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Social immunity and the expression of immune-related genes in the Eastern subterranean termite

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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SOCIAL IMMUNITY AND THE EXPRESSION OF IMMUNE-RELATED GENES IN THE EASTERN SUBTERRANEAN TERMITE

(Thesis format: Integrated Article)

by

Qi Gao

Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract

Individuals living within social groups may benefit from the efficiencies of division of labour, but on the other hand render themselves vulnerable to socially transmitted disease. This cost to social living should promote cooperative barriers to disease transmission, especially in eusocial taxa where spatial and genetic proximity to nestmates are characteristically pronounced. Though studies of the immunity at multiple levels in social species are becoming more common, little is known about how their sociality is deployed to resist contagion. By exposing the Eastern subterranean termite Reticulitermes flavipes to entomopathogenic fungi, I studied their immune responses at multiple levels. At the level of group, I found that group size and caste composition affected individual survivorship, and that these effects can be independent of any infection. That is, larger groups or groups of mixed caste simply lived longer. Upon infection, I found that only caste composition, and not group size, affected the group’s ability to survive. This effect from caste, but not from group size, suggests that the group-level immunity in R. flavipes is affected more by the nature rather than the number of social interactions. Within groups, individual termites appeared to express relatively few immune genes, but did so in response to specific fungi and social contexts. R. flavipes expressed specific combinations of immune-genes following exposure to various infective fungal spores. This pathogen-specific expression pattern indicates that R. flavipes maintains a high degree of immunological specificity in its innate immunity, and the degree of the specificity is subject to species-level. Furthermore, some immune genes tended to be less responsive within infected groups, compared to the response of singly infected individuals. But others showed up-regulation on infection regardless of social context. This socially-responsive expression pattern suggests that R. flavipes has the capacity to adjust its innate
immune response to its immediate social context. In summary, my behavioral and genetic analyses suggest an adaptive interaction between individual versus collective immunity in a subterranean termite.

Keywords

Social insects, innate immunity, social immunity, division of labour, gene expression, termites, entomopathogenic fungi.
Co-Authorship Statement

A version of Chapter 2 is published in *Acta Ethologica* with Dr. Michael J. Bidochka and Dr. Graham J. Thompson as co-authors. Dr. Bidochka provided the fungal strains and gave advice on fungal culturing. Dr. Thompson contributed significantly to the ideas and discussion, and provided editorial comments on the manuscript.

A version of Chapter 3 is published in *Archives of Insect Biochemistry and Physiology* with Sarah E. Tancredi and Dr. Graham J. Thompson as co-authors. Sarah Tancredi helped in the experimental design, and with the implementation of the real-time PCR experiment. Dr. Thompson contributed significantly to the ideas and discussion, and provided editorial comments on the manuscript.

Chapter 4 is currently in preparation for submission to *Journal of Invertebrate Pathology* with Dr. Graham J. Thompson as co-author. Dr. Thompson co-conceived the experiment and is helping me write the manuscript.

A version of Chapter 5 has been submitted to the journal *Insectes Sociaux* with Dr. Graham J. Thompson as co-author. Dr. Thompson co-conceived the experiment and is helping me write the manuscript.
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# Table of Contents

Abstract ............................................................................................................................................................................. ii  
Co-Authorship Statement .................................................................................................................................................. iv 
Acknowledgments ............................................................................................................................................................. v  
Table of Contents ............................................................................................................................................................ vii  
List of Tables ................................................................................................................................................................. xii  
List of Figures .................................................................................................................................................................. xiii  
List of Appendices .......................................................................................................................................................... xiv  
List of Abbreviations ....................................................................................................................................................... xv  
Chapter 1 General introduction ........................................................................................................................................ 1  
  1.1 Insect immunity ....................................................................................................................................................... 2  
      1.1.1 Physical barriers ............................................................................................................................................... 3  
      1.1.2 Immunological reactions ................................................................................................................................. 3  
      1.1.3 Immune pathways ............................................................................................................................................. 4  
      1.1.4 Immunological memory and specificity ......................................................................................................... 7  
      1.1.5 Social immunity ................................................................................................................................................ 8  
      1.1.6 Individual immunity versus social immunity ............................................................................................... 10  
  1.2 Why study termites? .................................................................................................................................................. 11  
      1.2.1 Non-Hymenopteran eusocial insect ............................................................................................................... 11  
      1.2.2 Immunity in termite species .......................................................................................................................... 13  
  1.3 Reticulitermes flavipes ............................................................................................................................................... 15  
  1.4 Entomopathogenic Fungi .......................................................................................................................................... 15  
  1.5 Thesis overview ......................................................................................................................................................... 16  
      1.5.1 Effect of social variables on individual immunity .......................................................................................... 16
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5.2 Immune gene diversity</td>
<td>17</td>
</tr>
<tr>
<td>1.5.3 Pathogen-specific immune gene expression</td>
<td>17</td>
</tr>
<tr>
<td>1.5.4 Effect of social context on individual immunity</td>
<td>18</td>
</tr>
<tr>
<td>1.6 References</td>
<td>19</td>
</tr>
<tr>
<td>Chapter 2 Effect of group size and caste ratio on individual survivorship and social immunity in a subterranean termite</td>
<td>28</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>28</td>
</tr>
<tr>
<td>2.2 Materials and methods</td>
<td>30</td>
</tr>
<tr>
<td>2.2.1 Termite collection and maintenance</td>
<td>30</td>
</tr>
<tr>
<td>2.2.2 Fungal culture and infectivity trials</td>
<td>30</td>
</tr>
<tr>
<td>2.2.3 Fungal virulence on <em>R. flavipes</em></td>
<td>31</td>
</tr>
<tr>
<td>2.2.4 Testing for social immunity</td>
<td>31</td>
</tr>
<tr>
<td>2.2.5 Group size assay</td>
<td>32</td>
</tr>
<tr>
<td>2.2.6 Caste ratio assay</td>
<td>32</td>
</tr>
<tr>
<td>2.2.7 Confirmation of mycosis</td>
<td>33</td>
</tr>
<tr>
<td>2.3 Results</td>
<td>33</td>
</tr>
<tr>
<td>2.3.1 Fungal virulence on <em>R. flavipes</em></td>
<td>33</td>
</tr>
<tr>
<td>2.3.2 Group size assay</td>
<td>33</td>
</tr>
<tr>
<td>2.3.3 Caste ratio assay</td>
<td>34</td>
</tr>
<tr>
<td>2.3.4 Confirmation of mycosis</td>
<td>37</td>
</tr>
<tr>
<td>2.4 Discussion</td>
<td>37</td>
</tr>
<tr>
<td>2.4.1 Effect of group size on disease resistance</td>
<td>40</td>
</tr>
<tr>
<td>2.4.2 Effect of group caste composition on individual immunity</td>
<td>41</td>
</tr>
<tr>
<td>2.5 Conclusions</td>
<td>42</td>
</tr>
<tr>
<td>2.6 Acknowledgments</td>
<td>43</td>
</tr>
</tbody>
</table>
Chapter 3 Identification of mycosis-related genes in the Eastern subterranean termite by suppression subtractive hybridization

3.1 Introduction ................................................................. 47

3.2 Materials and methods ................................................. 49
  3.2.1 Termite collection and maintenance ......................... 49
  3.2.2 Fungal challenge ....................................................... 49
  3.2.3 RNA extraction and cDNA synthesis ......................... 49
  3.2.4 Subtracted cDNA library construction ....................... 50
  3.2.5 Bioinformatic analyses .............................................. 50
  3.2.6 Relative quantification of candidate gene expression ..... 51
  3.2.7 Statistical analysis .................................................. 53

3.3 Results ........................................................................ 53
  3.3.1 Overview of immune-induced genes ......................... 53
  3.3.2 Gene Ontology Analysis .......................................... 56
  3.3.3 Quantitative RT-PCR Data ....................................... 56

3.4 Discussion .................................................................... 60
  3.4.1 Description of normalized, immune-relevant cDNA library 60

3.5 Acknowledgments .......................................................... 64

3.6 References .................................................................... 64

Chapter 4 Fungus infected termites show a pathogen-specific immune gene response... 68

4.1 Introduction .................................................................. 68

4.2 Materials and methods ................................................ 69
  4.2.1 Termite collection and maintenance ......................... 69
  4.2.2 Fungal infections ...................................................... 70
6.1.2 Pathogen-specific immune response ....................................................... 107

6.2 Social immunity in *R. flavipes* ........................................................................ 108
   6.2.1 The number of social interactions ........................................................... 109
   6.2.2 The nature of social interactions ............................................................ 110

6.3 Effect of social immunity on innate immune response in *R. flavipes* .......... 111

6.4 Antifungal immune pathway .......................................................................... 112

6.5 Conclusions and future directions ................................................................. 112

6.6 References ..................................................................................................... 115
List of Tables

Table 1.1 The main differences between Hymenoptera and Isoptera eusocial species......... 12

Table 2.1 Cox proportional hazard regression analysis for group size and caste ratio assay... 35

Table 3.1 Primers sequences for all candidate immune loci analyzed by qRT-PCR. .......... 52

Table 3.2 Bioinformatic analysis of ESTs from the subtracted cDNA library of R. flavipes. . 54

Table 3.3 Summary statistics for qRT-PCR analysis of candidate immune genes isolated from R. flavipes following fungal infection. .......................................................... 61

Table 4.1 Primers used for real-time PCR amplification of one reference gene and six candidate immune genes in R. flavipes.............................................................. 73

Table 4.2 Summary of statistical analysis for the effect of “colony” of origin on expression across the six candidate immune loci in R. flavipes ................................................... 77

Table 5.1 Primers used for real-time PCR amplification of one reference gene and six candidate immune genes in R. flavipes.............................................................. 91

Table 5.2 Factors affecting immune gene expression at two time points post infection (24 and 48 hrs), as evaluated from a multi factorial analysis of variance (MANOVA) in which fungal infection and social context are coded as fixed factors.................................................. 92

Table 5.3 Factors affecting immune gene expression on a locus-by-locus basis at two time points post infection (24 and 48 hrs), as evaluated by a one-way analyses of variance in which fungal infection and social context are coded as fixed factors................................. 96
List of Figures

Figure 1.1 Schematic overview of generalized insect innate immune pathways .......................5

Figure 1.2 Schematic overview of the levels of immune mechanisms in termite colonies ..........14

Figure 2.1 The association between treatment and subsequent survivorship (±SE) as a function of group size. ..........................................................................................................................36

Figure 2.2 The association between treatment and subsequent survival (±SE) as a function of group caste ratio (soldier: worker: nymph). ..............................................................................39

Figure 3.1 cDNA sequences and putative amino acid sequences of (A) tigger transposable element-derived protein 7-like and (B) leucine-rich repeat neuronal protein 2-like ..............57

Figure 3.2 Gene expression of candidate immune genes to fungal infection in control (white bars) and treated (gray bars) samples ......................................................................................59

Figure 5.1 Experimental design showing how termites are infected in social isolation (single termite) or in a social group of five (grouped termites), and how immune gene response was recorded after a relatively short (24 hrs) or long (48 hrs) time frame following infection. 88

Figure 5.2 Gene expression patterns at six immune-related loci in R. flavipes following exposure to the fungus M. anisopliae ...........................................................................................................95

Figure 6.1 Hypothetical antifungal pathway in R. flavipes. .........................................................114
List of Appendices

Appendix A: Permission to use the copyright material in Chapter 1 ......................... 120
Appendix B: Chapter 2 supplement material ............................................................... 121
Appendix C: Permission to reprint published material in Chapter 2 ......................... 122
Appendix D: Permission to reprint published material in Chapter 3 .......................... 123
List of Abbreviations

AMP: antimicrobial peptide

Dscam: Down syndrome cell adhesion molecule

EST: expressed sequence tag

GNBP: Gram-negative binding protein

Imd: Immune deficiency

JAK/STAT: Janus kinase/signal transducer and activator of transcription

JNK: c-Jun N-terminal protein kinases

lncRNA: long noncoding RNA

LRR: leucine-rich repeat neuronal protein 2-like

MHC: major histocompatibility complex molecules

PGRP: peptidoglycan recognition protein

PRR: pattern recognition receptor

qRT-PCR: quantitative real-time PCR

SSH: suppression subtractive hybridization

Tc7: tigger transposable element-derived protein 7-like
Chapter 1

1 General introduction

As the largest Class within the Invertebrata, insects are found in every land and freshwater habitat, and are most abundant in the tropics (Boucias and Pendland, 1998). This wide range of natural and urban habitats potentially exposes them to a diversity of disease-producing microorganisms, including bacteria, fungi, protozoa, nematodes and viruses (Boucias and Pendland, 1998; Schmid-Hempel, 1998; Vallet-Gely et al., 2008). This exposure can be a burden to afflicted individuals, and over time we expect host lineages to co-evolve with their pathogens (Thompson, 1994). The immune system in hosts is an evolutionary outcome in response to the selective pressure from pathogens. Hosts and pathogens enter into an evolutionary arm race, in which pathogens evolve to evade host defenses while host defenses evolve to circumvent such evasion. This permanent challenge between resistance mechanisms of host and pathogenicity of pathogens leads to a rapid evolution of the immune system (Lazzaro and Little, 2009).

