*

et al., 2006). In locusts, GNBP1 seems to perform differently. Recent comparisons of GNBP1 expression levels in the fat body of locusts injected with different types of PAMPs found an increase in GNBP1 gene expression in insects injected with laminarin (simulating fungal infection) and peptidoglycan (Wang*, et al.*, 2013). Similar to the results presented here, however, no upregulation of GNBP1 in response to LPS was detected. This study can now extend the same observations to other tissues with no demonstration of GNBP1 upregulation in any of the additional tissue-types examined.

Prophenoloxidase (ProPO)

The ProPO–activating system is an important component in the humoral immune response of both insects and crustaceans (Goldsworthy*, et al.*, 2002; Jang*, et al.*, 2011). Prophenoloxidase is the inactive zymogen of phenoloxidase, an enzyme produced in response to microbial invasion for the catalysis of several oxidation reactions, and primarily for the synthesis of melanin (Ashida and Brey, 1995). Phenoloxidase-associated local melanisation, cuticular sclerotisation, wound healing and pathogen sequestering are all central defence responses of insects (Jang*, et al.*, 2011). These responses, frequently associated with clotting and physical encapsulation of invading parasites, are all triggered by non-self recognition mechanisms (Cerenius and Söderhäll, 2004; Eleftherianos and Revenis, 2010). Phenoloxidases also serve an important role in insect developmental physiology, in particular exoskeleton regeneration during ecdysis (Cerenius and Söderhäll, 2004). Relatively little, however, is known about the molecular mechanisms that regulate the production and activation of its precursor ProPO.

In insects, the regulation of enzymes such as ProPO may be largely controlled by haemocytes (Franssens*, et al.*, 2008). Cerenius and Söderhäll (2004) suggest that

haemocyte-produced ProPO might infiltrate and accumulate in other body parts, therefore playing a large role in ProPO synthesis in many tissues. This could explain why comparable ProPO expression levels were detected across the different tissues assayed in this study. Interestingly, although variable ProPO expression across the seven different body parts was observed, upregulation following LPS-injection only occurred in gut sections. This suggests that while ProPO is constitutively expressed in most of the host's tissues, the gut may be one of the first organs to respond to infection. Since the gut provides a large window of opportunity for pathogen invasion (particularly through diet), this heightened ProPO expression could reflect one of several rapidly occurring primary responses to infection.

5.5.3 Conclusions and future work

This study has shown clear differences in locust immune gene expression across different tissue types as well as in response to an immune challenge with LPS. Interestingly, injection with LPS revealed gut-segments as the only tissues to show upregulation of ProPO expression, revealing a potentially newfound contribution of ProPO as a rapidly responding trait within the locust immune system. The results observed in this gene in particular, emphasise the importance of tissue selection when performing immune assays using RT-qPCR. Under these conditions, assays that tested more commonly used tissues (*e.g.,* fat body and haemolymph) would not have revealed an apparent response in *L. migratoria* between LPS- and saline injected treatments. A more general expression response to LPS was detected in PGRP-SA as expected. Significant increases in PGRP-SA expression across all tissue types indicated that this gene might have a broader capacity to recognise and activate immune pathways in response to invading microbes. GNBP1 showed a change in gene expression in response to LPS injection in one tissue only (*i.e.,* fat body). This change was counter-intuitive, being higher in saline controls than the

LPS challenged treatment. This unexpectedly demonstrated how the pathogen-associated molecular pattern of LPS (from the gram-negative bacteria *E. coli*) was ineffective at inducing the expected response from a gene encoding a gram-negative binding protein (*i.e.,* GNBP1).

From these experiments, responses detected in insect hosts are expected to depend specifically on tissues analysed, type of immune challenge and immune genes or traits quantified. Variation across insect species is also likely to occur, with many studies already revealing differences in immune pathway activation when exposed to similar types of pathogens. The occurrence of tissue-specific immune response adds a new level of complexity to the insect immune system. Changes in relative expression between different tissues, both constitutive and following an immune challenge, clearly demonstrates the prospective consequences of uninformed tissue or gene selection. Such complexities should be taken in particular consideration in future studies, particularly when attempting to gain underlying mechanistic insight in the context of comparative insect physiology.

For *L. migratoria* and other locusts, further studies that utilise these developed techniques will strengthen current understandings of immune function in this species. Of particular interest would be the use of a living gram-negative pathogen under similar experimental circumstances to determine whether the observed decrease in expression was a generalised response or a result of the PAMP selected. Such insights are particularly important in the pursuit for the development of effective and ecologically safe biological controls against this enormously destructive pest.

Chapter 6.

Locusts and their Bacteria

6.1 Summary

The bacterial communities that reside within the gastrointestinal tracts of animals are fast being recognised as an important element for the maintenance of immune function and overall fitness in hosts. Recent studies have found that, although locusts contain relatively few phylotypes in comparison to other species, the diversity of their gut bacteria greatly affects their capacity to withstand infection through colonisation resistance. This study investigated how the introduction of gut bacteria, isolated fortuitously from field specimens of *Chortoicetes terminifera* during a mass disease outbreak, influenced the constitutive immune function of the migratory locust, *Locusta migratoria*. Using a combination of conventional and molecular techniques, common trends in immune gene expression and lysozyme concentration were found, indicating that the presence of introduced gut bacteria is likely to have influenced locust immunity. Tissue specificity and assay selection are discussed as important considerations for such experiments, particularly in instances where differences between immune trait responses are observed. The implications of these results, as a starting point for future studies, suggest that commensal bacteria have more influence upon host immunity than perhaps previously thought. For locusts, a pest species of worldwide economic and agricultural concern, understanding the importance of host-microbe interactions is critical for the identification and development of integrated biological controls. In an investigation that began as a search for an unknown, yet prospective locust pathogen, this work is a reminder that any study can reveal unexpected, yet fundamental insight into ecological systems.

6.2 Introduction

6.2.1 Host infection outbreaks

Infection outbreaks in a population can reveal great insight into how immune function and defence responses occur within hosts. Such epidemics often occur unexpectedly, triggered by opportunistic pathogens under particular combinations of environmental conditions. The timeliness and extent of host response depends also on their environment, adaptive fitness and infection history (Schmid-Hempel, 2011).

Understanding the dynamics of disease epidemics or epizootics is of great importance, especially within pest species. Consequently, clues to pathogen infection mechanisms and how they overcome host defences are of great interest for the development of new pathogenic agents for biological control.

Plague in a plague: field locusts die in migratory band epizootic

In 2010, during a field trip to central New South Wales, individuals within several migratory bands of the Australian plague locust (*Chortoicetes terminifera*) were observed to be dying en masse from what appeared to be pathogen mediated. Post-death symptoms of locusts included dark brown to red coloration, a strong odour and soft, moist cadavers that decomposed rapidly. Such symptoms are commonly associated with bacterial infection (Zelazny*, et al.*, 1997). Since locusts are a primary cause for enormous agricultural and economic devastation worldwide, it was of particular interest to attempt to identify the exact cause of the observed disease outbreak.

Pyrosequencing data of 16S rDNA sequences, from collected field cadavers, revealed a high abundance of one particular bacterial genus, *Pseudomonas*. This comprised approximately 70-80% of the DNA in the gut samples analysed within

each migratory band (Table 6.1). As a prominent and well-studied bacterial genus, *Pseudomonas* can be found nearly everywhere within the environment (Lyczak*, et al.*, 2000). Some phylotypes are also known to be opportunistically pathogenic. One species, *Pseudomonas aeruginosa,* for example, is a known nosocomial pathogen and one of the top causes of secondary infection in humans whose immune systems have already been compromised (Rello*, et al.*, 2013). Unfortunately, uninfected conspecifics from the same migratory bands were unobtainable for comparison, since all individuals appeared infected. Consequently, it could not be conclusively determined whether *Pseudomonas* was the pathogen of interest, in light of its high abundance. Furthermore, pyrosequencing data also identified other known insect pathogens (*e.g., Bacillus* and *Serratia* sp.), thereby presenting other potential candidates, despite their lower abundance.

Attempts to culture bacteria from previously frozen field cadavers found no recoverable colonies from the *Pseudomonas* genus. Since the field epizootic occurred unexpectedly, ideal storage conditions for the preservation of viable colonies were not on hand and may account for why more phylotypes were not recoverable from these samples. A number of common acridid gut bacteria, however, were cultured including *Enterobacter*, *Pantoea* and *Citrobacter*. A potential pathogen, *Bacillus*, was also isolated. Each of these had previously been identified in the pyrosequencing results, spanning a range of relative abundances (Table 6.1). It was with some of these recovered gut microbiota that the following experiment was conceptualised.

6.2.2 Microbial communities in the gut

Understanding the interactions between hosts, their pathogens and other commensal microbes provides important insights into an organism's physiology, metabolism,

Table 6.1 Relative bacterial abundances of infected locusts (*C. terminifera*) collected from two migratory bands determined by 16S rDNA pyrosequencing of locust cadavers. Locusts collected had died of an unknown infection in the field. Bacterial abundance is shown as a percentage of the total bacteria present. The first twenty most abundant phylotypes are shown.