Understanding the evolution of the complex immune mechanisms in insects is a topic relevant to ecology, genetics, agriculture and even medicine (Schmid-Hempel, 2005; Decanini et al. 2007). For example, knowledge of insect immunity provides one avenue for understanding the conserved molecular components that may even be shared with the innate immune system of vertebrates. And we have already known that the genes encode intracellular signaling pathways leading from the cell surface to the activation of the transcription factors are well conserved in both vertebrate and invertebrate innate immunity (Salzet, 2001).

Similarly, an understanding of insect immunity can inform aspects of applied entomology – for example, as it relates to health of beneficial pollinators (Rolff and Reynolds, 2009) or eradication of insect pests (Chouvenc and Su, 2010). Bees play an important ecological function as pollinators. In recent years, their rapid population decline in many countries is at least in part a consequence of compromised immunity may due to the abuse of pesticides (Rolff and Reynolds, 2009). Therefore, studying the immune system of insects can lead to useful knowledge for the improvement of biological pesticides.
Finally, since some human diseases are vector by insect intermediaries, an understanding of how pathogens infect insects can be important to human disease control (Rolff and Reynolds, 2009). For example, *Anopheles gambiae* is the principle vector of malaria, one of the major infectious diseases affecting the health of hundreds of millions of people (Breman et al., 2001). The parasite has to live in mosquito blood for part of its life cycle, all the while exposed to the host immune system. A successful parasite has to avoid the immune system or be able to defend against it. Understanding how a pathogen can survive might result in ways to disrupt the transmission of diseases.

Furthermore, as models of discovery, insects are relatively easy to experiment with. They can be reared easily in controlled conditions and in large numbers, and are not subject to the same ethical restrictions as vertebrates. Moreover, the infection process in insects is much quicker relative to vertebrates, and yields results more rapidly (Scully and Bidochka, 2006). For many insect species there is a growing database of genomics information, ranging from transcriptomics, proteomics, complete genomes, to massive molecular tools and resources beyond the assembly itself. For some taxa, such as *Anopheles* and *Drosophila*, these resources are extensive, and it is for this reason that these species in particular have been adopted as models in the study of immunity.

### 1.1 Insect immunity

Insects show remarkable diversity in mechanisms of pathogen defense, which include physical barriers (Vincent and Wegst, 2004), immunological reactions (Iwanaga and Lee, 2005), behavioral responses (Siva-Jothy et al. 2005) and even cooperative defenses (Cremer et al., 2007; Evans and Spivak, 2010). For example, the chitinous cuticle can protect a host from physical injury, primitive immunological reactions mainly combat and eliminate the foreign invaders *in vivo*, and some behavioral adaptations such as self- and allo-grooming can provide a good hygienic environment in insect colonies. We are at a point where we understand at least some of what insect immune responses are, but how they are interrelated and interact on each other on disease resistance is still not very clear.
1.1.1 Physical barriers

Before any molecular immune factors are triggered, physical barriers, such as the exoskeleton and the digestive lining, serve as a first-line of defense. These barriers serve as prophylactic barrier to keep host cells and tissues away from pathogenic invaders (Vincent and Wegst, 2004; Ferrandon et al., 2007). These potential breaches to host immunity can simply be repelled or arrested on the epithelial surface, or even deactivated by antimicrobial secretions (Brey et al., 1993). For example, venom glands in bees and wasps (Baracchi et al., 2012), metapleural glands in ants (Schluns and Crozier, 2009) and salivary glands in termites (Bulmer et al., 2012) can produce external secretions with antimicrobial activity. The spread of antimicrobial secretions on the host cuticle can be considered as a primary barrier to infection (Zasloff, 2002; Sadd and Schmid-Hempel, 2006).

1.1.2 Immunological reactions

Once this physical defensive line has been breached, individuals can mount a series of immediate immunological reactions to cope with the foreign invaders (Schmidt et al., 2008). Unlike vertebrates, which have evolved an ‘adaptive’ immune system that is characterized by a type of immunological memory and high level of pathogen specificity, insects rely on an evolutionarily conserved immune system that provides individuals with a broad-spectrum of protection from infection (Little et al., 2005; Rowley and Powell, 2007; Schmidt et al., 2008). This evolutionarily conserved innate immune system consists of both cellular and humoral immune mechanisms (Tsakas and Marmaras, 2010), which mostly rely on germline-encoded receptors and effectors for recognizing and eliminating the foreign invaders (Janeway and Medzhitov, 2002).

The cellular response relies on highly differentiated immune cells, like plasmatocytes, lamellocytes or granulocytes (collectively, ‘hemocytes’), to activate the cell-mediated reactions (Gillespie et al., 1997; Schmid-Hempel, 2005; Siva-Jothy et al., 2005; Strand, 2008). The major cell-mediated mechanisms of the cellular response are: *encapsulation*, in which lamellocytes can form a multi-layered capsule around larger invading objects (i.e., parasites, protozoa and nematodes), *phagocytosis*, in which plasmatocytes can internalize and destroy a
variety of small targets (i.e., bacteria, yeast, double stranded RNA), and nodulation, in which hemocytes can aggregate and entrap bacteria (Pham and Schneider, 2008; Strand, 2008; Tsakas and Marmaras, 2010).

The humoral response, by contrast, functions to induce the expression of antimicrobial molecules. The antimicrobials are synthesized in the fat body and ultimately function to disrupt cell membranes and interfere enzyme activity of invasive microorganisms (Bulet et al., 1999). The majority of these humoral immune response molecules can be classified into one of three functional categories based on their roles within immune pathways (Fig. 1.1). These categories are: pattern recognition receptors (PRRs) which recognize conserved structural features of foreign microbes, signaling molecules, which regulate the pathway-mediated response, and effector molecules, which kill and clear disease agents (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007).

In general, PRRs are either soluble within the circulating hemolymph, or are membrane-bound on the surface of the fat body. PRRs can recognize the unique structures (‘patterns’) of invading microorganisms. The recognition signal is then modulated and amplified within the cytoplasm by multiple signaling molecules, and leads to activation of transcription factors that enter the nucleus of fat body cell and turn on expression of antimicrobial peptides (AMPs) (Fig. 1.1). AMPs are released into the hemolymph where they can destroy the molecular structure of the pathogenic microbes (Broderick et al., 2009).

1.1.3 Immune pathways

Four principal immune pathways are inferred from insect models (Figure 1.1), though the number and type of immune molecules associated with each pathway can vary among species and taxonomic order (Kafatos et al., 2009). These pathways are the Toll pathway, the Immune deficiency (Imd) pathway, c-Jun N-terminal protein kinases (JNK) pathway and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (Hoffmann, 2003; Brennan and Anderson, 2004; Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007).
Figure 1.1 Schematic overview of generalized insect innate immune pathways. (Illustration is provided by Robert M. Brucker 2011, and presented here with permission)
Each pathway is partially redundant but to some extent specialized to respond to infection by a particular class of pathogen.

In *Drosophila*, for example, Toll and Imd pathways are the two major immune pathways (Hoffmann, 2003). Several peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs) are involved in the recognition process of Toll pathway (Brennan and Anderson, 2004). The presence of Gram-positive bacteria and fungi *in vivo* can be sensed by PGRP-SA, PGRP-SD, GNBP1, and GNBP3. Then, the binding of recognition can activate an extracellular proteolytic cascade that lead to the cleavage of cytokine protein Spätzle. The Toll receptor, a transmembrane protein with an extracellular leucine-rich repeat domain and an intracellular signaling domain, can receive the upstream signal and activate an intracellular signaling cascade that recruits three Death domain-containing proteins, which are MyD88, Tube and Pelle, and leads to the phosphorylation and degradation of Cactus. The Rel transcription factors Dorsal and Dif are finally translocated into the nucleus to activate or up-regulate the expression of the antibacterial peptides Defensin or the antifungal peptides Drosomysin and Metchnikowin (Broderick et al., 2009; Valance et al., 2011). In Imd pathway, PGRP-LC can directly bind with Gram-negative bacteria, and activate an intracellular signaling network that associates with several singling molecules such as Imd adaptor, dFADD, and Dredd (Hoffmann, 2003; Brennan and Anderson, 2004). As in the down stream of the Toll pathway, once Relish is phosphorylated and the Rel domain translocates into the nucleus, then the antibacterial peptides Attacins, Cecropins, Drosocin and Diptercins are finally expressed against the infection of Gram-negative bacteria (Broderick et al., 2009; Myllymaki et al., 2014).

Compared to the Toll and Imd pathways, our knowledge of JNK and JAK/STAT pathways is limited. The JNK pathway can also be activated in response to Gram-negative bacteria, and regulate the expression of AMP. Finally, the JAK/STAT pathway is mainly associated with stress, injury or viral response, and may be involved in communication from the hemocytes to the fat body (Pham and Schneider, 2008; Broderick et al., 2009;).
1.1.4 Immunological memory and specificity

In vertebrate adaptive immunity, immunological memory refers to the ability of the immune system to raise an enhanced (faster and stronger) response against the repeat challenge of the same pathogen (Little and Kraaijeveld, 2004; Kurtz, 2005). Immunological specificity can be simply expressed in terms of the degree to which immune responses differentiate between pathogens. Clearly, the immunological memory and specificity are bound to the activation of adaptive immunity against a specific induction (Schmid-Hempel and Ebert, 2003; Kurtz, 2005; Pham and Schneider, 2008).

For many years, it was believed that the insect immune system was not capable of maintaining an immunological memory of prior infection, or of mounting a highly specific response to a particular pathogen. Insect immunity does not feature the necessary lymphocytes (B and T cells) and immunoglobulins (major histocompatibility complex molecules, MHC), which enable memory and specificity in adaptive immune systems (Kurtz, 2004; Little et al., 2005). In other words, insect innate immunity was expected to exhibit only broad specificity to distinguish between different classes of pathogens, and respond identically to repeated challenges (Hoffmann, 2003; Pham and Schneider, 2008).

However, growing experimental evidence shows that invertebrate innate immunity can exhibit some degree of memory and specificity comparable to that of vertebrates (Kurtz and Franz, 2003; Pham and Schneider, 2008). A previous exposure can improve the chance of an individual withstanding infection. This immunological mechanism known as ‘immune priming’ occurred in response to a variety of pathogens has been successively reported in several insect species. In social insects, for example, after a previous challenge with heat-killed bacteria or fungal spores, the dampwood termite *Zootermopsis angusticollis* survived longer when subsequently exposed to the activated bacteria or fungal spores in a second infection (Rosengaus et al., 1999). Bumblebee *Bombus terrestris* workers showed a higher survivorship upon second exposure with the same bacteria (Sadd and Schmid-Hempel, 2006). In solitary insects, the yellow mealworm beetles *Tenebrio molitor* previously injected with an immune-inducing lipopolysaccharide (the outer membrane of Gram-negative bacteria) were better protected against a new fungal infection (Moret and Siva-Jothy, 2003). When
previously exposed to Gram-positive bacteria *Streptococcus pneumoniae* or fungal pathogen *Beauveria bassiana*, the fruitflies (*Drosophila melanogaster*) exhibited an enhanced disease resistance to a second infection of the same pathogens (Pham et al., 2007). The red flour beetles (*Tribolium castaneum*) exposed to previous priming with heat-killed bacteria were likely to survive a subsequent exposure to live bacteria that were homologous to the priming (Roth et al., 2009). The indian meal moths (*Plodia interpunctella*) previously exposed to low doses of DNA virus were subsequently less susceptible to the same challenge (Tidbury et al., 2011). A challenge with prior Gram-negative bacteria *Serratia marcescens* provided long-term protection to wood tiger moths (*Parasemia plantaginii*) against re-infection (Mikonranta et al., 2014). Among those insects, some instances, such as bumblebees (Sadd et al., 2005), yellow mealworm beetles (Moret, 2006), red flour beetles (Roth et al., 2010) and indianmeal moths (Tidbury et al., 2011), can even transfer this ‘acquired immunity’ to their offspring, a mechanism known as ‘trans-generational immune priming’. All these findings suggest insects do show memory and specificity in their innate immune responses. However, without the involvement of B cells, T cells or MHC molecules, the mechanism underlying these phenomena in insect innate immunity may be developed under different genetic or cellular process (Little, 2005; Rowley and Powell, 2007).

The discovery of a hypervariable receptor, Down syndrome cell adhesion molecule (Dscam), in insect models such as *Drosophila* (Watson et al., 2005) and *Anopheles* (Dong et al., 2006) suggests a cellular mechanistic basis for immunological specificity (Das et al., 2009). The alternative splicing of *Dscam* generates a receptor repertoire that is sufficiently diverse for discriminating a large variety of pathogens (Das et al., 2009). As a consequence, the induced cellular immune response such as phagocytosis can be highly specifically directed against specific pathogen genotypes (Kurtz, 2005; Rowley & Powell 2007).

### 1.1.5 Social immunity

In solitary insects, individuals defending themselves against infection rely mainly on their own individual immune system that consists of physical barriers, cellular and humoral reactions. However, when living in groups, as social insects do, the members of social groups gain a further level of protection in addition to the individual-level defences by collectively
defending against pathogen invasion, that is their social immune mechanisms (Boomsma et al., 2005; Cremer et al., 2007; Cotter and Kilner, 2010).

The Hymenoptera (ants, bees, wasps) and Isoptera (termites) contain many social species, some of which are fully social and thus maintain a high level of organization in their societies (Wilson, 1971). In order to sustain the long-term, healthy and steady development of their colony, social insects cooperate on many tasks related to survival and reproduction, including brood care, nest construction, and provisioning (Oster and Wilson, 1978). Social group members derive great benefit from these cooperatively or mutually performed behaviors that allow for task specialization and parallel processing of those tasks (Schmid-Hempel, 1998; Cremer and Sixt, 2009).

Despite these advantages there are potential disadvantages of living in a social group. Being a member of a large, densely populated colony with genetically related individuals, social insects are especially vulnerable to the spread of contagion (Boomsma et al., 2005; Schmid-Hempel, 2005; Fefferman et al., 2007; Naug, 2008). Therefore, maintaining a high level of organization in their societies seems to be a burden on individual immunity. Grouped individuals may therefore need to invest more into an effective immune system that can provide them with a continuous protection from the potential invasion of foreign microorganisms. For example, in bees, the strength of antimicrobial response is positively correlated with the level of social complexity (i.e. solitary, semisocial and eusocial) (Stow et al., 2007). Similarly, social wasp species exhibit significant higher antimicrobial activity on their cuticles than do solitary species (Hoggard, et al., 2011). These density-dependent effects on antimicrobial activity indicate that grouped individuals are at increased risk from pathogen threat and have responded by maintaining a high level of antimicrobial secretions.

Group living seems to be a selective force that can shape immune investment at the individual-level, however, immune costs may be offset to some degree by behavioral adaptations that are not available to solitary insects. ‘Social immunity’ is described as a series of group-enabled behaviors that can suppress the probability of exposure and transmission of infectious disease within a population (Cremer et al., 2007). The key idea of this definition is that by acting collectively, individuals from the social groups are better able to mount
defenses against infection, that would be impossible were they to act independently (Cremer et al., 2007; Wilson-Rich et al., 2009). Therefore, social immunity is crucial to prevent disease transmission at the group-level.

For the best-studied eusocial model honey bee, *Apis mellifera*, examples of social immunity include simple grooming behavior in which an individual grooms its nestmates to remove foreign microbes (Evans and Spivak, 2010). Honey bee adults can increase the temperature of brood comb in response to infection by *Ascosphaera apis*. This social ‘fever’ is an example of social immunity that can prevent the further development of this fungal disease (Starks et al., 2000). Honey bee workers can detect and remove diseased brood from the nest (Rothenbuhler, 1964; Arathi et al., 2000). This type of hygienic behavior is one of the best-known examples of social immunity, and has long been known to have a genetic basis (Oxley et al., 2010). Similarly, the removal of dead adults from the nest by undertaker specialists helps to control disease in social insect colonies (Visscher, 1983). All these typical behavioral adaptations provide the social members an extra protection beyond the individual-level from disease contamination (Cremer et al., 2007; Cremer and Sixt, 2009; Wilson-Rich et al., 2009).

### 1.1.6 Individual immunity versus social immunity

The combination of individual and social defences may help to offset the increased disease pressures of social living (Boomsma et al., 2005; Cremer et al., 2007). However, very little is known about how social immunity has affected the evolution of the innate immune system.

A few recent genome-wide analyses from the Hymenoptera, including honey bee, *Apis mellifera* (Evans et al., 2006), and ants *Pogonomyrmex barbatus* (Smith et al., 2011a) and *Linepithema humile* (Smith et al., 2011b) reveal that these eusocial insects have a reduced number of immune genes relative to the solitary insect models such as *Drosophila* and *Anopheles*. Sociality is initially proposed as the cause of the loss of immune genes in these eusocial genomes. Genes involved in individual immunity may have been lost during the development of social immunity. However, the annotation of another two solitary genomes show that the non-social wasp *Nasonia vitripennis* (Werren et al., 2010) and the pea aphid, *Acyrthosiphon pisum* (Gerardo et al., 2010) also maintain fewer immune genes compared to Dipteran models. This discovery indicates that the reduction in the number of immune genes
is not unique to eusocial insects. Contrary to the previous prediction, the latter evidence suggests that the loss of immune genes in some taxa is clearly more than a matter of eusociality.

1.2 Why study termites?

1.2.1 Non-Hymenopteran eusocial insect

Most eusocial insects are from the order of the Hymenoptera (ants, bees, wasps) and Isoptera (termites). But eusociality evolved independently within these two orders (Thorne et al., 2000; Howard and Thorne, 2011). The earliest fossil evidence of evolution of sociality in Hymenoptera is a fossil nest of social vespids in the upper Cretaceous. In comparison, the oldest described fossil termite, which is an apparent worker caste individual, indicates that the termite may be the first insect that evolved sociality in the upper Jurassic or lower Cretaceous period (Thorne et al., 2000; Howard and Thorne, 2011).

Without any solitary or primitively social species, all of the existing termite species are eusocial. Unlike the haplodiploid Hymenoptera having holometabolous development, termites are diploid and have hemimetabolous development (Roisin, 2000; Lo and Eggleton, 2011). In termite colonies, male and female individuals can be found in all castes, and bear the same social tasks in their societies. The youngest termites resemble the adults in appearance with little morphological change as they develop, except size and colour. They can participate in social tasks at an early age (Howard and Thorne, 2011).

Given all these differences between Isoptera and Hymenoptera (Table 1.1), termite species can be a good non-Hymenopteran eusocial model for studying the evolution of immunocompetence and social adaptations for reducing disease susceptibility within complex insect societies. Furthermore, termites are noted for their ability to digest cellulose-based materials, thus they are considered as one of the most destructive pests that can cause economic damage in urban and agricultural settings (Logan et al., 1990; Rust and Su, 2012). They also have considerable ecological importance in improving soil quality, recycling of wood material and other plant matter in numerous ecosystems (Nutting, 1990). Therefore, finding an effective way to reduce the economic damage caused by these group-
Table 1.1 The main differences between Hymenopteran and Isopteran eusocial species

<table>
<thead>
<tr>
<th></th>
<th>Hymenoptera</th>
<th>Isoptera</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eusocial species</strong></td>
<td>all ants, some bees,</td>
<td>all termites</td>
</tr>
<tr>
<td></td>
<td>some wasps</td>
<td></td>
</tr>
<tr>
<td><strong>Evolution of sociality</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Numbers</strong></td>
<td>at least 7 times</td>
<td>once</td>
</tr>
<tr>
<td><strong>The earliest fossil record</strong></td>
<td>a fossil nest of social vespids</td>
<td>a worker caste individual</td>
</tr>
<tr>
<td><strong>Time</strong></td>
<td>within the upper Cretaceous</td>
<td>the upper Jurassic or lower Cretaceous period</td>
</tr>
<tr>
<td><strong>Development</strong></td>
<td>Holometabolism</td>
<td>Hemimetabolism</td>
</tr>
<tr>
<td><strong>Chromosome system</strong></td>
<td>haplodiploid</td>
<td>diploid</td>
</tr>
</tbody>
</table>
living insects, but conform to a minimum environmental impact becomes very important (Su, 2002).

1.2.2 Immunity in termite species

Because of the pest status, some termite species have become the focus of immune studies, owing to the potential to exploit immunity as a means of eradicating them (Chouvenc et al., 2011). Some initial researches have indicated the existence of a multi-level defensive mechanism in termite immunity.

At the level of the individual (Fig. 1.2), certain species of termites can mount an immune response by releasing antimicrobial molecules (Lamberty et al., 2001; Da Silva et al., 2003; Thompson et al., 2003; Bulmer and Crozier, 2004, 2006; Rosengaus et al., 2007) or by activating cellular encapsulation in their hemolymph (Chouvenc et al., 2009). Although a few immune relevant genes have been discovered in termites, not many of them with a clear immune function can be found in on-line databases (e.g., GenBank) with a full length of cDNA sequences. Four of these are PRRs: the gram-negative bacteria binding proteins 1 and 2 (GNBP1, GNBP2) (Bulmer and Crozier, 2006), a termite-unique antifungal peptide called termicin (Lamberty et al., 2001; Da Silva et al., 2003), and a $NF_{κB}$-like transcription factor that is homologous to Drosophila’s relish (Bulmer and Crozier, 2006).

At the level of the group (Fig. 1.2), certain termite species can complement their innate immunity by expressing a type of social immunity (Chouvenc and Su, 2010; Rosengaus et al. 2011). For example, they can mutually groom nestmates contaminated with fungal conidia (Rosengaus et al., 1998a; Shimizu and Yamaji, 2003), respond to and communicate information about hygienic breaches to the colony (Rosengaus et al., 1999; Myles, 2002), limit the spread of disease by sequestering or cannibalizing infected nestmates (Rosengaus and Traniello, 2011; Chouvenc et al., 2008), produce antifungal fecal pellets to coat the inside of their nest (Rosengaus et al., 1998b), and may even be able to transfer their immunity to nestmates (Traniello et al., 2002). All these behavioral adaptations suggest that termites can take advantage of their persistent and repeated interactions with kindred to mutually bolster immunity or mutually reduce susceptibility to disease.
Figure 1.2 Schematic overview of the levels of immune mechanisms in termite colonies.
1.3 *Reticulitermes flavipes*

The Eastern subterranean termite *Reticulitermes flavipes* (Rhinotermitidae) is used as the eusocial insect model in my thesis. This species is widely distributed in North America including in southern Ontario, Canada. The laboratory maintained colonies that I use in my thesis are collected from Point Pelee National Park and Pelee Island regions in southern Ontario during 2009-2010 (Raffoul et al., 2011). Like other termite insects, these Canadian *R. flavipes* populations are generally organized into the two non-reproductive castes, the worker and the soldier, and one reproductive caste, the brachypterous nymph. In northern populations of *R. flavipes* there is no sexual alate (imago) caste, possibly due to the shortened season that renders this mode of colony founding and reproduction too costly (Clarke, et al., 2013). The worker caste, making up the majority of the colony, are primarily responsible for building and repairing the subterranean galleries, caring for eggs and young, foraging and for feeding other members, and also participate in colony defense. The primary function of the soldier castes is colony defense. Soldiers have specialized mandibles so are not capable of feeding themselves. They have to rely on the worker termites to provide them with regurgitated food. As the only reproductive castes in Canadian populations, *R. flavipes* nymphs resemble workers except that they have a larger body size and wing-buds on their backs, and perform the colony tasks as workers.

1.4 Entomopathogenic Fungi

In order to understand what kind of immune strategies termites may apply against infection, we need to find an infective disease agent with which to effectively inoculate termites. As a major class of insect pathogen, entomopathogenic fungi produce conidia (asexual spores) that can attach on the surface of the host cuticle. They apply a combination of secreted enzymes and mechanical force to degrade the host cuticles. After germination, the fungal hyphae penetrate through the cuticle, develop *in vivo* and eventually kill the host, typically within a few days (Myles, 2002; Siva-Jothy, et al., 2005). In the termite literature, there are numerous reports of various entomopathogenic fungi that have the potential to infect and kill termites in the laboratory (Myles, 2002; Rath, 2000; Chouvenc et al., 2011). Among them *Metarhizium anisopliae* and *Beauveria bassiana* are the most frequently used for directly inoculating
termites. They have shown virulence against various termite species, including harvester termites (Hodotermitidae), dampwood termites (Termopsidae), drywood termites (Kalotermitidae), subterranean termites (Rhinotermitidae), and higher termites (Termitidae) (Chouvenc et al., 2011). These fungi are known to induce an immune response in termites, most often via topical application, and the response is typically measured as a function of survivorship (Rosengaus et al., 1998b; Traniello et al., 2002), defensive behaviours (Myles, 2002; Shimizu and Yamaji, 2003; Chouvenc et al., 2008; Yanagawa et al., 2008), cellular encapsulation (Chouvenc et al., 2009), or up-regulation of immune transcripts (Thompson et al., 2003; Rosengaus et al., 2007; Hussain et al., 2013).