Migratory band 1		**Migratory band 2**	
Bacteria	**% Abundance**	**Bacteria**	**% Abundance**
Pseudomonas veronii	48.901	*Pseudomonas veronii*	41.185
Pseudomonas sp	34.777	*Pseudomonas* sp	33.975
Achromobacter sp	4.960	*Stenotrophomonas* sp	7.822
Stenotrophomonas sp	4.776	*Enterobacter* sp	4.094
Delftia sp	2.226	*Achromobacter* sp	3.391
Pseudomonas fluorescens	1.284	*Serratia* sp	2.444
Enterococcus sp	0.600	*Citrobacter* sp	1.436
Pseudomonas fragi	0.231	*Delftia* sp	1.283
Pseudomonadales sp	0.222	*Delftia acidovorans*	0.519
Delftia acidovorans	0.166	*Pseudomonas fragi*	0.428
Rhodococcus sp	0.166	*Salinivibrio* sp	0.397
Alcaligenes sp	0.157	*Pantoea* sp	0.397
Allobaculum stercoricanis	0.139	*Rhodococcus* sp	0.367
Bacillus sp	0.111	*Pseudomonas fluorescens*	0.336
Pseudomonas antarctica	0.083	*Stenotrophomonas maltophilia*	0.244
Enterobacter sp	0.074	*Pseudomonadales* sp	0.214
Escherichia sp	0.074	*Pseudomonas antarctica*	0.122
Serratia sp	0.065	*Moorella* sp	0.122
Collimonas sp	0.065	*Pseudomonas libanensis*	0.092
Alcaligenaceae sp	0.065	*Klebsiella* sp	0.092

nutrition, behaviour and immune function (Schulenburg*, et al.*, 2009). Consequently, the field of ecological immunology has seen a recent shift in focus from studies that look solely at the environmental impacts on host immunity to the integration of an organism's internal and external ecosystems (O'Hara and Shanahan, 2006).

With most animals under constant exposure to microbes, it is not surprising that this co-occurrence has resulted in the evolution of numerous symbiotic relationships (Pancer and Cooper, 2006; Lee and Mazmanian, 2010; McFall-Ngai*, et al.*, 2013). With a fast reproduction rate, and capacity to rapidly adapt to a range of environments, bacteria have been able to successfully occupy almost every existing ecological niche on the planet (Lee and Mazmanian, 2010). The gastrointestinal tracts (GITs) of most animals are no exception, with a stable, nutrient-rich environment making this organ ideal for microbe colonisation (Lee and Mazmanian, 2010; Hooper*, et al.*, 2012).

The benefits of host-microbe symbiosis

Investments in maintaining microbial communities within the GIT confer many benefits to both host and microbe. The gut provides constant growth conditions and nutritional supply for microbes (Lee and Mazmanian, 2010; Hooper*, et al.*, 2012). In return, commensal bacteria assist in the breakdown of complex carbohydrates and provide essential nutrients to hosts, thereby enhancing digestive efficiencies (Dillon and Charnley, 2002; Lee and Mazmanian, 2010; Hooper*, et al.*, 2012). Microbial contributions to host angiogenesis and fat storage have also been described (Stecher and Hardt, 2008).

Gut microbiota also play a large role in host immune function, producing anti-inflammatory and antimicrobial compounds, thereby improving colonisation resistance against competing or pathogenic bacteria (Dillon and Charnley, 2002;

Stecher and Hardt, 2008; Cerf-Bensussan and Gaboriau-Routhiau, 2010). Germ-free animals (produced using sterile rearing techniques, antibiotics or irradiation) have been shown to suffer substantial disadvantages from a microbe-free gut compared to microbe-harbouring conspecifics. Decreases in longevity, vascularity, digestive enzyme activity, muscle wall thickness and several other physiological effects have all been described in hosts that lack enteric microbes (O'Hara and Shanahan, 2006).

Gut microbiota have been found to influence the tissues, cells and molecular profile of a host's gastrointestinal immune system (Lee and Mazmanian, 2010). With colonisation and regulation of gut microbiota occurring throughout the lifetime of an animal, the importance of gut bacteria and its role in host immune function is becoming increasingly recognised (Cerf-Bensussan and Gaboriau-Routhiau, 2010).

6.2.3 Insect gut microbiota

The evolutionary success of insects, alongside the opportunistic (and often unique) bacteria that occupy them, provides an ideal model system in which to study the relationship between commensal and host symbionts. As with most organisms, insects are in perpetual contact with microorganisms, often in a range of variable environments. Consequently, many insects carry highly dense and diverse communities of microorganisms that often exceed the total cell number of hosts themselves (Dillon and Dillon, 2004).

As with most animals, the introduction of new microbes to the insect gut occurs primarily through diet, where nutrition has a substantial impact on its microbial populations and vice versa (Douglas, 2009). Introduction of new foods to the GIT can also introduce new species, enabling the colonisation of a greater diversity of microbes (Maslowski and Mackay, 2011).

Density versus diversity of gut microbiota in locusts

For locusts, environmental variation has enormous implications for food availability. Locusts in the field are frequently found in a state of nutrient deprivation, causing juveniles to march in search of new resources (see Chapter 1). During such states, the nutritional priorities of individuals may lead to the redistribution of resources toward physiology, metabolism and immune function (Simpson and Raubenheimer, 2000). Changes in gut microbe density and diversity have also been shown to respond to starvation in locusts, where reduced peristalsis and food passage is thought enable increased bacterial colonisation (Dillon*, et al.*, 2010).

Previous work has found that the diversity of resident commensal bacteria, not solely their density, is critical in the regulation of host immune function (Dillon and Dillon, 2004). In the desert locust (*Schistocerca gregaria*), diverse microbial communities within the gut have been shown to prevent invasion and colonisation of the pathogenic bacteria, *Serratia marcescens* (Dillon*, et al.*, 2005). As commensal diversity increased, so too did colonisation resistance to *S. marcescens*. Interestingly, however, compared to the enteric diversity in other organisms, locust gut microbial communities are considered to be relatively simple (Dillon*, et al.*, 2008).

6.2.4 Aims

The original aim of this chapter was to identify and isolate the pathogen responsible for the mass deaths observed in field *C. terminifera*. Unforseen circumstances, however, meant that this was not possible. Consequently, this study made use of the gut bacteria that were able to be cultured from these insects.

The primary aim of this chapter was therefore to identify how the introduction of gut bacteria influences the constitutive immune function of the migratory locust, *Locusta*

migratoria. As this was performed without *a priori* knowledge of the resident enteric communities found within these insects, these experiments served primarily as a pilot study.

Gene expression assays (developed in Chapter 5) were used to quantitatively compare expression levels of three immune genes with results from three conventional immunological assays used previously in this thesis (Chapters 2 and 4). This aimed to describe how different techniques and trait measurements demonstrate variation in the perceived immune status of hosts. This also enabled the differences between immune gene and tissue type selection to be described.

6.3 Materials and methods

6.3.1 Field locust bacteria isolation, purification and identification

Gut bacteria were isolated from collected field specimens of *C. terminifera*. Migratory bands were located near Hillston, New South Wales (33° 29.118' S, 145° 31.968' E) in the summer of 2010. Insect cadavers collected were in their 5th stadium and frozen at -20°C until long-term storage at -80°C was obtained.

Thawed locusts were dissected aseptically by cutting ventrally along the abdomen and removing the entire GIT. Gut and remaining cadavers of each locust were plated separately on sterile lysogeny broth (LB) agar and incubated for 3 d at 30°C. Several spot samples were taken from various locations from the confluent growth on each plate and spread aseptically onto fresh LB agar. These were again incubated to obtain single colonies. Forty colonies were randomly selected and subcultured by streaking and incubating once more to ensure pure cultures. Fresh colonies were then aseptically selected with a sterile toothpick and incubated overnight at 30°C in 5 mL of liquid LB media.

Genomic DNA extraction

Fresh liquid cultures (2 mL) were centrifuged to obtain a pellet and the supernatant discarded. Bacterial genomic DNA was extracted from remaining pellets using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. DNA concentration and purity were quantified using nano-drop spectrophotometry.

Bacterial PCR and sequencing

Genomic DNA extractions, from purified bacterial cultures, were diluted 10-fold using sterile Milli-Q water and used as templates for PCR amplification. Universal forward and reverse 16S rDNA primers, GM3 (5'-AGAGTTTGATCMTGGC-3') and GM4 (5'-TACCTTGTTACGACTT-3'), were used for PCR amplification (Muyzer*, et al.*, 1995). These primer sequences corresponded to positions 8-23 and 1492-1477 bp on *Escherichia coli* respectively (Brosius*, et al.*, 1981).

PCRs were carried out in a total volume of 50 μL, consisting of 1.25 U Taq polymerase (Qiagen), 1x Qiagen buffer (containing 0.5 mM $MgCl_2$), 0.4 μM of each primer and 0.2 mM of each dNTP. Thermal cycling conditions used an initial denaturing step of 10 min at 94°C, followed by 25 cycles of denaturing (94°C, 1 min), annealing (58°C, 1 min) and extension (72°C, 1 min). A final extension step of 72°C for 5 min completed the amplification process.

Unpurified PCR products were sequenced by Macrogen Inc. (Korea) and the results obtained were compared to the GenBank database using NCBI BlastN searches (Altschul*, et al.*, 1997). Sequences obtained ranged between 400 and 650 bp, from which 100% identity was matched to four genera: *Enterobacter, Pantoea, Citrobacter* and *Bacillus*. Of these, *Enterobacter* and *Pantoea* were selected for gut

diversity experiments, as they are known to exist in the gut of several acridid species including *L. migratoria* (Dillon*, et al.*, 2008).

6.3.2 Determination of bacterial doses

In order to calculate bacterial dosage, growth curves and colony enumeration of freshly cultured bacteria were performed. Most studies that utilise living bacteria for inoculation treatments typically use cultures within the log phase of growth. This is when bacteria are growing exponentially in the presence of excess nutrients and minimal inhibitory compounds or waste (Monod, 1949).

Bacterial growth assays

Growth curves were performed by measuring the change in optical density in 50 mL of bacterial culture (diluted 1000-fold and incubated in liquid LB media at 30˚C). Spectrophotometry measurements were taken hourly at 600 nm until readings reached a plateau, indicating the stationary phase of the culture (where all available nutrients have been exhausted). Growth progress was compared against an uninoculated volume of LB media to ensure no contamination throughout the growth time-course. Growth curves for *Enterobacter* and *Pantoea* are shown in Figure 6.1.

Colony counts: concentration determination

During the exponential growth phase (determined to be approximately OD_{600} = 0.26 and 0.22 for *Enterobacter* and *Pantoea* respectively), bacterial cultures were serially diluted to a concentration range of 10^{-1} to 10^{-9}. Aliquots of each dilution were spread evenly on triplicate plates of LB media and incubated for 2 d at 30˚C. Plates were photographed digitally to enable accurate colony enumeration using the program ImageJ (Schneider*, et al.*, 2012). For statistical accuracy, only plates with 30-300 colony-forming units (CFU) were used for calculations. Bacterial concentrations

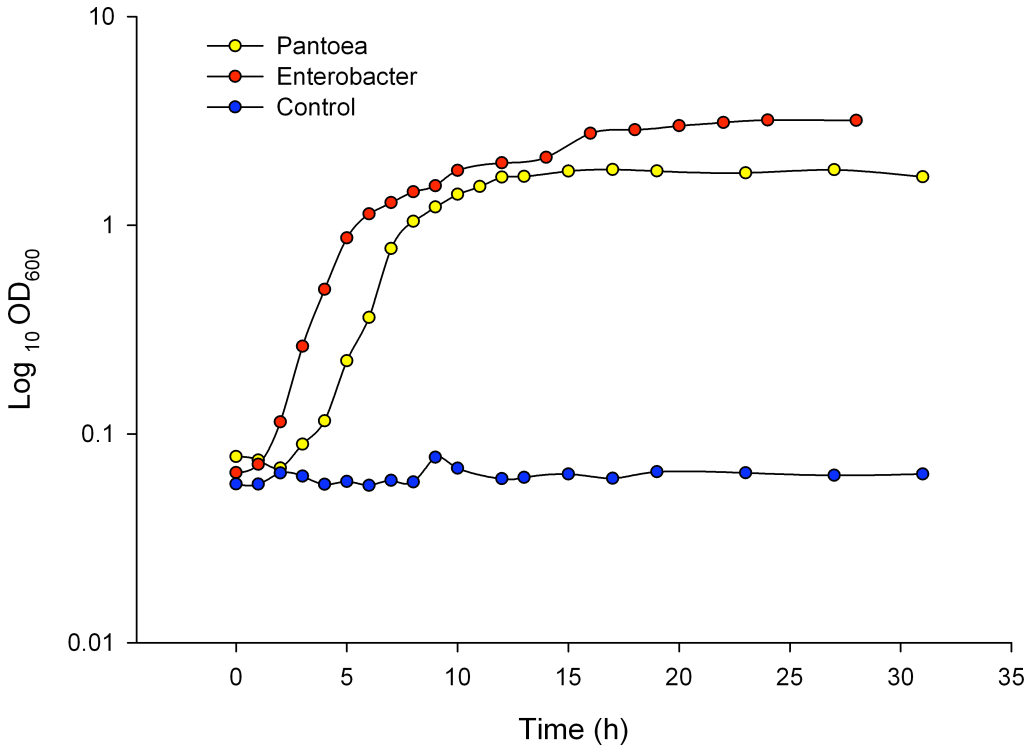

Figure 6.1 Growth curves of two isolated gut bacteria from field-collected cadavers of *C. terminifera*. Optical density at 600nm was measured at regular intervals in liquid cultures containing *Enterobacter* (red) and *Pantoea* (yellow) until reaching stationary growth. A bacteria-free control (blue) verified no contamination. The y-axis is shown with a $\log_{10}$ scale.

were determined as $28x10^4$ and $26x10^4$ CFU/μL for *Enterobacter* and *Pantoea* respectively.

6.3.3 Experimental insects and gut bacteria treatments

Locusts (*L. migratoria*) were obtained from a long-term laboratory culture at the University of Sydney as described earlier (Chapter 5). Newly moulted adults were reared to 7 days maturity under gregarious conditions and fed *ad libitum* on fresh wheat grass and wheat germ. Since *L. migratoria* were used in the development of gene expression assays (Chapter 5), the same species was adopted for current experiments.

Pure cultures of bacteria were set up in the same way as described above and brought to a log growth phase of OD_{600} = 0.26 (*Enterobacter*) and 0.22 (*Pantoea*). At these known concentrations, bacteria cultures were diluted to make three inoculation solutions: i.) *Enterobacter*, ii.) *Pantoea*, and iii.) an equal mixture of the two. All solutions contained the same total cell concentration ($1.2x10^6$ CFU/μL).

6.3.4 Gut bacteria inoculation

Locusts were deprived of food and water for 6 h prior to inoculation. Individuals were force-fed with one of the three bacteria inoculation treatments (outlined above) by pipetting 50 μL ($6x10^7$ CFU) into their oral cavity using a micropipette. This dose followed that used in previous experiments (Dillon*, et al.*, 2005). A control treatment group was inoculated with sterile LB media only. Locusts that regurgitated their stomach contents during or immediately following force-feeding were discarded resulting in a final sample size of n=10 for each treatment group.

Following inoculations, locusts were transferred to individual cages and maintained without food or water for 20 h to allow gut bacteria the opportunity to establish

within the GIT. After this period, insects were fed with fresh wheat grass and wheat germ *ad libitum* for another 20 h prior to sample collection.

6.3.5 Haemolymph and tissue sampling

Locusts were bled and dissected 40 h post inoculation. Haemolymph was obtained by piercing the arthrodial membrane of the left hind leg between the coxa and metathorax using a sterile needle. The maximum volume of obtainable expelled haemolymph per locust was transferred to microcentrifuge tubes using a micropipette and immediately frozen at -80˚C. Locusts were then dissected to obtain the fat body and midgut section of the GIT, two tissues previously shown to have specific and different levels of constitutive and responsive immune gene expression (Chapter 5). Midguts were obtained by sectioning the gut at the gastric caeca and malpighian tubules. They were dissected open, contents removed and washed in phosphate buffered saline (PBS). After dissection, tissues were immediately placed in RNA*later*® (Life Technologies) and held at 4˚C overnight before long-term storage at -80˚C.

6.3.6 Immune function and gene expression assays

Locust immune gene expression was measured in fat body and midgut tissues from all gut bacteria treatment groups using reverse transcription quantitative polymerase chain reaction (RT qPCR) assays. Immune genes analysed included one coding for the prophenoloxidase (ProPO) enzyme, a gram-negative binding protein (GNBP1) and a peptidoglycan recognition protein (PGRP-SA). Primers, reference genes and protocols (described in Chapter 5) were developed either independently or adapted from previous studies (Chapuis*, et al.*, 2011; Ponton*, et al.*, 2011b). Methods used conformed to the suggested guidelines for minimum information for publication of quantitative real-time PCR experiments (MIQE) (Bustin*, et al.*, 2009).

In addition, two conventional immune parameters; lysozyme activity and ProPO activity, and on host condition assay; protein concentration, were measured in locust haemolymph to identify trait variation in response to changes in gut bacterial diversity. Detailed protocols for each assay are outlined in Chapter 2.

6.3.7 Data analysis

Gene expression assays

Immune gene expression was analysed by normalising target genes against the geometric mean of two reference genes, Actin and Elongation factor 1a (EF1a) (Vandesompele, et al., 2002). Expression levels were taken as the number of amplification cycles required to reach the exponential phase of the RT qPCR reaction; termed the quantification cycle value (Cq) (Walker, 2002). Reaction efficiencies were used to transform Cq values using a standard curve (Vandesompele*, et al.*, 2002). Normalised expression levels were compared between treatment groups with two-way ANOVAs using the statistical software SPSS (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY). Since no significant interactions between tissue type and gut microbiota treatments were observed for any of the genes analysed (*i.e.,* $p>0.05$), the analyses were repeated with the interaction terms removed (Engqvist, 2005).

Conventional immunological assays

All data for haemolymph ProPO activity, lysozyme and protein concentrations were analysed with a one-way ANOVA using the statistical software SPSS (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY).

6.4 Results

6.4.1 Immune gene expression

Prophenoloxidase gene expression levels were significantly higher in fat body than midgut tissue types (Figure 6.2; two-way ANOVA, $F_{(1,79)}$=136.695, p<0.001). No significant difference, however, was found between gut bacteria inoculation treatments (two-way ANOVA, $F_{(3,79)}$=1.402 p=0.249). Despite this, an increasing trend in ProPO expression was apparent in bacteria inoculated treatments in fat body tissues compared to uninoculated controls (Figure 6.2a). Midgut tissues indicated a possible upregulation of ProPO expression in *Enterobacter* inoculated locusts only, however, a large variation between samples meant that this result was not significant (Figure 6.2b). There was no significant interaction between inoculation treatment and tissue type for ProPO expression.