1.5 Thesis overview

The aim of my doctoral study is to expand our knowledge of the proximate and ultimate factors affecting the evolution of immunity in social insects. Specifically, I seek to test how social immunity among individuals, if present, might affect the innate immune response within individuals in termite species. There are four data chapters in my thesis (Chapters 2 - 5). They are independently prepared as the research manuscripts for publication. Two of the data chapters have been published (Chapters 2 and 3) and the other two are currently under revision (Chapters 3 and 4).

1.5.1 Effect of social variables on individual immunity

In Chapter 2, I tested the ability of *R. flavipes* to withstand infection as a function of group size and group composition. To this end, I quantified the extent to which individual immunity is mediated by both the number and the nature of social interactions. I used survivorship as a measure of ability for individuals within groups to withstand *M. anisopliae* infection. To test whether group membership affects this ability, I measured individual survivorship as a function of variation in two socially important traits: group size and caste composition. Specifically, I measured differences in survivorship between treated and control groups across a range of group sizes for a fixed caste ratio, and across a range of caste ratios for a fixed group size. If individual immunity is affected by the frequency of social interactions, then survivorship should vary with manipulations in group size. Alternatively, if individual
immunity is affected by the nature of interactions, then survivorship should vary as a function of different caste ratios. I found that both group size and caste composition directly affected the survivorship of individuals within groups, but only caste composition moderated survivorship upon immune challenge. This study provided no evidence for a density-dependent social immune response in *R. flavipes*. However, it did suggest that the caste-specific nature of interactions might be more essential for controlling disease in a social context.

1.5.2 Immune gene diversity

In Chapter 3, I evaluated the immune genes diversity associated with an antifungal pathway (the Toll pathway) in *R. flavipes*. To this end, I applied the suppression subtractive hybridization (SSH) technique to generate a subtracted normalized cDNA library of genes excessively expressed upon fungal infection. At 24 hrs postinfection with *M. anisopliae*, the library revealed 182 expressed sequence tag (EST) clones that potentially represent immune responsive genes. The nucleotide sequence from a majority (97%) of ESTs assembled into a small number (n = 13) of contiguous sequences, with the remainder (n = 6) representing singletons. This screen therefore captured as many as 19 different mRNAs highly expressed in response to the fungal pathogen. Primary sequencing of all loci revealed that approximately half (n = 10) contained open reading frames with significant similarity to known proteins. These clones represent nuclear and mitochondrial coding genes, as well as putative long noncoding RNA genes. This pattern suggested that either the cDNA library failed to sample the complete diversity of immune gene activity within infection assay or that *R. flavipes* employs relatively few loci to combat fungal infection, at least as can be measured at 24 hrs postinfection. Two genes identified here: leucine-rich repeat neuronal protein 2-like coding gene (abbr. *LRR*) and tigger transposable element-derived protein 7-like coding gene (abbr. *Tc7*) were studied further in the following researches.

1.5.3 Pathogen-specific immune gene expression

In Chapter 4, I chose the most promising candidate immune genes identified from my subtractive screen (Chapter 3), along with some known termite immune genes (*GNBP1,*
and verified their expression against various entomopathogenic fungi. The fungi chosen represent a range of genetic distances, from congeneric (*Metarhizium anisopliae*, *Metarhizium brunneum*, *Metarhizium guizhouense* and *Metarhizium robertsii*) to phylogenetically distant (*Aspergillus flavus*, *Beauveria bassiana*). Using quantitative real-time PCR (qRT-PCR), I tested whether the immune response varies as a function of locus, and as a function of pathogen type. If the specificity of the immune-gene response of *R. flavipes* is limited to a pathogen class-level, then I predict a similar expression pattern across all fungi – that is, across all six immune-infection assays regardless of genus or species. If, by contrast, *R. flavipes* is more finely tuned to specific fungal infections, then I predict a more specific response at the genus or species level. In this study, I found that *R. flavipes* showed distinct immune-gene expression patterns following exposure to spores of *Metarhizium* spp., *Beauveria* sp., and *Aspergillus* sp.. Of the six immune genes screened, five (*GNBP1, GNBP2, Relish, Termicin* and *LRR*) were up-regulated in response to *M. brunneum* and *M. guizhouense*. *Relish* and *Termicin* were responsive to all *Metarhizium* spp. infections, while *GNBP1* and *LRR* were response to *B. bassiana* and two *Metarhizium* spp. (*M. anisopliae* and *M. robertsii*) infections. Finally, *GNBP2* was responsive only to two *Metarhizium* spp. infections. These pathogen-specific expression patterns suggest that a high degree of immunological specificity do exist in subterranean termite innate immunity, and the degree of this specificity is subject to species-level.

### 1.5.4 Effect of social context on individual immunity

In Chapter 5, I investigated how social response to infection might impact the innate response of individual *R. flavipes*. To test this idea, I challenged *R. flavipes* with *M. anisopliae* and then measured immune gene expression as a function of social context. I predicted that *R. flavipes* exposed to fungal infection would either ramp up immune gene expression within groups owing to the socially amplified potential for pathogen transmission, or individual termites would temper innate immune genes expression in response to a socially mitigated pathogen load. In this study, I found that immune loci tend to be less responsive within infected groups, compared to the response of singly infected individuals. For example, three immune genes (*GNBP1, GNBP2* and *LRR*) were up-regulated upon infection of singletons, but not disregulated upon grouped infection. Other immune loci (*relish, termicin*) showed up-
regulation on infection regardless of social context, or were non-responsive (Tc7) in this assay. The socially-responsive pattern of some immune loci detected here suggested that R. flavipes had the capability to adjust its innate immune response to its immediate social context. The observation of the ‘trade-off’ between two sets of immune gene expressions was consistent with a socially mediated immune system in which interacting individuals can mitigate each other’s response to disease.

1.6 References


Chapter 2

2 Effect of group size and caste ratio on individual survivorship and social immunity in a subterranean termite

A version of this chapter has been published in Acta Ethologica and is presented here with permission.


2.1 Introduction

The Hymenoptera (ants, bees, wasps, sawflies) and Isoptera (termites) contain many social species, some of which are fully eusocial (all termites) and thus have a permanent helper caste and strong reproductive division of labour (Wilson 1971). Eusocial species typically live in large, densely populated colonies of closely related and frequently interacting individuals. These demographic attributes are predicted to make social insects vulnerable to the social transmission of disease (Alexander 1974; Hamilton 1987; Schmid-Hempel 2011). Although group living is a burden on individual immunity, innate immune costs may be offset by behavioural adaptations that are not available to solitary insects. For example, social taxa can take advantage of their frequent interactions for mutual grooming or exploit other group-enabled behaviours that may reduce pathogen load (Cremer et al. 2007; Wilson-Rich et al. 2009).

In the honey bee Apis mellifera examples of so-called ‘social immunity’ include hygienic behaviour (Lapidge et al. 2002), the raising of offspring in sterile nurseries (Burgett 1997), social ‘fever’ in response to disease (Starks et al. 2000), cadaver disposal (Visscher 1983) and heightened risk-taking by infected individuals (Schmid-Hempel 2005). With respect to the study of termite social immunity, however, initial research has indicated that at least one dampwood (Termopsidae) species will increase mutual grooming in the presence of infectious spores (Rosengaus et al. 1998b), communicate information about the presence of disease to nestmates (Rosengaus et al. 1999), cannibalise-infected nestmates (Rosengaus and Traniello 2001), produce antifungal faeces and other body exudates to control infection within the nest.
(Rosengaus et al. 1998a), and even transfer immunity from immunised to previously unexposed nestmates through a process called ‘social vaccination’ (Traniello et al. 2002). These observations from *Zootermopsis angusticollis* suggest that termites in general may also have a well-adapted social immune system whereby behavioural interactions help resist the spread of disease.

The ability for termites or other insects to mount a social defence will likely depend on the average frequency of interactions within their societies. Careful studies on *Z. angusticollis* do support the notion that termite social immunity is density dependent - for example, the survivorship of nymphs was higher when exposed to disease in small groups compared to those exposed in isolation (Rosengaus et al. 1998b). In addition, the effectiveness of socially enabled defences may also depend on the nature of these interactions - for example, as mediated by different task specialists or castes. Termite soldiers may be specialised to protect the colony against macroorganisms like predatory ants (Prestwich 1984), but compared to workers may be poorly adapted to mechanically defend against pathogenic microorganisms. At any rate, behavioural repertoires of termite castes differ (Watson et al. 1985), as they do for all social insects, and thus group caste composition may also affect a colony’s ability to coordinate a social defence.

In this study, I tested the potential for a common pest species of termite, the Eastern subterranean termite *Reticulitermes flavipes* (Rhinotermitidae), to mount a social defence by quantifying the extent to which the number and nature of social interactions mediate individual survivorship in the face of disease. Specifically, I challenged groups of termites with a generalist entomopathogen and monitored their survivorship against untreated controls as a function of group size and group caste composition. If individual immunity is affected by the frequency of social interactions, then I expected the treatment (immune challenge) effect on survivorship to vary as a function of group size. Moreover, if individual immunity is affected by the nature of inter-individual interactions, then I expected the treatment effect on survivorship to vary as a function of caste ratio. In both cases, the mechanisms involved are unknown, so I could not make directional predictions about what specific group size or caste ratio ought to be important for social immunity in *Reticulitermes*. 
2.2 Materials and methods

2.2.1 Termite collection and maintenance

In the summer (June-August) of 2009, I collected termites from three genetically unrelated and well-characterised field colonies (Raffoul et al. 2011) in Point Pelee National Park (Essex County, ON, Canada), transported them back to the laboratory at the Biotron Environmental Research Facility at the University of Western Ontario, Canada, and reared within plastic containers (roughly 60×40×15 cm) within an environmental chamber set to 26°C, 85% relative humidity (RH) and 24 hrs darkness. To perpetuate each colony, I fitted each container with moist play sand as substrate, and provided plywood and single-face, two-ply cardboard as food. I checked colonies regularly for health and vigour, and provided additional water via a saturated sponge and vaporizer, as necessary. Each field-collected laboratory-maintained colony initially consisted between 1,000 and 2000 individuals and contained all three of the species-typical castes - that is, nymphs and functionally sterile workers and soldiers (Lainé and Wright 2003). For northern populations of this species, the alate (imago) caste is rare (Myles 1999) and was not observed in our samples.

2.2.2 Fungal culture and infectivity trials

I used an entomopathogenic strain of *Metarhizium anisopliae* (strain number 2575, from USDA collection, Ithaca, NY, USA) to infect groups of termites. This native fungus is known to cause significant mortality in termites; it uses hydrolytic enzymes to penetrate the insect cuticle, and subsequently the hyphae ramify within the haemocoel (Bidochka and Khachatourians 1994). First, I plated conidia (asexual spores) of *M. anisopliae* onto potato dextrose agar (PDA) media plates, and cultured them at 25 °C in darkness (Myles 2002). After approximately 14 days, I harvested the conidia by washing each plate with a sterile 0.01% Tween 80 solution, and then estimated the concentration of conidia in suspension using a conventional haemocytometer (Hausser Scientific). From these washes, I made dilutions of known concentration (10^3, 10^5, and 10^7 spores per mL), and stored these (4 °C) for no more than 1 week prior to using them in infectivity trials. I used these serial dilutions to first establish a dose for this strain that is sufficient to induce mycosis. I treated groups of termites
by allowing them to walk within a plastic Petri dish (100×15 mm) lined with filter paper (Whatman qualitative no. 5) impregnated with 1 mL of conidia suspension (Thompson et al. 2003). I established control groups from the same procedure but using sterile, conidia-free Tween solution. I could not precisely control for age of termites. Because termites are hemimetabolous, however, age is correlated with instar and size. I used only late instar (large) workers, late instar (brachypterous) nymphs, and soldiers (a terminal caste) in our analyses. Beyond this, I assumed a random distribution of ages with respect to treatment. Our sampling into Petri arenas may have included individuals that would not otherwise be interacting (for example, if they were spatially separated) but I assume they do not interact any more or less as a consequence. After 1 h, I transferred treated and control groups of termites to new Petri dishes lined with clean filter paper and damp cardboard. I then monitored the survivorship of each group (maintained at 26°C, 85% RH and 24 hrs darkness, as above) daily, for a maximum of 15 days.

2.2.3 Fungal virulence on *R. flavipes*

To establish an appropriate dose for this strain that is sufficient to induce mycosis and impose an immune challenge, I tested a series of spore dilutions (spores per mL) for their effect on termite survivorship. For this preliminary analysis, I simply compared the survivorship of treated versus control groups using a Kaplan-Meier survivorship analysis. I used a fixed caste ratio (all workers) and fixed group size (n=40).