Fat body showed significantly higher PGRP-SA expression than midgut tissues across all inoculation treatments (two-way ANOVA, $F_{(1,79)}$=48.319, p<0.001). Both tissue types showed a similar trend of slight PGRP-SA downregulation within both inoculation treatments that used *Enterobacter* (Figures 6.3a and 6.3b). Once again, however, large sample variation meant that these differences between inoculation treatments were not statistically significant (two-way ANOVA: $F_{(3,79)}$=1.406, p=0.248). Inoculation treatment and tissue type did not show a significant interaction.

Expression levels for GNBP1 were significantly higher in fat body than for midgut tissue types (two-way ANOVA, $F_{(1,79)}$=75.629, p<0.001). Similar to ProPO results, fat body tended to show increased expression of GNBP1 in locusts that were inoculated with gut bacteria compared to uninoculated controls (Figure 6.4a). Midgut again showed GNBP1 upregulation in locusts inoculated with *Enterobacter* only

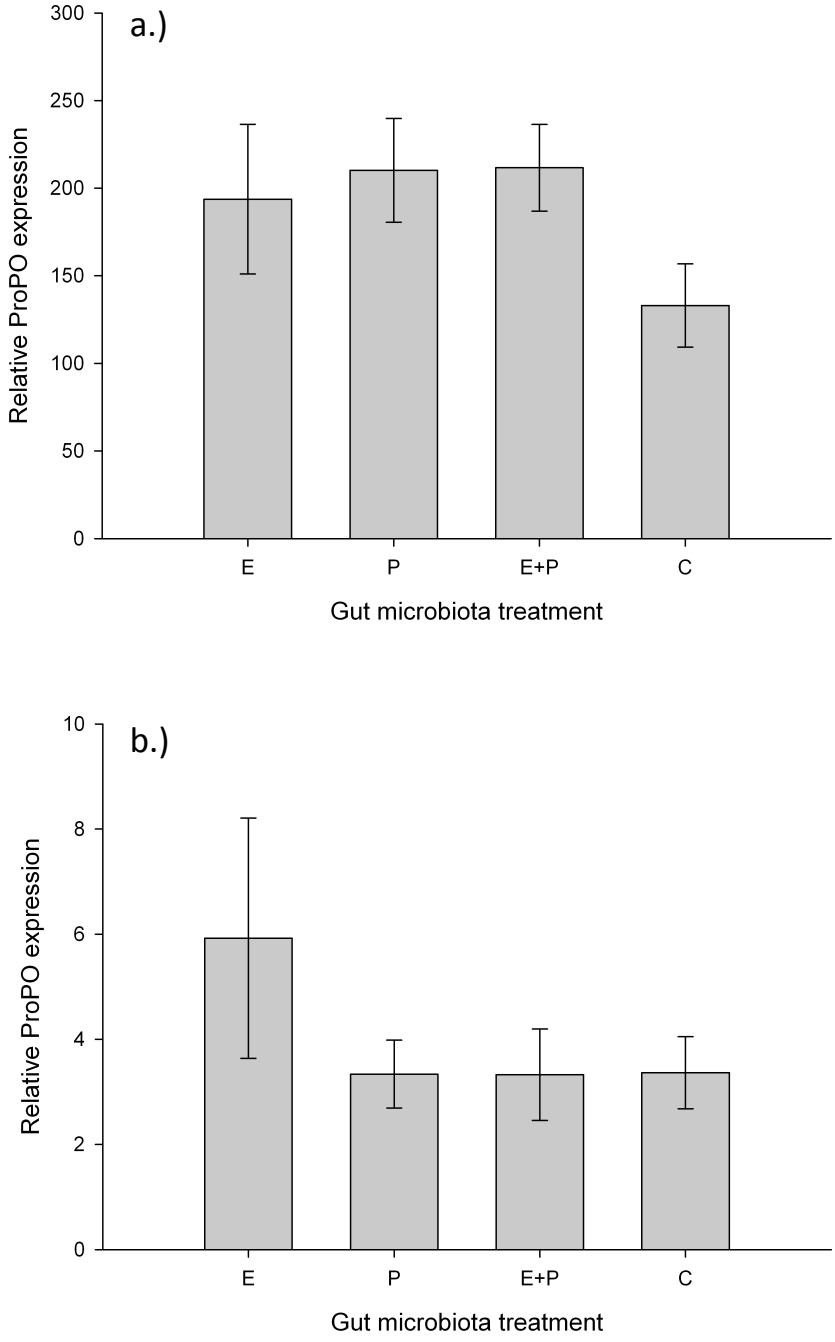

Figure 6.2 Prophenoloxidase gene expression in the a.) fat body and b.) midgut of *L. migratoria.* Individuals were fed with one of three gut bacteria inoculation treatments: *Enterobacter* (E), *Pantoea* (P), or a combination of the two (E+P). Total concentrations of bacteria were constant for each of the three treatment groups. Relative expression levels were compared to a no bacteria control treatment (C). Error bars represent ± SEM.

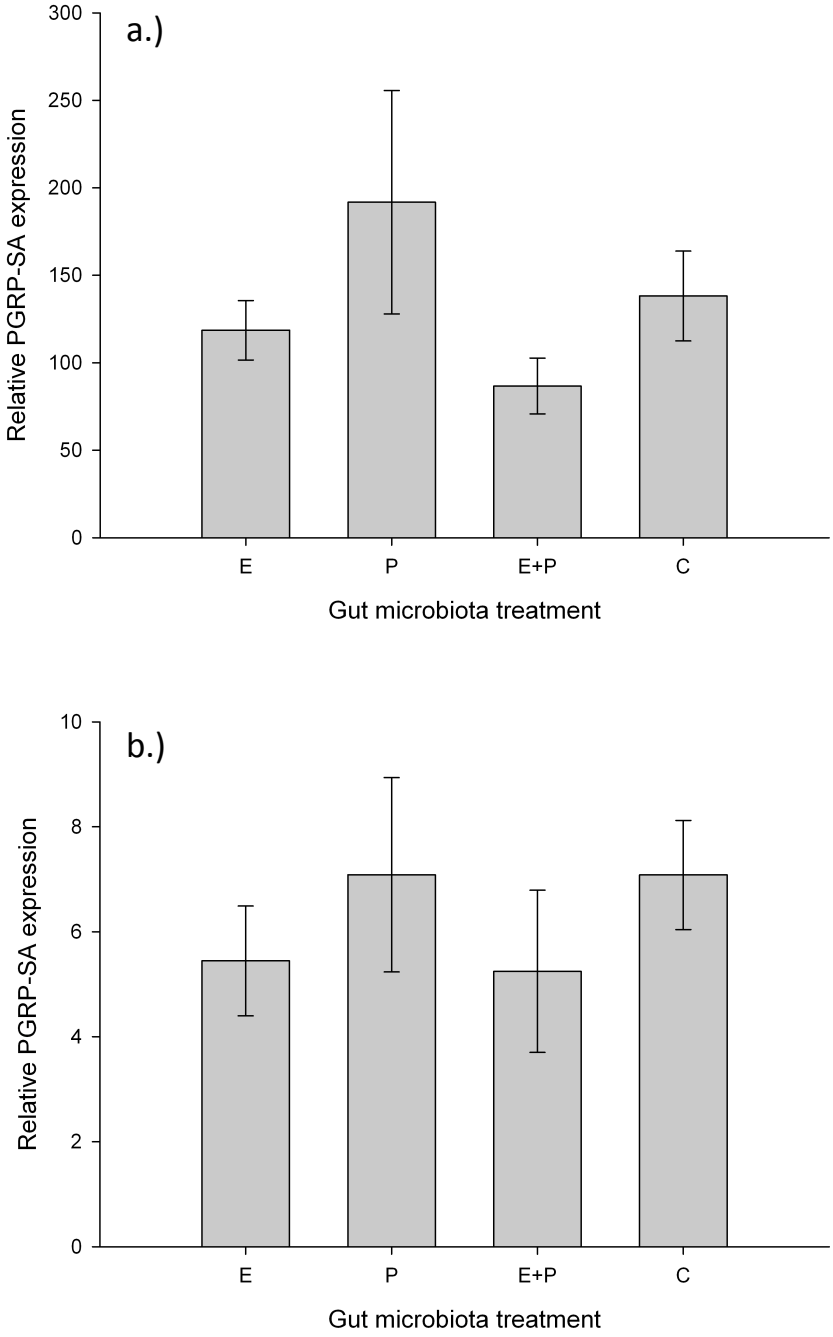

Figure 6.3 Relative expression levels of the gene encoding a peptidoglycan recognition protein (PGRP-SA) in a.) fat body and b.) midgut of *L. migratoria.* Individuals were fed either with *Enterobacter* (E), *Pantoea* (P) or a combination of the two (E+P). Bacteria concentrations were constant for each of the three treatment groups. Relative expression levels were compared with a no bacteria control treatment (C). Error bars represent ± SEM.