2.2.4 Testing for social immunity

I used survivorship (time to death) as a measure of the ability for individuals within groups to withstand infection. To test whether group membership affected this ability, I measured individual survivorship as a function of variation in two socially important traits, group size and caste composition. Specifically, I measured differences in survivorship between treated and control groups across a range of group sizes for a fixed caste ratio (all workers), and across a range of caste ratios for a fixed group size. I assume that variation in group size (in effect, density) will affect the number of social interactions within groups, while variation in caste composition will affect the nature of social interactions within groups. Thus, if *R.*
flavipes expresses a type of social immunity whereby the nature or number of interactions affects an individual’s ability to withstand infection, then I expect a significant interaction effect of treatment × group size or treatment × caste ratio on survivorship, respectively.

2.2.5 Group size assay

I established groups of treated and control termites across a range of size classes: n=5, 10, 20, and 40 workers. These group sizes are small compared to the mature colony sizes of free-living colonies, but are experimentally tractable and reflect the size range of small parties and of incipient colonies. For each size class, I established a total of three biological replicates whereby each replicate was from an unrelated field colony. Here, relatedness was previously estimated from microsatellite DNA analysis (Raffoul et al. 2011). I recorded survivorship of each replicate daily and used these data to estimate the baseline hazard function via Cox proportional hazard regression (Cox 1972), as implemented in PASW Statistics (version 18.0 for Mac). Significant departures in shape of the hazard function were tested via step-wise addition of the factors ‘treatment’ (treated vs. control), ‘grouping factor’ (4 levels: 5, 10, 20, 40), and their interaction term to the model and comparing the fit (log likelihood) against the baseline model in which these parameters were not included. I tested goodness-of-fit between models using likelihood ratio tests.

2.2.6 Caste ratio assay

I established groups of treated and control termites across a range of caste ratios. Caste ratios vary widely in northern populations of R. flavipes, such that some colonies are replete with sterile soldiers (exceeding 10%) while others are all but void of the (potentially) reproductive nymph caste (GJT, personal observation; Husby 1980). This variation in caste composition probably reflects each colony’s plastic response to resources and subsequent investment into defence (soldiers) versus reproduction (nymphs), which may be erratic at the northernmost extreme of its range. Using a fixed group size (n=10), I experimentally manipulated the proportion of soldier/worker/nymph. From an observed average starting ratio of 2:6:2 (averaged from three laboratory colonies), I adjusted the proportion of workers to represent mild (3:4:3 and 4:2:4) or extreme (5:0:5) deficits in the worker caste. Likewise, I established
groups representing a mild (1:8:1) or an extreme (0:10:0) excess of the worker caste, relative to our starting ratio. Though arbitrary, I chose to manipulate the proportion of workers because the functional versatility of this caste makes it a good candidate to enact a social immune response. As above, I established a total of three biological replicates for each caste category and recorded survivorship daily as input for Cox regression analysis.

2.2.7 Confirmation of mycosis

Though *M. anisopliae* is a confirmed entomopathogen of a wide range of insects, including termites (Zoberi 1995; Rath 2000), I made an effort to verify the cause of mortality within our own trials. As termites died daily, I removed each cadaver and surface sterilised them individually with 95% alcohol. I then transferred each cadaver into a 0.25 mL Eppendorf tube containing PDA medium. I incubated each tube at 25 °C for 7 days (Rosengaus and Traniello 1997). If termites died from fungal infection, then treated termites will likely grow visible mycelia and produce spores. Meanwhile, if termites died from other factors, then cadavers are not likely to show such growth.

2.3 Results

2.3.1 Fungal virulence on *R. flavipes*

Concentrations of $10^3$ and $10^5$ conidia per milliliter were insufficiently virulent to affect survivorship, as indicated by a basic Kaplan-Meier survivorship analysis (log rank $\chi^2$: 0.45 and 0.10 respectively, $P$ value>0.05 in both cases, $df=1$, n=40). A concentration of $10^7$ conidia per milliliter was, however, sufficient to infect groups of termites and affect survivorship relative to controls (log rank $\chi^2=7.76$, $df=1$, $P=0.005$, n=40). At this concentration, the 2575 fungal strain is therefore sufficiently virulent to induce mycosis via topical exposure. I used this concentration for conducting the assays.

2.3.2 Group size assay

There were significant effects of treatment and social grouping on termite survivorship (Table 2.1). Both infection with *M. anisopliae* and group size directly affected individual termite
lifespan. Challenged termites had a hazard ratio of death 8.8 times higher (95% CI 6.6-11.8, Wald statistic=226.01, df=1, P<0.001) than did untreated termites, after controlling for variation in group size. Likewise, the least-densely grouped termites (in group size 5) had a hazard ratio of death 1.9 times higher (95% CI 1.3-2.9, Wald statistic=9.55, df=1, P=0.002) than the most-densely grouped termites (in group size 40), after controlling for treatment. This social grouping effect on survivorship was, however, not related to disease resistance: survivorship increased with group size in both infected and uninfected (control) groups, providing no evidence that individual Eastern subterranean termite have increased resistance in larger groups. That is, the treatment × group size interaction effect on survivorship was not significant (Table 2.1). Termites living within larger social groups simply live longer, but are not necessarily better (or worse) at resisting infection as a consequence (Fig. 2.1).

2.3.3 Caste ratio assay

There was a significant effect of treatment and social grouping on termite survivorship (Table 2.1). Both infection with *M. anisopliae* and group caste composition directly affected individual termite lifespan. Infected termites had a hazard ratio of death 8.5 times higher (95% CI 3.26-11.6, Wald statistic=172.76, df=1, P<0.001) than untreated termites, after controlling for variation in caste ratio. The group caste ratio (soldier/worker/nymph) with the lowest survivorship (1:8:1) had a hazard of death 4.4 times (95% CI 2.9-6.8, Wald statistic=44.79, df=1, P<0.001) higher than that of the group caste ratio with the highest survivorship (4:2:4), after controlling for treatment. Further, variation in caste composition does appear to be related to disease resistance, as evidenced by a significant treatment × caste composition effect on survivorship (Table 2.1). This moderating effect of social grouping on individual disease resistance was pronounced for three caste configurations (4:2:4 and 2:6:2 and 5:0:5) in which treatment and caste effects were not strictly additive. Instead, for these groups survivorship following exposure to pathogen was significantly higher (4:2:4 Wald
Table 2.1 Cox proportional hazard regression analysis for group size and caste ratio assay. In the group size assay, the factors are treatment (treated vs. control) and social grouping factor (4 levels: 5, 10, 20, 40 individuals), as well as their interaction term (treatment × group size). In the caste ratio assay, the factors are treatment (treated vs. control) and social grouping factor (nymph/worker/soldier ratios), as well as their interaction term (treatment × caste ratio).

<table>
<thead>
<tr>
<th>Factor</th>
<th>df</th>
<th>−2 Log likelihood</th>
<th>Chi-square</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group size assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>3,564.67</td>
<td>263.54</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Group size</td>
<td>3</td>
<td>3,551.10</td>
<td>13.60</td>
<td>0.004</td>
</tr>
<tr>
<td>Treatment × group size</td>
<td>3</td>
<td>3,548.85</td>
<td>2.26</td>
<td>0.521</td>
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<tr>
<td><strong>Caste ratio assay</strong></td>
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</tr>
<tr>
<td>Treatment</td>
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<td>188.07</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Caste ratio</td>
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<td>52.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment × caste ratio</td>
<td>5</td>
<td>2,751.91</td>
<td>30.61</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Figure 2.1 The association between treatment and subsequent survivorship (±SE) as a function of group size. A Cox proportional regression model confirmed that treatment (dashed lines) on monobast groups (all workers) is highly significant ($\beta=-2.181$, $SE=0.145$) and that group size is positively related to survivorship (relative to group size 5, group size 10 $\beta=-0.192$, SE=0.240, Wald=0.640, $P=0.424$; group size 20 $\beta=-0.531$, SE=0.222, Wald=5.708, $P=0.017$; group size 40 $\beta=-0.647$, SE=0.209, Wald=9.546, $P=0.002$). The interaction between group size and treatment on survivorship is, however, not significant (Wald=2.265, $df=3$, $P=0.519$). Solid line controls
statistic=13.15, df=1, P<0.001; 2:6:2 Wald statistic=11.49, df=1, P=0.001, 5:0:5 Wald statistic=10.29, df=1, P=0.001, respectively) than the most susceptible group (1:8:1; Fig. 2.2).

2.3.4 Confirmation of mycosis

Within social grouping factor, the effect of fungal infection on survivorship was highly significant across all group sizes and across all caste ratios (log-rank chi-square range 18.76—95.94, df=1 in all cases, P<0.001 in all cases). For treated groups of termites, the confirmation rate for *Metarhizium* infection was high. Of 529 cadavers collected from treated and control groups over the 15-day census period, 312 (59%) tested positive for live mycelia, and all of these were retrieved from the treated groups. No cadaver testing positive for mycosis was retrieved from control (untreated) groups.

2.4 Discussion

In this study, I used variation in group size and caste composition to characterise termite susceptibility to infection and tested whether social context modifies this susceptibility. Using the Eastern subterranean termite *R. flavipes* as a model, I did not find a group size effect on susceptibility to infection (Table 2.1; Fig. 2.1). Our assay therefore provides no evidence for a density-dependent social immune response, at least not to an appreciable degree over the range of size classes tested (5-40) against the common fungal pathogen *M. anisopliae*. I did, however, find an alternative social grouping effect: variation in the caste composition of groups did moderate individual susceptibility to infection, as evidenced by a strong treatment × caste composition effect on survivorship following exposure to the fungal pathogen (Table 2.1; Fig. 2.2). The interaction means that the treatment effect size depends on the particular caste ratio of the group. I suggest that for Eastern subterranean termites, it may not simply be the sheer number of interactions, as is important for social immunity in other termite species (Rosengaus et al. 1998b), but rather the caste-specific nature of these interactions that can help control disease in a social context.
Figure 2.2 The association between treatment and subsequent survival (±SE) as a function of group caste ratio (soldier/worker/nymph). A Cox proportional regression model confirmed that treatment (dashed lines) on mixed-caste groups is highly significant ($\beta=-2.134$, SE=0.162) and that caste ratio is related to survivorship (relative to 1:8:1, ratio 5:0:5 $\beta=-0.651$, SE=0.200, $Wald=10.531$, $P=0.001$; ratio 0:10:0 $\beta=-0.754$, SE=0.194, Wald=15.056, $P<0.001$; ratio 3:4:3 $\beta=-0.797$, SE=0.192, Wald=17.128, $P<0.001$; ratio 2:6:2 $\beta=-1.141$, SE=0.210, Wald=29.449, $P<0.001$; ratio 4:2:4 $\beta=-1.488$, SE=0.222, Wald=44.791, $P<0.001$).

Further, the interaction between caste ratio and treatment on individual survivorship is significant ($Wald=27.07$, $df=5$, $P<0.001$). The effect of treatment was highest for a ratio mildly in excess of workers (1:8:1) and lowest for a ratio mildly deficient in workers (4:2:4). Different lower case alphabetical letters denote significant differences in the survival distribution relative to the most susceptible reference (ref) population.

Solid line controls
2.4.1 Effect of group size on disease resistance

The use of a relatively small number of termites in laboratory containers does not approach the complexity of large colonies and subterranean habitat (Lenz 2009). Nonetheless, several comparable laboratory studies have demonstrated the potential for a group density effect on disease resistance. Specifically, nymphs reared in isolation show a lower resistance to infection than do nymphs reared in a group (Rosengaus et al. 1998b; Traniello et al. 2002). Dealates (imagoes) of this species were also better able to resist fungal infection when kept in male-female pairs, than individuals of either sex kept in isolation (Rosengaus et al. 2000). Data from subterranean termite species has been less forthcoming, but preliminary studies from R. speratus have also been used to suggest a group size effect on immunity: workers reared in groups of 10 were apparently more resistant to infection than were singletons (Shimizu and Yamaji 2003), and the related subterranean pest Coptotermes formosanus showed a nearly identical pattern whereby group membership seemingly conferred a higher level of resistance (Meikle et al. 2005; Yanagawa and Shimizu 2007). One issue with this latter group of studies is, however, that all termites are treated, with no comparison to untreated controls. As such, the treatment effect is confounded with the social grouping effect. From these preliminary studies, it is not known therefore whether termites in crowded groups may simply survive better, with or without treatment. To test for a social effect on resistance (not just survivorship) it is the treatment × group size interaction term that is informative.