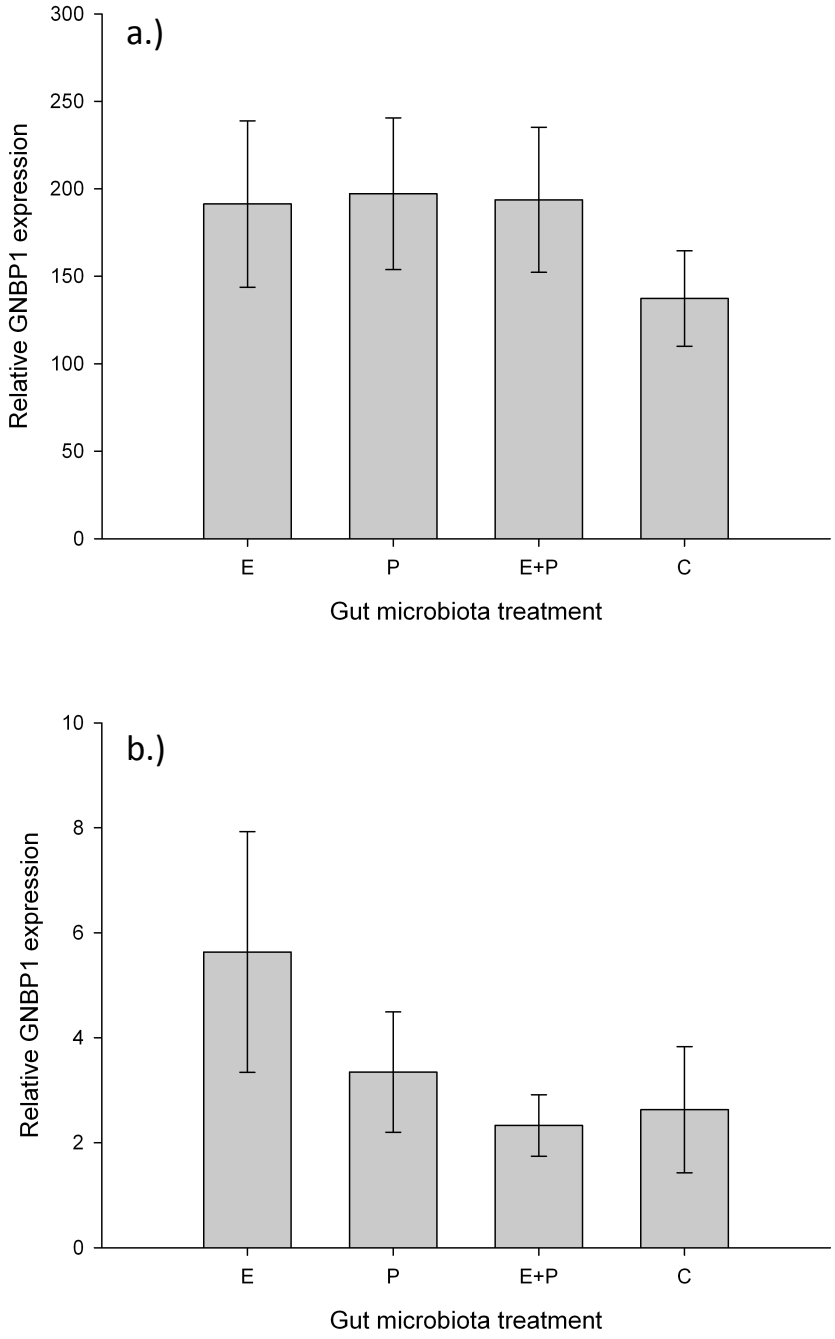

Figure 6.4 Gram-negative binding protein 1 (GNBP1) expression levels in two tissue types of *L. migratoria;* a.) fat body, and b.) midgut. Locusts were inoculated either with *Enterobacter* (E), *Pantoea* (P) or a combination of both bacteria (E+P). Total bacteria concentrations were constant in each treatment group. A no bacteria treatment (C) was used as a comparative control. Error bars represent ± SEM.

(Figure 6.4b). As above, the lack of statistically significant results between inoculation treatments may be due to the wide variation observed across samples (two-way ANOVA, $F_{(3,79)}$=0.511, p=0.676). GNBP1 showed no significant interaction between inoculation treatment and tissue type.

6.4.2 Conventional immune assays

Inoculation treatments had no significant effect on ProPO activity (Figure 6.5; one-way ANOVA, $F_{(3,40)}$=0.498, p=0.686). Haemolymph protein concentration was also not significantly different across treatments indicating no variation in individual protein reserves (Figure 6.6; one-way ANOVA, $F_{(3,40)}$=1.590, p=0.209). Lysozyme activity appeared to increase in bacteria inoculated treatments compared to uninoculated controls, however, this pattern was not statistically significant (Figure 6.7; one-way ANOVA, $F_{(3,40)}$=1.202, p=0.323).

6.5 Discussion

Although the initial enteric composition of *L. migratoria* was unknown, the introduction of gut bacteria to the GIT of *L. migratoria* in this study still may influence their immune function. Though not statistically significant, both ProPO and GNBP1 expression levels in the fat body appeared slightly higher in locusts fed with additional gut microbiota compared to uninoculated controls. The same pattern was mirrored in conventional lysozyme assay results.

Both *Enterobacter* and *Pantoea* are part of the group Enterobacteriaceae, a large family of gram-negative bacteria, which may account for an upregulation response of GNBP1 (a gene encoding gram-negative binding protein 1) within the fat body. Activation of ProPO cascades in *L. migratoria* and *S. gregaria,* by the lipopolysaccharides of gram-negative bacteria, have also been previously

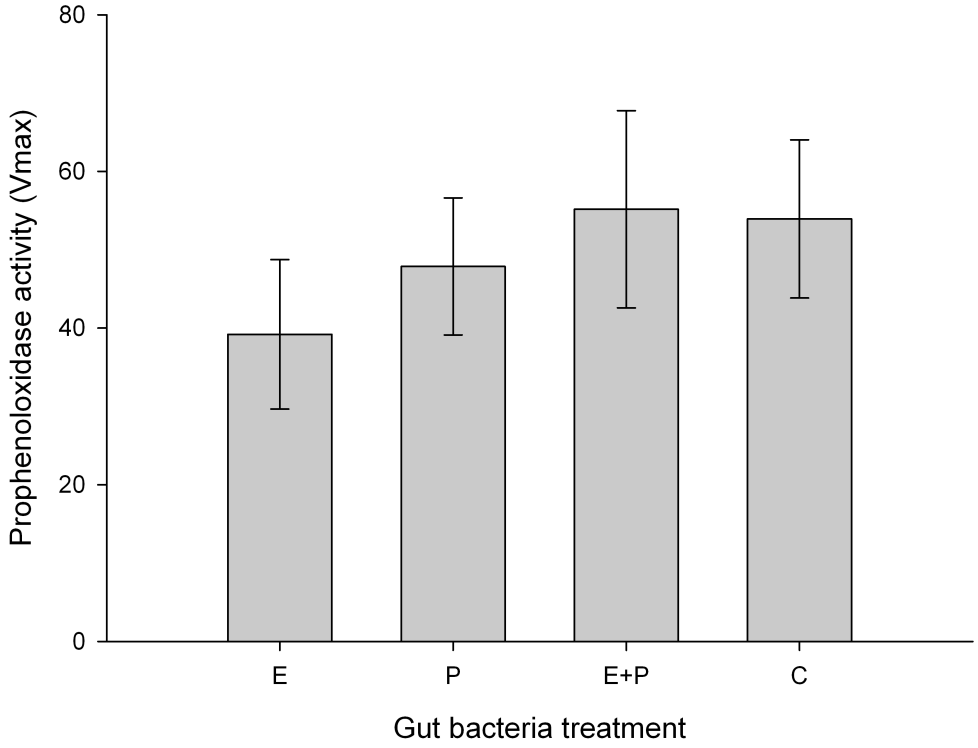

Figure 6.5 Prophenoloxidase activity of *L. migratoria* haemolymph inoculated with one of three gut bacteria feeding treatments or media only controls; E=*Enterobacter*, P=*Pantoea*, C=Control (no bacteria). Equivalent bacteria concentrations were used for all microbiota treatments. Error bars represent ± SEM.

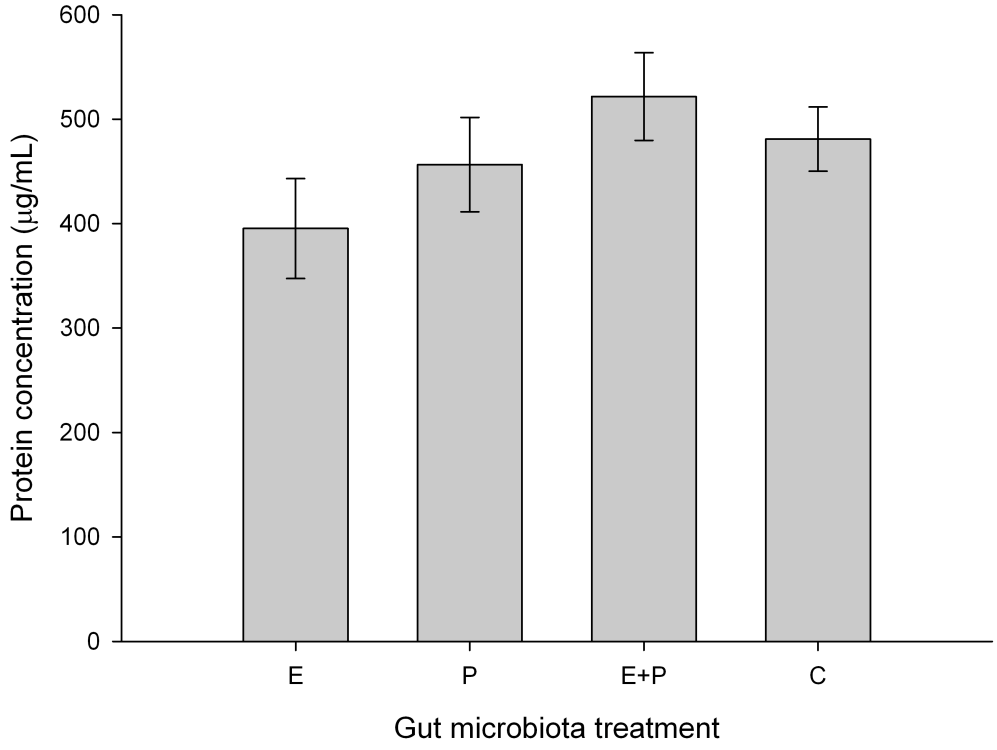

Figure 6.6 Haemolymph protein concentration of *L. migratoria* fed with three different bacteria feeding treatments: *Enterobacter* (E), *Pantoea* (P), or a combination of the two (E+P). No bacteria controls (C) were inoculated with sterile culture media only. Individuals received the same concentration of gut bacteria within all treatments. Error bars represent ± SEM.