I do find a strong effect of social group size on survivorship (Table 2.1), but this effect was not related to resistance. Termites living in larger groups live significantly longer, confirming that for many species of termites group size is positively correlated with survivorship (Lenz and Barrett 1984; Rosengaus et al. 1998b). I do not rule out the possibility of a density effect on resistance, and future studies could increase power to detect it over previous studies with larger sample sizes and increased ranges in size classes, perhaps spanning three or more orders of magnitude. Moreover, one could measure the magnitude or frequency of the group-enabled mechanism directly, if it were known. In natural colonies, the population size of R. flavipes is measured in the hundreds of thousands, if not millions of individuals. Thus, the potential for inter-individual interaction is vast, and even small effects on each other’s health may confer large additive, multiplicative, or even exponential effects on overall colony hygiene. Future
studies seeking to test for a density-dependent social immune response in *R. flavipes* should do so by looking for a modulatory effect of group size on the treatment effect. As a case in point, even *Z. angusticolis* in which density-dependent immunity is frequently reported (Rosengaus et al. 1998b; Traniello et al. 2002) shows no such effect when infection and social grouping are treated as independent factors (Pie et al. 2005). This and the present negative result highlight the need to carefully test density-dependent social immunity from a factorial design, or even a grouped factorial design that accommodates the population structure that is typical of social insects. The present study does not use a grouped factorial design and therefore does not take the potential non-independence of colony members into account.

### 2.4.2 Effect of group caste composition on individual immunity

How social interactions among individuals change as a function of caste composition within groups is difficult to quantify. Indeed, few studies have tested for caste effects on group immunity. Rosengaus et al. (2000) report a caste composition effect on disease resistance in two species of *Nasutitermes* (Termitidae) whereby worker monospecific groups outperform mixed caste or soldier monospecific groups. In their study, however, the caste groups also varied in size so it is not yet clear to what extent caste versus group size contributed to the observed survivorships. Rath (2000) provides contrasting survivorship data, in this case showing that soldier-worker mixed caste groups of *Nasutitermes exitiosis* resist *M. anisopliae* infection better than do monospecific groups of either caste. In our assay, I do find that individual survivorship varies directly with caste composition in *R. flavipes* and, intriguingly, this variation is related to disease resistance (Fig. 2.2). I speculate that pathogens are either (1) interacting differently with the natural ability of each caste to withstand infection or (2) the division in labour afforded by castes has a moderating effect on disease risk. If the former is true, anatomical or physiological characteristics of certain castes (nymphs and soldiers, as the case may be) may retard the onset of disease, even in the absence of explicitly hygienic behaviour interactions. If the latter, then caste diversity may sharpen behavioural divisions in labour and improve disease resistance through transfer of caste-specific physiological defences or sharing of complimentary behavioural defences (Boomsma et al. 2005; Elliot and Hart 2010). Our study cannot differentiate between these two possibilities. The association between social immunity and caste ratio that I observe is not dependent on the proportion of
workers (0-100%). Instead, disease resistance peaked with particular combinations of workers, soldiers and nymphs (Fig. 2.2). I speculate that soldiers and nymphs in combination are important to the colony in pathogen defence, and that pathogen-mediated selection for particular caste ratios, or even instar or gender ratios within castes (Rosengaus and Traniello 2001; Fefferman et al. 2007), may be an important aspect of termite social evolution. Soldiers have specialised mandibles and so might be inefficient groomers, but well equipped to remove or guard cadavers away from the colony. Mankowski et al. (2005) showed that for Coptotermes spp. the presence of workers with soldiers significantly reduced soldier mortality, and at least one study on Z. angusticolis (Rosengaus et al. 2007) showed that the soldier caste can upregulate antimicrobial proteins that are more effective at reducing conidial viability than are antimicrobials expressed by pseudergates (a worker-like caste found in some species). Soldiers may therefore represent an important element of communal defence against pathogenic microorganisms.

2.5 Conclusions

By using a behavioural assay, I showed that communal defence in termites may not simply be a matter of number of social interactions as inferred from group density, but may depend on the nature of these interactions as inferred from group composition. This finding is significant because it suggests that termites have greater resistance to disease than would be predicted from the frequency of interactions alone, and further indicates that the social immune response in termites may be subject to divisions in labour such that certain caste combinations are required for optimal function (Cremer et al. 2007), as are so many other aspects of colony growth, survival and reproduction. By extension, because group size and group caste ratio are uniquely social variables that are not applicable to the demographics of solitary animals, our results imply that R. flavipes expresses a type of ‘social immunity’ analogous to that found in some other social insects, including the honey bee A. mellifera (Evans et al. 2006) and dampwood termites Z. angusticolis (Rosengaus et al. 1998b). While density is expected to increase the average number of interactions among individuals within a society, caste-based divisions in labour are expected to reduce the overall number of interactions (Wilson 1971; Jeanne 1999; Ratnieks and Anderson 1999). Models that describe social interaction as a function of group size suggest that social connectivity increases with group density per se, but
that in natural colonies an increase to the number of individuals imposes spatial constraints that actually make complete mixing less likely (Naug and Gadagkar 1999; Pie et al. 2004). This localization of behavioural interactions with increases to colony size probably promotes behavioural specialisation and division of labour (Jeanson et al. 2007). It is therefore not obvious how variation in the number (density) and nature (caste) of social interactions will interact to affect total immunity in natural populations. Future studies should try to estimate this interaction effect through manipulation of both variables simultaneously within a single, fully factorial experiment.

2.6 Acknowledgments

I thank Beth MacDougall-Shackleton, Bryan Neff, Yolanda Morbey, Rebeca Rosengaus and all members of the Social Biology Group at the University of Western Ontario for useful discussion and comments, and R Greg Thorn for expert advice on fungal culturing. This work is part of a Doctoral thesis project for QG, and is funded in part by a Natural Sciences and Engineering Research Council Discovery (NSERC) grant to GJT.

2.7 References


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Chapter 3

3 Identification of mycosis-related genes in the Eastern subterranean termite by suppression subtractive hybridization

A version of this chapter has been published in Archives of Insect Biochemistry and Physiology and is presented here with permission.


3.1 Introduction

Insects inhabit a wide range of habitats that expose them to a diversity of microbial pathogens (Boucias and Pendland, 1998). Therefore, insects have evolved a variety of mechanisms to mitigate the impact of pathogen load and defend themselves against infection (Siva-Jothy et al., 2005). A principal mechanism involved in this defense is the innate immune system that is widely conserved in its main features across disparate taxa (Gillespie et al., 1997). For example, Dipteran immune models such as Drosophila (Lemaitre and Hoffmann, 2007) and Anopheles (Christophides et al., 2002) show four principal pathways (Toll, Imd, JNK, and JAK/STAT) that are preferentially stimulated by different classes of pathogens. These same pathways are functionally conserved across other insect orders from Hymenoptera (Evans et al., 2006; Tian et al., 2010) to Coleoptera (Zou et al., 2007) and Lepidoptera (Tanaka et al., 2008). One pattern to emerge from such broad comparisons is that the number of genes that makeup each pathway varies between species, with A. gambiae (Christophides et al., 2002), for example, showing roughly three times as many innate immune genes as Apis mellifera (Evans et al., 2006).

For termites (Isoptera) the genetics of immunity are less well studied, but several small-scale studies have begun to uncover termite immune-gene diversity. For example, termicin is a small, cysteine-rich antifungal peptide that shares structural similarities with the antimicrobial
insect defensins (Lamberty et al., 2001), and spinigerin is a 25 amino acid linear cysteine-free antibacterial peptide with structural similarities to the antimicrobial peptide cecropin A (Lamberty et al., 2001). Further, Thompson et al. (2003) isolated a transferrin gene up-regulated against fungal infection in the termite *Mastotermes darwiniensis*, and two gram-negative binding protein genes (GNBP1, GNBP2) and *Relish* were sequenced from *Nasutitermes* (Bulmer and Crozier, 2006). Finally, an enhancement of constitutive proteins in the hemolymph, as well as induction of novel proteins, are known from *Zootermopsis angusticollis* when exposed to an immunizing dose of fungal conidia (Rosengaus et al., 2007). Beyond these targeted studies, however, our understanding of the molecular immune response from termites is limited.

Using the Eastern subterranean termite *Reticulitermes flavipes* (Rhinotermitidae) as a model, I challenge groups of workers with a naturally occurring fungal pathogen, *Metarhizium anisopliae*, which is sometimes used in the context of pest control (Chouvenc et al., 2008). The fungal spores of *M. anisopliae* are known to cause mycosis in the same Ontario populations of *R. flavipes* that I use in the present study (Gao et al., 2012). Our goal is to identify immune-responsive genes from *R. flavipes* that potentially mediate an individual’s response to fungal challenge. For northern populations of this species, the alate (imago) caste is rare and was not observed in our samples. I therefore focus on the worker caste. To this end, I construct a normalized cDNA library for genes expressed on fungal infection. First, I infect a test population with a topical application of *M. anisopliae*. I then compare the gene expression profiles of infected and non-infected groups of workers via a subtracted cDNA library designed to isolate transcripts that are differentially expressed postinfection. From this library, I selected multiple clones for sequencing, and assign to each of these a predicted function via homology to previously characterized genes from termites and other taxa. Finally, I validate individual genes for differential expression between treated and control groups using quantitative reverse-transcription polymerase chain reaction (qRT-PCR) normalized to a set of endogenous reference genes.
3.2 Materials and methods

3.2.1 Termite collection and maintenance

I collected multiple, unrelated colonies of R. flavipes from Point Pelee National Park, Ontario, Canada, as previously described (Raffoul et al., 2011). From these field collections, I reared four laboratory colonies within large (36 L) plastic containers that were maintained within environmental chambers (26 °C, 85% relative humidity, and 24 hrs darkness) within the Biotron Institute for Experimental Climate Change Research (Western University, Canada). I fit each container with moist play-sand, plywood, and single-faced two-ply cardboard that the termites use as food and shelter. Generally, laboratory colonies of R. flavipes form stable breeding societies and are easily perpetuated for months or even years without decline.

3.2.2 Fungal challenge

I challenged termites by exposing them to a topical pathogen for 1 h. Specifically, I followed Thompson et al. (2003) and allowed groups of five workers to walk within a plastic Petri dish (100 × 15 mm) lined with filter paper (Whatman qualitative no. 5) impregnated with 1 mL of $10^7$ conidia/ml of M. anisopliae (strain number 2575, from USDA collection, Ithaca, NY) suspended in 0.01% Tween 80 solution. I chose this dose because it is known to induce a sublethal immune response (Gao et al., 2012). Unexposed (control) termites, by contrast, were treated to the same procedure but using a sterile, conidia-free Tween solution. Following exposure, I transferred treated and control termites to new Petri dishes lined with clean filter paper and damp cardboard. These termites were maintained under the same environmental conditions as above for 24 hrs. Finally, I flash froze all termites in liquid nitrogen prior to RNA extraction.

3.2.3 RNA extraction and cDNA synthesis

I extracted total RNA using the Trizol reagent (Invitrogen, Burlington, Canada) protocol, but performed the RNA wash and RNA elution steps using the columns from an RNeasy mini Kit (Qiagen, Toronto, Canada) (Koywiwattrakul et al., 2005). From treated and control groups, I pooled RNA extracted individually from the whole bodies of five treated or five control
workers to create infected and control RNA populations. I then used approximately 850 ng of RNA from each pool as template to synthesize first- and second-strand cDNA. For cDNA synthesis, I used the Super SMART PCR cDNA Synthesis Kit (Clontech, Mountain View, CA).

3.2.4 Subtracted cDNA library construction

I used the PCR-Select cDNA Subtraction Kit (Clontech) to generate a cDNA library of genes differently expressed upon fungal infection. Briefly, I defined the infected sample as the “tester” and control sample as the “driver.” I digested tester and driver cDNAs with Rsa I endonuclease to yield blunt ends appropriate for ligation. I subdivided the digested tester sample into two portions, and ligated each with a different adaptor (Adaptor 1 or 2R from Clontech kit). I then performed two hybridizations; first, I added an excess of driver sample to two separate tester samples, then hybridized again by adding a fresh volume of driver. Because cDNAs presented in both the tester and driver populations reannealed to form double-stranded cDNAs, any remaining single stranded molecules should represent uniquely expressed genes. These single stranded tester cDNAs are then targeted for PCR enrichment via adaptor-mediated priming (Diatchenko et al., 1996). I then cloned the PCR products by ligating amplified DNA fragments into a TOPO vector (pCR2.1-TOPO), then transforming recombinants into Escherichia coli (strain DH5α-T1) using the TOPO TA Cloning kit (Invitrogen). Finally, I used a GeneJET Plasmid Miniprep kit (Fermentas, Burlington, Canada) to blue/white screen recombinant clones prior to sequencing. An initial 215 clones were sequenced from the M13 forward primer using an Applied Biosystems 3730 analyzer.