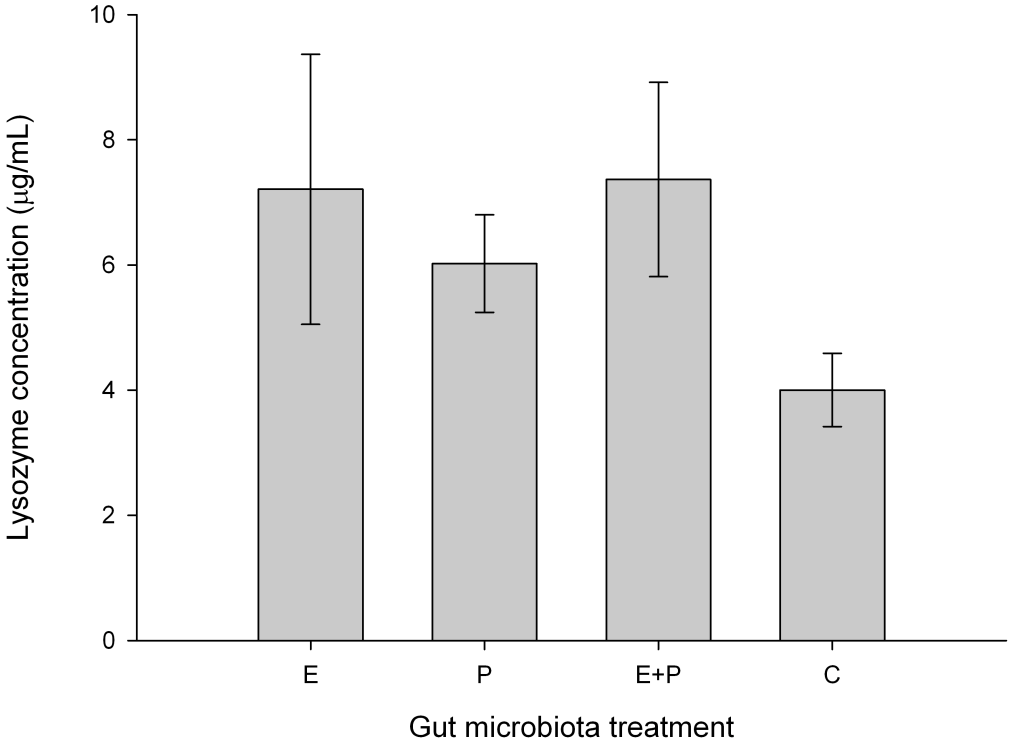

Figure 6.7 Lysozyme activity of *L. migratoria* fed with different gut bacteria inoculation treatments: E=*Enterobacter*, P=*Pantoea*, C= no bacteria control. Total bacterial concentrations were constant across all treatment groups. Error bars represent ± SEM.

demonstrated (Ratcliffe*, et al.*, 1992). This may explain the similar increasing pattern of ProPO expression observed in fat body in this study. Interestingly, midgut tissues showed heightened (though again non-significant) expression of both GNBP1 and ProPO in *Enterobacter* inoculated treatments only. This may reveal differential immune gene expression across tissue types, suggesting a more localised response of the midgut to the addition of *Enterobacter* compared to the broad response demonstrated by fat body to both bacteria. For PGRP-SA, heightened but non-significant expression levels were consistent in *Pantoea* inoculated treatments across both tissue types. This suggests that *Pantoea* may have a greater influence on this gene alone than seen in the other inoculation treatments.

These initial results suggest that the introduction of additional gut bacteria may indeed influence locust immune function. Whether the increasing trends seen in the current study are indicative of potential changes in constitutive immune function, or are merely a response to the introduction of new phylotypes, will require further investigation using pathogen resistance assays and verification of successful inoculation. Previous studies on *S. gregaria* have demonstrated an increase in colonisation resistance against the pathogen *S. marcescens,* as a result of increased enteric diversity (Dillon*, et al.*, 2005). Understanding precisely how similar gut bacteria contribute to *L. migratoria* immune function requires further detailed investigations.

6.5.1 Experimental considerations

This is the first known study to compare how molecular and conventional approaches reveal the effects of gut bacteria introduction on locust immunity. Consequently, this initial work has identified several experimental components that may facilitate future studies. Both *Enterobacter* and *Pantoea* have previously been reported to occur

naturally in the gut of several wild acridid species including *L. migratoria* (Dillon*, et al.*, 2008). Although not tested in this study, these genera may have already been present in the laboratory insect culture meaning that the introduction of these phylotypes would not have altered locust gut diversity. Nonetheless, an effect of microbial manipulation (by manipulating bacterial density) was still expected, particularly at the concentration chosen.

Acridid commensal diversity is known to range between three and twelve phylotypes (Dillon*, et al.*, 2008). The addition of up to two in this study was therefore expected to substantially change gut community structure, especially in the short term. Although no significant variation in immune traits were detected, initial evidence supporting an immune effect of commensal manipulation was still observed. Comprehensive identification of enteric populations in the *L. migratoria* culture (pre- and post-inoculation) would therefore benefit future investigations.

Competition for space and nutritional resources within the gut by microbial communities is known to impact both host and bacteria (Sekirov*, et al.*, 2010). However, interactions between resident microorganisms and introduced species within hosts are still largely unclear. Previous studies on colonisation resistance in *S. gregaria* allowed one week for introduced colonies to become established (Dillon*, et al.*, 2005). Constitutive responses, however, are likely to occur more rapidly and so these experiments performed sampling at 40 h post inoculation. The introduced bacteria in this study may have been outcompeted by established colonies, thereby enabling hosts to avoid any necessary adaptive changes in host immune investment. In some instances there is a small window of opportunity to sample and detect active immune responses. Therefore a similar approach, where immunity is assayed across a longer time-course, may enable better tracking of constitutive changes. Greater sample sizes may also reduce the large variation observed between individuals. This

approach would provide particular insight into the reaction time-course and robustness of *L. migratoria* immunity.

6.5.2 Assay diversity: technical sensitivity and subtle clues

Molecular techniques have fast become a major tool in uncovering the mechanisms involved in insect immune function (Kang*, et al.*, 1996; Khush and Lemaitre, 2000; Tzou*, et al.*, 2000; Schmid-Hempel, 2005). Through the analysis of three immune genes, this work provides an early indication of gene-specific variation in response to the manipulation of commensal bacteria. Significant differences between fat body and midgut expression levels support the tissue-specific results seen in Chapter 5 and further demonstrate the importance of assay, tissue and gene selection when conducting insect immune studies.

Throughout the literature, suggestions towards a multifaceted approach when investigating immune function in animals are becoming increasingly common (Adamo, 2004a; Cotter*, et al.*, 2004a). The assumption that just one or two immunological assays will accurately represent a host's overall immune state is generally unreliable, particularly when viewing from an ecological context (Martin*, et al.*, 2006). Environmental variation and immune challenges can often result in trait-specific responses meaning that different immune traits will often tell different stories when measured in parallel (Adamo, 2004a). Caloric distribution and investment into different immune traits play a significant role in host response, particularly where resources are limited (Sheldon and Verhulst, 1996; Wilson*, et al.*, 2003). With complex interactions between host and environment, the measurement of multiple traits almost always provides wider insight into the underlying mechanisms of immune function, as was demonstrated in this work (Cotter*, et al.*, 2004a; Martin*, et al.*, 2006).

6.5.3 Ecological relevance of microbial diversity

As mentioned above, the bacteria used in this study fit within the small range of phylotypes already known to exist in the gut of the *L. migratoria* (Dillon*, et al.*, 2008). Thus far, however, the overlap of gut microbiota between *C. terminifera* and *L. migratoria* is unknown.

Although it appears that relatively simple communities exist within the acridid gut, the specificity these communities hold to individual species is still poorly understood. The presence of few and sometimes unique commensal bacteria often indicates a strong interaction between insect host and bacteria (Koch and Schmid-Hempel, 2011b; McFrederick*, et al.*, 2013). Similar to locusts, several other insects (*e.g.,* bumblebees, honeybees and flies) have been shown to harbour few, yet highly specific, resident gut bacteria that play vital roles in immune defence and food preservation (Koch and Schmid-Hempel, 2011b). The genetic diversity within honeybee microbiota, however, has been found to be surprisingly high, revealing the importance of microbial diversity as well as density within the GIT (Martinson*, et al.*, 2011; Wong*, et al.*, 2011; Engel*, et al.*, 2012).