3.2.5 Bioinformatic analyses

I manually trimmed all gene sequences to eliminate vector and adaptor sequence. To guard against contamination and false positives, I only considered as genuine sequences those that contained the Rsa I cut site (GT ↓ AC) and both adaptor sequences. For genuine transcripts, I used SEQUENCHER software (v5.0.1; Gene Codes) with custom setting (greater than 90% identity over a minimum of 50 bps) to assemble individual sequences into contigs. Contigs and any remaining singleton sequences were then submitted as query sequences against
nonredundant GenBank databases. I used BLASTn and BLASTx criteria to assess homology, and the conserved domains of sequences were derived from the CDD database on NCBI in silico (Marchler-Bauer et al., 2011). The “best-hit” was selected from each query under both BLAST algorithms. And the predicted open reading frames (ORFs) of the assembled clusters were further identified using EMBOSS Transeq (www.ebi.ac.uk/Tools/emboss/transeq). I also used the Gene Ontology (www.geneontology.org) annotation database and Blast2 GO software (www.blast2 go.de) to further infer the function of expressed sequence tags (ESTs).

### 3.2.6 Relative quantification of candidate gene expression

I used qRT-PCR to verify expression levels of the most promising candidate immune loci, as well as two other immune loci known from termites: GNBP2 (Bulmer et al., 2010) and Relish (Bulmer and Crozier, 2006). For this analysis I designed primers from cDNA sequence using PRIMER3 software (v0.4.0), and otherwise employed a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA) with qScriptTM One-Step SYBR® Green qRT-PCR Kit (Quanta Biosciences, Gaithersburg, MD). The primer sequences for all loci analyzed by qRT-PCR are shown in Table 3.1. The PCR thermoprofile was as follows: 95 °C for 3 min, followed by 45 cycles of 95 °C for 10 s, 55-60 °C (depending on the locus) for 30 s, and 72 °C for 10 s. Finally, I performed a melt curve analysis (from 65 to 95 °C) to confirm specificity of primer annealing. In all cases, I used groups of n = 10 (five treated, five control) workers sampled from each of eight unrelated field-caught colonies as biological replicates. In addition, I included three technical replicates per treated and per control sample.

I normalized all expression-fold calculations at target loci against a set of endogenously expressed reference genes. In a pilot study, I screened multiple candidate reference genes (\textit{GADPH, NADH, HSP70, Amylase, 18 S, β-actin}) using the GENORM utility (Hellemans et al., 2007) within qbasePLUS2.0 software (Biogazelle, Zwijnaarde, Belgium). For each locus, GENORM calculates an index of expression stability (\textit{M}-value) and, by this criterion, identifies the most stably expressed gene given the experimental conditions (Vandesompele et al., 2002). From this process, \textit{18 s, GADPH}, and \textit{β-actin} were deemed stably expressed (i.e.,
Table 3.1 Primers sequences for all candidate immune loci analyzed by qRT-PCR.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Tm (°C)</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>59</td>
<td>AGCGGGAAATCGTGCCTGAC</td>
<td>CAATAGTGATGACCTGGCCGT</td>
</tr>
<tr>
<td>Leucine-rich repeat neuronal protein 2-like</td>
<td>56</td>
<td>TTGCCTCTCATCTCTCTCTG</td>
<td>TCCTTGGGTTTGATGATG</td>
</tr>
<tr>
<td>NADH dehydrogenase subunit 5</td>
<td>60</td>
<td>GTTAGGTTGGGATGGTTTG</td>
<td>CATACCACCGCCATAAGACC</td>
</tr>
<tr>
<td>Tigger transposable element-derived protein 7-like</td>
<td>57</td>
<td>GGACCTGGCTGCTCAATATG</td>
<td>GGTTTTTACCCCTTTCACC</td>
</tr>
<tr>
<td>Cytochrome b</td>
<td>59</td>
<td>CGCGGAAACACAAATTCTACC</td>
<td>CGGGCTCAAATCAAGTAAG</td>
</tr>
<tr>
<td>IS58 mitochondrion gene</td>
<td>57.5</td>
<td>TTCTGTGCTTTTTAACTTGG</td>
<td>TCCAATACTTCATGTCATACCC</td>
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<tr>
<td>GNB2</td>
<td>56</td>
<td>CCAGGGGGGCTCTTATCTTT</td>
<td>GAGGGACCAGTTTGATGT</td>
</tr>
<tr>
<td>Relish</td>
<td>57</td>
<td>GTATGGGCATCATAACACAGC</td>
<td>AGGTTCATCCTCTTAGCCTCCAG</td>
</tr>
</tbody>
</table>
\( M\)-value < 1), with \( \beta\)-actin being the most stable. I therefore used \( \beta\)-actin as an endogenous reference gene to normalize all expression-fold calculations against any systematic bias. Finally, to estimate the gene expression difference between treated and control samples, I used the 2\(^{-\Delta\Delta CT}\) method of Livak and Schmittgen (2001).

### 3.2.7 Statistical analysis

I coded treatment and colony as fixed and random variables respectively in a two-way ANOVA. To assess their effect on gene expression I used only real-time data that showed a single peak on the melt curve analysis. I performed all statistical calculations using PASW Statistics (version 18.0 for Mac).

### 3.3 Results

#### 3.3.1 Overview of immune-induced genes

A total of 215 clones were randomly selected for sequencing from the subtracted library, resulting in 182 ESTs (> 80 bps) with high sequence quality available for further analysis. Using SEQUENCHER software (5.0.1), I assembled these sequences into 19 unique clusters, comprising 13 contigs and six singleton sequences (Table 3.2). The average length of all clusters was 433 bp (range = 100 - 1,097 bp). In addition, the redundancy of those EST sequences was approximately 97% (number of ESTs in contigs/ total number of ESTs), which suggests that our sequencing effort covers most of the transcripts present in the SSH library. The largest two contigs (contig245: 59 ESTs and contig246: 66 ESTs) contained the majority (total \( n = 125\)) of ESTs, and these two sets of ESTs had a similar average length (992 and 1,097 bp). Intriguingly, both of the large contigs are characterized by a long noncoding region that includes a ~20 bp repeat (5’ - ACCACTGCTTACTCTGCGTTGAT- 3’) that is typical of long noncoding RNA (lncRNA). In addition, these two sequences show few or no ORFs, suggesting that our library has captured a mix of protein coding and noncoding (RNA) transcripts. I have deposited all 19 cluster sequences into GenBank with accession numbers JK747866–JK747884.
Table 3.2 Bioinformatic analysis of ESTs from the subtracted cDNA library of *R. flavipes*.

<table>
<thead>
<tr>
<th>Cluster ID</th>
<th>ESTs (NO.)</th>
<th>Size (bp)</th>
<th>Best hit</th>
<th>Predict ORF</th>
<th>E-value</th>
<th>Organism</th>
<th>Sequence description</th>
<th>GO attributes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Singletons</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>T2-1-611</td>
<td>1</td>
<td>100</td>
<td>No match</td>
<td>Yes</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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</tr>
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<td>TN60-123</td>
<td>1</td>
<td>214</td>
<td>YP_001256899</td>
<td>Yes</td>
<td>4×10^{-25}</td>
<td><em>Reticulitermes flavipes</em></td>
<td>NADH dehydrogenase subunit 1</td>
<td>Catalytic activity, cell respiratory</td>
</tr>
<tr>
<td>TN16-123</td>
<td>1</td>
<td>241</td>
<td>YP_001256895</td>
<td>Yes</td>
<td>6×10^{-42}</td>
<td><em>Reticulitermes flavipes</em></td>
<td>NADH dehydrogenase subunit 4</td>
<td>Catalytic activity, cell respiratory</td>
</tr>
<tr>
<td>T2-19-611^a</td>
<td>1</td>
<td>322</td>
<td>XP_001949288</td>
<td>Yes</td>
<td>1×10^{-20}</td>
<td><em>Acyrthosiphon pisum</em></td>
<td>Leucine-rich repeat neuronal protein 2-like</td>
<td>Protein-protein interaction, protein binding</td>
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<td>T8-65</td>
<td>1</td>
<td>557</td>
<td>CP000226</td>
<td>Yes</td>
<td>0.12</td>
<td><em>Drosophila melanogaster</em></td>
<td>Unknown</td>
<td>N/A</td>
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<td>T7-6-724^a</td>
<td>1</td>
<td>603</td>
<td>ABN10451</td>
<td>Yes</td>
<td>3×10^{-81}</td>
<td><em>Reticulitermes flavipes</em></td>
<td>NADH dehydrogenase subunit 5</td>
<td>Catalytic activity, cell respiratory</td>
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<tr>
<td>ContigN02</td>
<td>2</td>
<td>240</td>
<td>No match</td>
<td>Yes</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Contig251</td>
<td>2</td>
<td>382</td>
<td>No match</td>
<td>Yes</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<td>Contig267</td>
<td>2</td>
<td>481</td>
<td>EFN62404</td>
<td>Yes</td>
<td>0.84</td>
<td><em>Camponotus</em></td>
<td>Unknown</td>
<td>N/A</td>
</tr>
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<td>Contig</td>
<td>EST #</td>
<td>EST Length</td>
<td>Accession</td>
<td>Differential</td>
<td>Log2 Fold Change</td>
<td>Species</td>
<td>Gene Name</td>
<td>Function</td>
</tr>
<tr>
<td>----------</td>
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<td>Contig277</td>
<td>2</td>
<td>545</td>
<td>XP_802449</td>
<td>Yes</td>
<td>$3 \times 10^{-14}$</td>
<td><em>floridanus</em></td>
<td><em>Trypanosoma cruzi</em></td>
<td>Lipoprotein</td>
</tr>
<tr>
<td>Contig284</td>
<td>2</td>
<td>549</td>
<td>XP_001604845</td>
<td>Yes</td>
<td>2.8</td>
<td><em>Nasonia vitripennis</em></td>
<td><em>Reticulitermes flavipes</em></td>
<td>Similar to receptor tyrosine phosphatase</td>
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<tr>
<td>ContigN01</td>
<td>3</td>
<td>290</td>
<td>AAY83391</td>
<td>Yes</td>
<td>$2 \times 10^{-64}$</td>
<td><em>Reticulitermes flavipes</em></td>
<td><em>Nematostella vectensis</em></td>
<td>Exoglucanase-type cellulase</td>
</tr>
<tr>
<td>ContigN04</td>
<td>4</td>
<td>258</td>
<td>EF206314</td>
<td>Yes</td>
<td>$8 \times 10^{-123}$</td>
<td><em>Reticulitermes flavipes</em></td>
<td>Isolate IS13 mitochondrion</td>
<td>Catalytic activity, cell respiratory</td>
</tr>
<tr>
<td>Contig271</td>
<td>5</td>
<td>546</td>
<td>XP_001632369</td>
<td>Yes</td>
<td>0.11</td>
<td><em>Nematostella vectensis</em></td>
<td><em>Reticulitermes flavipes</em></td>
<td>Predicted protein</td>
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<td>Contig266$^a$</td>
<td>5</td>
<td>602</td>
<td>XP_003242975</td>
<td>Yes</td>
<td>$8 \times 10^{-30}$</td>
<td><em>Acyrthosiphon pisum</em></td>
<td>Tigger transposable element-derived protein 7-like</td>
<td>Regulation of transcription, DNA binding</td>
</tr>
<tr>
<td>ContigN07$^a$</td>
<td>7</td>
<td>263</td>
<td>YP_001256898</td>
<td>Yes</td>
<td>$5 \times 10^{-39}$</td>
<td><em>Reticulitermes flavipes</em></td>
<td><em>Cytochrome b</em></td>
<td>Catalytic activity, cell respiratory</td>
</tr>
<tr>
<td>Contig255$^a$</td>
<td>17</td>
<td>140</td>
<td>EF206317</td>
<td>Yes</td>
<td>$3 \times 10^{-64}$</td>
<td><em>Reticulitermes flavipes</em></td>
<td>Isolate IS58 mitochondrion</td>
<td>Catalytic activity, cell respiratory</td>
</tr>
<tr>
<td>Contig245</td>
<td>59</td>
<td>992</td>
<td>No match</td>
<td>No</td>
<td>N/A</td>
<td>No match</td>
<td>No match</td>
<td>N/A</td>
</tr>
<tr>
<td>Contig246</td>
<td>66</td>
<td>1097</td>
<td>No match</td>
<td>No</td>
<td>N/A</td>
<td>No match</td>
<td>No match</td>
<td>N/A</td>
</tr>
</tbody>
</table>

NB: EST #, number of expressed sequenced tags assembling to that cluster; N/A, not available (no homology detected).