Bacteria have the capacity to evolve and specialise throughout the life of their host, an adaptation that may benefit both symbionts (Sekirov*, et al.*, 2010; Engel and Moran, 2013). Suggestions that locusts are capable of self-regulating their gut microbiota, based on the estimated virulence of introduced populations, are a topic of growing interest (Dillon*, et al.*, 2008). By demonstrating successful inoculation of *L. migratoria* with gut microbiota isolated from *C. terminifera*, this work has opened the door to such studies.

6.5.4 The lost pathogen

As stated above, the original aim of this work was to isolate and identify the pathogen responsible for the observed *C. terminifera* disease outbreak in Hillston in 2010. Although this was not achieved, the experiments presented demonstrate how unexpected information can provide insight into new aspects of host biology. In this case, the isolation of known locust gut bacteria, rather than the suspected pathogen (*i.e., Pseudomonas*), redirected this study toward investigating the impact of their introduction to the GIT of *L. migratoria*.

Future studies will seek to conclusively identify and recover the pathogen of interest, ultimately aiming to examine its potential as a new form of locust biocontrol. Whilst pyrosequencing results indicated a strong likelihood that *Pseudomonas* was the responsible bacteria, other phylotypes must also be considered. Bacterial colonies from the known pathogen *Bacillus*, for example, were also identified in pyrosequencing data and successfully isolated from field cadavers. Bacteria from the genus *Serratia* was similarly identified in pyrosequencing results, though (as with *Bacillus*) was relatively less abundant than *Pseudomonas* and was not recoverable from field samples. The potential for either of these less abundant (yet potentially more infective) phylotypes to be the responsible pathogen cannot be discounted. Initial focus on more ecologically dominant species, however, may be a more sensible approach.

6.5.5 Conclusions and future work

The contribution of enteric bacteria to pathogen defence is being increasingly demonstrated across several orders of insects (Dillon and Charnley, 2002; Egert*, et al.*, 2005; Raymond*, et al.*, 2008; Engel*, et al.*, 2012). In this study, no significant effect of microbial introduction on the constitutive immune function of *L. migratoria*

was found, however, several positive trends were still observed in response to bacteria inoculation. Considering the experimental restrictions, this greatly encourages the need for continued investigation. Through the application of RT qPCR, observed trends between immune gene expression and inoculation treatments may have been uncovered in their early stages, a response less likely to be observed using conventional approaches. The availability and sensitivity of such technologies provide new incentives to pursue studies that focus on the relationships between commensal bacteria and their hosts. With new opportunities to apply a diverse range of molecular and conventional approaches, immunological studies such as this are able to gain a broader view of host-microbe interactions. For locusts, a model organism with many ecological and economic impacts, understanding the interactions and mechanisms between their internal and external environments is critical. Without such information, pursuits to identify and develop new biocontrol agents may ultimately limit their application.

Chapter 7.

General Discussion

“Science never solves a problem, without creating ten more.”

George Bernard Shaw

7.1 Overview

A strong immune system is crucial for the prevention and negotiation of infection. Adaptive and innate defences are complex and shaped largely by host ecology, life history and physiology (Sadd and Schmid-Hempel, 2009). The growing body of work from the field of ecological immunology therefore emphasises the importance of considering host environment when studying immune function (Schulenburg*, et al.*, 2009; Martin*, et al.*, 2011). The goal of this thesis was to address some of the major environmental factors that are likely to influence host-parasite interactions in Australian locusts. In particular, this included variation in population density, encounters of cannibalism, responses to infected conspecifics and the impact of gut microbiota to constitutive immune function. By employing an ecological perspective, and a comprehensive range of techniques, this study has provided an in-depth view into the physiological and behavioural responses and trade-offs that locusts exhibit under a range of environmental circumstances.

7.2 Locust ecology and immunity

The evolution of polyphenism in locusts has greatly contributed to their ecological success, particularly their capacity to plastically respond and adapt to environmental change (Nijhout, 2003; Simpson*, et al.*, 2011). The phenomenon of density-dependent phase polyphenism, for instance, causes locusts to react behaviourally, physiologically and morphologically to changes in population density (see Chapters 1 and 2) (Pener and Simpson, 2009). Locust immune function has been similarly found to flexibly respond to changes in population dynamics. With many shared traits between vertebrate and invertebrate systems, locusts provide a unique model through which to study innate immune function. Their standing as a major global agricultural pest makes such studies particularly important.

As described in Chapter 1, the life cycle of a locust encompasses a suite of physiological and developmental changes that occur across five nymphal stadia before eclosion to adult (Chapman*, et al.*, 2013). These processes are greatly influenced by variation in nutritional resources and population dynamics, which profoundly influence locust physiology, development and behaviour (Raubenheimer and Simpson, 1999; Behmer*, et al.*, 2003). Nutritional constraints, and spatial organisation of resources within natural environments, commonly lead to the aggregation of conspecifics, causing behavioural gregarisation of individuals (Bazazi*, et al.*, 2011). Under increased localised population density, incidence of cannibalism and competition for resources increases dramatically (refer to Figure 1.1). Such conditions are often opportunity for pathogens (or other microbes) to colonise and reproduce within hosts.

7.3 Summary of findings

In nature, locusts commonly experience changes in population density, a variable that has been shown to trigger a collection of behavioural and physiological changes in individuals, through the process of density-dependent phase polyphenism (Pener and Simpson, 2009). How locust immune state changes in response to shifts in behavioural phenotype between solitarious and gregarious phases, however, is still a matter of contention throughout the literature (Wilson*, et al.*, 2002; Miller*, et al.*, 2009a; Miller and Simpson, 2010). Experiments in Chapter 2 investigated the density-dependent prophylaxis (DDP) hypothesis in *Chortoicetes terminifera*; a theory that predicts elevated immune investment following local population-density increase and heightened risk of infection (Wilson and Reeson, 1998). Using a multifaceted approach, locust immune response was found to vary between gregarious and solitary-reared individuals. Whether these findings supported or contradicted predictions of the DDP hypothesis, however, changed according to the

trait examined as well as the infection status of individuals. This suggests that immune traits may be differentially prioritised in response to such variables, a complex pattern that has been similarly observed in a number of other organisms (Cotter*, et al.*, 2004b; Adamo and Parsons, 2006; Piesk*, et al.*, 2013).

In addition to an increased risk of pathogen transmission, locusts within high-density populations also have a greater likelihood of cannibalistic encounters, primarily when alternative nutritional resources are unavailable. Experiments from Chapter 3 found that these encounters can be dramatically influenced when the targets of cannibalism (*i.e.,* conspecifics) have a fungal infection. In the absence of alternative food sources, newly-dead and sporulating victims (infected with *Metarhizium acridium*) were found to significantly deter potential cannibals, causing behavioural avoidance and ultimately death by starvation. Interestingly, fungal-infected victims that showed no infection symptoms were not avoided, but rather cannibalised with equal likelihood to uninfected cadavers.

The mechanisms by which locusts detect and differentiate infected from uninfected conspecifics, in particular the recognition of unique cuticular hydrocarbon signatures, are likely contributors to the observed patterns. The deterrence exhibited by locusts, possibly using these conspecific cues, was an efficient means to escape pathogen transmission through cannibalism, and is perhaps an evolved response that facilitates the avoidance of infection altogether.

In addition to behavioural avoidance, this thesis also explored whether similar exposure to fungal-infected conspecifics also induced physiological immune responses in locusts. Chapter 4 examined the constitutive immunity of *C. terminifera* exposed by proximity or direct contact to conspecifics at various stages of infection. Unexpectedly, these experiments found no plastic immune anticipation response to

any mode of infection exposure, a finding that contrasted the immune upregulation observed in similar experiments on ants (Konrad*, et al.*, 2012). Locusts preparing for ecdysis, however, were found to exhibit clear immune upregulation, presenting evidence of a hard-wired immune component of locust development. Once again, these experiments used a multifaceted approached, employing a series of conventional immunological assays to identify specific changes in individual immune traits. Perhaps unsurprisingly, due to the complex interactions between locust immunity and environment, the comparative outcomes of individual traits were often inconsistent. These experiments revealed the intricate network of trait resource investment within host immune systems, highlighting the challenges for immunological studies, particularly when attempting to comprehensively describe patterns of overall host immunity.

As an additional means to quantify locust immune function, gene expression assays were developed using *Locusta migratoria* (Chapter 5). This approach compared the expression of three immune genes across a range of tissue types to characterise how constitutive and responsive immune function are differentially expressed at the gene level. As expected, tissue-specific immune gene expression was found across the seven tissue-types examined. This variation also changed according to locust immune state (*i.e.,* immune challenged versus controls), demonstrating the importance of tissue, gene and pathogen selection when studying immune function.

The premise of experiments in Chapter 6 began with the unexpected epizootic of *C. terminifera* migratory bands in Hillston, New South Wales, in 2010. Attempts at identifying and isolating the bacterial pathogen responsible for these mass deaths were overall unsuccessful, with further investigation falling beyond the scope of this project. Initial pyrosequencing data, however, suggested that *Pseudomonas* (the most

abundant phylotype) might have been the bacteria responsible. Unfortunately, colonies from this genus were unrecoverable from locust cadavers.

Throughout the culturing attempts described above, a number of known gut bacteria were successfully isolated and cultured from the infected locust cadavers. Consequently, Chapter 6 went on to test the capacity of *L. migratoria* to respond to changes in their gut microbiota. Using a combination of gene expression and conventional assays, these experiments found preliminary evidence of constitutive immune variation in response to the introduction of gut bacteria to the gastrointestinal tract. Interestingly, not all measured traits revealed the same patterns or sensitivity to treatments, again indicating the importance of a multifaceted approach. The trends identified, though not statistically significant, showed common positive increases across several assays with the addition of one or more phylotypes. Whether this was a defensive response to the recognition of invading cells, or a constitutive increase as a result of enteric manipulation, remains unclear. Such results, however, do reveal the complex interactions that occur between resident gut microbiota and host immunity, providing compelling grounds for further study.

7.4 Plague management and locust biocontrol

Locust plagues have devastating agricultural and economic impacts worldwide (A.P.L.C, 2014; F.A.O, 2014). Understanding their behaviour, physiology and overall biology is therefore critical in learning how to better predict and manage outbreaks in the field. Gaining insight into the mechanisms of locust immune response is similarly crucial for the development of efficient and safe biological controls. The use of *M. acridium* in this study (a favoured yet still relatively poorly understood pathogen) has provided valuable insight into how this acridid-specific fungus interacts with its host under different conditions.

Survivorship experiments in Chapter 2 found no effect of phase on the mortality of *C. terminifera* regardless of fungal-inoculation dose. Although evidence for DDP was found in some of the conventional measures applied, the overall resistance of locusts to *M. acridium* did not differ with phase. This compares to other studies on locusts, which similarly found variable survival rates and immune responses across crowd- and solitary-reared locusts (Wilson*, et al.*, 2002; Miller*, et al.*, 2009a; Miller and Simpson, 2010). These results suggest that while solitarious and gregarious *C. terminifera* differentially invest in trait response, it may not necessarily impact their survivorship. In natural systems, it is therefore possible that locusts at high population densities may have no survivorship advantage over their solitarious counterparts.

Cannibalism was found to be an unsuccessful mode of secondary transmission of *M. acridium* between locusts (Chapter 3). Similar to observations in a recent study on four other orthopteran species (Jaronski, 2013), individuals were completely deterred from the consumption of symptom-bearing conspecifics. This indicates that whilst *M. acridium* may indeed be transmissible via cannibalism, the adaptive behaviour of locusts to avoid infection makes this highly improbable. The tendency for locusts to succumb to death by starvation, rather than consume infected conspecifics, indicates severe nutritional deprivation in such circumstances. Previous works have demonstrated the importance of nutrition for effective immune defence (Feder*, et al.*, 1997; Lee*, et al.*, 2008; Adamo*, et al.*, 2010). Although not tested in this study, nutritional deprivation may be an additional indirect benefit for locust biocontrol.

The increase in constitutive immune investment with stadium progression was proposed in Chapter 4 to be an evolved hard-wired innate anticipatory response to locust development. Altered nutrient intake across nymphal stadia has been

demonstrated in other studies, confirming that locusts experience physiological shifts in the lead up to ecdysis (Miller*, et al.*, 2009b). Previous studies have similarly found increases in host infection resistance across insect development (Wago and Ichikawa, 1979; Gillespie*, et al.*, 1997). Developmental changes are similarly expected to enable stronger immune protection in locusts against *M. acridium* (or indeed any pathogen) during a time of elevated vulnerability. In the context of locust biocontrol, these results suggest that targeting migratory bands comprised of early stage nymphs may lead to higher rates of mortality than treating bands comprised of older individuals. Accordingly, such approaches may also allow for the application of less concentrated spore solutions.

Unlike the hard-wired anticipatory responses observed in the lead up to ecdysis, plastic immune upregulation was not detected in locusts exposed to infected conspecifics. This was different to previous studies on ants, where fungal transfer between conspecifics elevated antifungal activity in self-immunising individuals (Konrad*, et al.*, 2012). Locusts exposed to infected conspecifics in the field are therefore unlikely to upregulate their constitutive immunity, and show no immune preparation against commonly encountered pathogens. This outcome therefore has positive bearings on the use of *M. acridium* in the field.

Preliminary evidence of upregulated constitutive immunity, following the introduction of gut bacteria to *L. migratoria,* was found in Chapter 6. Although not tested in this work, these initial results suggest a potential increase in colonisation resistance following the addition of new phylotypes. Previous studies have shown that germ-free locusts are more susceptible to infection than conspecifics with higher commensal diversity (Dillon*, et al.*, 2005), a pattern supported by the findings of Chapter 6. By pursuing a better understanding of the role of gut microbiota in host immune defence, future studies may be able to exploit enteric bacteria for the

development of more targeted biocontrol agents. The initial data and techniques provided by this study have added to the potential of such investigations.

Preliminary evidence in Chapter 6 was also obtained in the search for a prospective new locust biocontrol agent. Following the analysis of locust cadavers, collected from a mass epizootic in the field, *Pseudomonas* was the most abundant bacteria identified. *Pseudomonas aeruginosa*, frequently used in insect immune studies, is a known opportunistic pathogen and potential candidate as a new biocontrol agent (Jander*, et al.*, 2000; Lyczak*, et al.*, 2000; Tan and Ausubel, 2000). With initial steps already taken toward the identification of the pathogen in question, these initial results provide a launch pad for further investigations into the feasibility of this pathogen as a new biological insecticide.

Overall, this study has made clear the importance of considering locust ecology when investigating new biocontrol strategies. Ideally, pathogens used against plagues will be customised, not only at a taxonomic level (*e.g.,* Acridids) but also in a way that accounts for developmental stage, population dynamics and nutritional state. This could mean optimising the dosage used on a case-by-case basis, or employing combinations of agents using an integrated approach. For bio-agents, such as *M. acridium*, where the wider environmental impacts are still unclear, this is particularly important.

7.5 Conclusions and future work

Until recently, there has been limited interest on the impact that the environment has on host immunity. The complex interactions between locust immune function and ecology have been clearly demonstrated in this thesis, with many new questions arising from each experiment presented. The importance of assay and tissue selection in immune studies, in particular how different traits change when subject to varying

conditions, has also been shown. With several techniques available to quantify locust immune function, including the development of gene expression assays for three immune-associated genes, this study has provided valuable insight into the ways in which subsequent studies should approach similar questions.

This thesis has provided insight into the complex nature of locust immunity. Immune defences have been found to differ in the nature of their response at the level of the individual, through behaviour and physiology, and on a smaller level still, within tissues and genes. Such variation was found to be either flexible and adaptable to environmental change, or hard-wired in anticipation of developmental processes.

Resource investment toward individual immune traits appears to greatly define how locusts respond to ecological change. Opposing results in response to single treatments were frequently observed across conventional assays. Population density and infection, for instance, were clearly found to induce immune trait prioritisation, demonstrating the costly nature of immune defence. Variation in constitutive and responsive immune gene expression was also demonstrated, showing clear tissue, gene and infection state specificity. The comparison of conventional and molecular approaches similarly revealed inconsistent results between assays. Overall, the use of multifaceted approaches throughout this work has successfully revealed the shortcomings of relying only on individual assays to better understand host immunity. This ultimately suggests that resources may be frequently reprioritised and distributed across immune traits.

Future studies focusing on locust immunology would benefit from a more comprehensive look at the effects of phase change on host immunity. Gene expression techniques developed in Chapter 5, for instance, could provide a better platform to tease-apart population density and locust phase in the context of the DDP

hypothesis. With the addition of more sensitive techniques, underlying responses to population density may be better able to characterise how phase polyphenism influences locust immunity on a molecular level. These techniques may be similarly informative in cannibalism experiments to provide a better understanding of the underlying triggers of deterrence responses and the adaptive significance of such infection avoidance behaviours.

With many studies having now identified the importance of symbiotic gut microbiota for the regulation of host immune function, this discipline has become a topic of particular interest. Within locusts, a group of animals with relatively few resident gut bacteria (Dillon*, et al.*, 2008), descriptions of gut composition, microbe diversity and their influence on host immunity, are still in its infant stages. Further studies, looking at the role of individual phylotypes, within a greater commensal diversity will help to strengthen current knowledge.

Finally, further works are needed to identify the pathogen responsible for the epizootic-like disease outbreak observed in the field in 2010. Having resulted in the mass eradication of individuals in their natural ecosystem, finding this pathogen and evaluating its potential in its use as a new biological control is an especially important step. Though further analysis is needed, this opportunistic pathogen presents as a promising candidate in locust biocontrol.

Overall, this thesis has presented a broad view of the ecological immunology of Australian locusts. As a dynamic and adaptable organism, it is clear that much work is still needed to describe the responses and adaptations exhibited by these insects. By looking simultaneously into several aspects of locust ecology, this thesis has clearly demonstrated the significance of how varied immune investment can change and influence observed response. With the evolved capacity to respond to

unpredictable surroundings, via a suite of physiological and behavioural responses, it is unsurprising that locusts have impacted human populations on such a broad scale. Attempts to better understand the immune function of these insects will not only enable their exploitation as a model system for other organisms, but also allow better integration of current and future biocontrol agents.

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