$^a$ Differential expression of these genes is confirmed via qRT-PCR.
In total, 10 of 19 clusters showed homology to GenBank sequences. Of these, 10 were significant (E-value < 10−5) matches to proteins known from other invertebrates, including some that are homologous to *R. flavipes* (the focal species) and *Acyrthosiphon pisum* (pea aphid). According to the “best hits” these 10 matches correspond to the following genes: NADH dehydrogenase subunit 1, NADH dehydrogenase subunit 4, NADH dehydrogenase subunit 5, cytochrome *b*, leucine-rich repeat neuronal protein 2-like, tigger transposable element-derived protein 7-like, isolate IS13, IS58 mitochondrion gene, exoglucanase-type cellulose, and a lipoprotein (Table 3.2). The remaining nine clusters are apparently unique sequences that show no obvious homology to sequences in the nr database.

### 3.3.2 Gene Ontology Analysis

I was able to assign GO terms to all clusters that showed significant BLAST matches (Table 3.2). These terms are grouped into five functional categories: (i) “catalytic activity components in respiration and energy metabolism” (TN60-123, TN16-123, T7-6-724, ContigN04, ContigN07, and Contig255), (ii) “transcriptional factor in regulation of transcription” (Contig266), (iii) “cellular component in protein-protein interaction” (T2-19-611), (iv) “cellular component in glucan catabolic process” (ContigN01), and (v) “cellular component in signal transduction” (Contig277). Notably, the EST (T2-19-611) shows significant homology \( (E\text{-value} = 1 \times 10^{-20}) \) to a predicted transmembrane protein: leucine-rich repeat neuronal protein 2-like from the pea aphid (Fig. 3.1A). An additional BLAST query against the specialized LRRML database (version 1.6; Wei et al., 2008) indicates that the gene’s LRR C-terminal domain is very similar \( (E\text{-value} = 8.13 \times 10^{-9}) \) to a structural motif (ID: LRR 683; LQYLRLNDNPWVCDCRARPLWAWLQKFR) with immune function.

### 3.3.3 Quantitative RT-PCR Data

The quantitative analysis confirms that six coding genes from our cDNA library are up-regulated postinfection (Fig. 3.2). That is, the genes coding NADH dehydrogenase subunit 5, cytochrome *b*, leucine-rich repeat neuronal protein, tigger transposable element-derived protein, and IS58 mitochondrion gene are statistically up-regulated following fungal infection. The lipoprotein coding gene is not detectable by qRT-PCR despite repeated efforts with five
Figure 3.1 cDNA sequences and putative amino acid sequences of tigger transposable element-derived protein 7-like (A) and leucine-rich repeat neuronal protein 2-like (B). Tc5 transposase DNA-binding domain and DDE endonuclease domain tigger transposable element-derived protein are indicated by gray shading with either dashed line or solid line; LRR domain from leucine-rich repeat neuronal protein is indicated by gray shading. Stop codon is shown as an asterisk.
**Figure 3.2** Gene expression of candidate immune genes to fungal infection in control (white bars) and treated (gray bars) samples; (A) *Leucine-rich repeat neuronal protein 2-like*, (B) *NADH dehydrogenase subunit 5*, (C) *Tigger transposable element-derived protein 7-like*, (D) *Cytochrome b*, (E) *IS58 mitochondrion DNA*, (F) *GNBP 2*, (G) *Relish*. Significant differences were observed between control and treated groups at all loci, except *Relish* (Table 3.3). Termite colony ID (from left to right): C1-9, C1-13, C2-9, C2-12, C2-13, C3-9, C3-12, and C3-13 as control samples; T1-9, T1-13, T2-9, T2-12, T2-13, T3-9, T3-12, and T3-13 as treated samples. There was no significant effect of colony of gene expression (Table 3.3). Error bars are ± 1 SE.
different custom primer pairs. The gene showing the most pronounced up-regulation is leucine-rich repeat neuronal protein, which is expressed at approximately 4.63-fold higher in treated groups as against control groups (Table 3.3), depending on the colony (Fig. 3.2). The gene showing the least pronounced expression is cytochrome b, which is up-regulated approximately 2.3-fold on infection. I found that other loci screened via qRT-PCR showed intermediate levels of expression.

Furthermore, though not derived from our own library, I found that GNBP2 is up-regulated by approximately 4.47-fold change postfungal infection, whereas Relish is apparently not dysregulated in our assay (Fig. 3.2).

### 3.4 Discussion

In this study, I constructed an EST library to capture genes relevant to immunity in a pest subterranean termite. The library, constructed via suppression subtraction hybridization, yielded ~100 transcripts apparently up-regulated at 24 hrs postfungal infection, as evidenced by their clonal capture in the forward subtracted library. By design these cloned ESTs represent candidate immune relevant genes, subject to verification. One pattern to emerge from this screen is that isolated transcripts correspond to relatively few loci; they assemble into a total of 19 unique sequences that correspond to protein-coding and noncoding (RNA) genes. Prominent within our library, was an inferred leucine-rich repeat neuronal protein 2-like represented by a singleton clone (T2-19-611). This sequence shows a LRR domain that suggests a role in protein-protein interaction, and a potential function as signal transduction mediator involved in sensing the extracellular ligands, activating the intracellular signaling cascade, and eventually regulating the expression of relevant genes (Kobe and Kajava, 2001). If so, the gene represented by this clone may play an important role in termite immunity.

### 3.4.1 Description of normalized, immune-relevant cDNA library

Our library uncovered many clones but few genes. There is therefore a high degree of redundancy among the ESTs captured in our transcriptomic screen; most ESTs assembled into single-gene contigs (Table 3.2). This pattern suggests that either our library failed to sample the complete diversity of immune gene activity within our infection assay or that *R. flavipes*
Table 3.3  Summary statistics for qRT-PCR analysis of candidate immune genes isolated from *R. flavipes* following fungal infection.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Fold-change by factor</th>
<th>ANOVA</th>
<th>Relative fold-change in expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>df</td>
</tr>
<tr>
<td>Leucine-rich repeat neuronal protein 2-like</td>
<td>Treatment</td>
<td>21.67</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Colony</td>
<td>1.23</td>
<td>7</td>
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<tr>
<td>NADH dehydrogenase subunit 5</td>
<td>Treatment</td>
<td>14.77</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Colony</td>
<td>0.78</td>
<td>7</td>
</tr>
<tr>
<td>IS58 mitochondrion gene</td>
<td>Treatment</td>
<td>11.28</td>
<td>1</td>
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<td></td>
<td>Colony</td>
<td>1.56</td>
<td>7</td>
</tr>
<tr>
<td>Tigger transposable element-derived protein 7-like</td>
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<td>Colony</td>
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<td>7</td>
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<tr>
<td>Cytochrome b</td>
<td>Treatment</td>
<td>35.82</td>
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<td></td>
<td>Colony</td>
<td>1.67</td>
<td>7</td>
</tr>
<tr>
<td>GNBP2</td>
<td>Treatment</td>
<td>7.42</td>
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</tr>
<tr>
<td></td>
<td>Colony</td>
<td>1.71</td>
<td>7</td>
</tr>
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<td>Relish</td>
<td>Treatment</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>Colony</td>
<td>1.09</td>
<td>7</td>
</tr>
</tbody>
</table>
employs relatively few loci to combat fungal infection, at least as can be measured at 24 hrs postinfection. Previous gene capture studies performed for *R. flavipes* have reported relatively high levels of gene ontology (GO) function redundancy together with relatively low numbers of clear homologues to known genes (Scharf et al., 2003; Scharf et al., 2005; Steller et al., 2010). Moreover, there is some precedence for below expected gene diversity in at least one comparable immune gene screen. Gerardo (2010) used suppression subtractive hybridization (SSH) to study immune gene expression in the pea aphid challenged with E. coli, and did not detect several genes predicted for the immune deficiency (IMD) pathway thought important to the bacterial response. They suggested that the pea aphid might have a reduced immune gene repertoire compared to other insects like fruit flies (Lemaitre and Hoffmann, 2007), mosquitoes (Christophides et al., 2002), and beetles (Zou et al., 2007). It is not possible from our study to tell if termites have reduced immune gene counts in general, or merely express few genes that I was able to detect from SSH.

Termite immunity has coevolved with their social behavior. Like other social insects, termites have evolved multiple levels of defensive mechanisms that can be deployed to withstand infection, including social immunity; a group-level trait that can potentially bolster individual immunity (Gao et al., 2012). It is conceivable that social taxa can take advantage of their frequent social interactions to minimize pathogen load (Cremer et al., 2007). These group-level defenses can potentially compensate individual immunity and eventually lead to loss of immune genes (Evans et al., 2006). However, this idea remains speculative, and the present study does not provide an adequate test of this idea for termites. I suggest, however, that if termites do prove to have reduced immune gene diversity, it may be due to compensatory social defenses.

Of the genes I did find, I infer their function in catalytic activity (cytochrome b, NADH dehydrogenase subunit 5, and IS58 mitochondrion DNA), regulation of transcription (tigger transposable element-derived protein 7-like), protein-protein interaction (leucine-rich repeat neuronal protein 2-like), and signal transduction (lipoprotein). Comparable studies have similarly identified genes involved in transcription and translation (Vogel et al., 2011), respiration (Riddell et al., 2011), and energy metabolism (Altincicek et al., 2008; Anselme et
al., 2008) as associated with the insect immune response. NADH dehydrogenase and cytochrome b coding transcripts have been previously reported from immune studies of *Rhodnius prolixus* (Ursic-Bedoya and Lowenberger, 2007) and *Pieris rapae* (Fang et al., 2010). Our finding of these mitochondrial genes suggests that the immune response is energetically demanding. Tigger transposable element-derived protein contains a Tc5 transposase DNA-binding domain and a DDE endonuclease domain (Fig. 3.1B), which are necessary for efficient DNA transposition (Collins and Anderson, 1994). This transposable element may indirectly influence the regulation of immune gene expression in many hypothetical ways, such as, by inserting transcription start site and transcription factor binding site, reorganizing the existing regulatory elements, or even translating a new protein isoform (Feschotte, 2008).

Clones from our library assembled to reveal two large contigs (contig245: 59 ESTs and contig246: 66 ESTs) that encompassed a large number of ESTs (total n = 125). Intriguingly, these two sets of ESTs have a similar length (992 and 1,097 bp) and contain tandem repeats of about 23 bps (5’ - ACTCTGCGTTGATACCACTGCTT - 3’) with little or no ORFs. Their lack of homology precluded us from inferring a molecular function from GO analyses but I suggest they represent lncRNAs, which are characterized as noncoding transcripts longer than 200 bps (Dinger et al., 2008) and generally function to regulate the expression of protein-coding genes at different levels such as chromatin modification, transcription, and post-transcriptional processing (Mercer et al., 2009). Peng et al. (2010) recently implicated lncRNAs in mouse immunity.

For the first time, a predicted leucine-repeat neuronal protein-coding gene has been found in a termite species. The leucine-rich repeat (LRR) transmembrane domain is one of the most common structural motifs for molecular recognition. Its role in pathogen recognition is exemplified by the Toll-like receptor, a key component conserved across Animalia for initiating intracellular signaling events in innate immunity (Takeda and Akira, 2005).

SSH is useful for identifying genes with differential expression pattern from two closely related DNA populations. In particular, this gene-finding technique has proven useful for studies in insect immunity (Altincicek et al., 2008; Riddell et al., 2011; Vogel et al., 2011).
For future studies on termites, the number of immune genes found could increase with modification to our experimental design. For example, screening at multiple time points (i.e., 12, 36, 48 h) after infection, or at different developmental stages (i.e., larval instars) or from different castes (i.e., soldiers, workers, reproductives), may increase the diversity of clones retrieved, as might infection with different types of entomopathogens. An immune gene library can help determine if termites possess genes from the same conserved immune pathways known to combat infection in other insects. Characterizing immune genes may likewise help determine if termites possess different numbers of immune genes compared to other social and nonsocial insects.

3.5 Acknowledgments

I thank Michael Bidochka for generously providing the fungal strain. I also thank Shawn Garner, Alanna Backx, and Alison Camiletti from the Social Biology Group at the University of Western Ontario for help and discussion throughout all aspects of this project.

3.6 References


Chapter 4

4 Fungus infected termites show a pathogen-specific immune gene response

This paper is currently in preparation for submission to *Journal of Invertebrate Pathology*.

4.1 Introduction

Termites have the rare ability to digest cellulose, and as such are an important decomposer in tropical and other ecosystems (Nutting, 1990). Moreover, termites are highly social and thus have a labour force that can actively manipulate the environment to feed, protect and propagate their colonies (Wilson, 1971). These two qualities render termites effective pests of wood products in urban and agricultural settings (Logan et al., 1990; Rust and Su, 2012), and some species have thus been the focus of immune studies - owing to the potential to exploit immunity as a means of eradicating them (Vargo and Husseneder, 2009).

Termite immunity is becoming well-understood. First, group-living termites can perform a series of collective defenses, such as mutual grooming (Rosengaus et al., 1998; Shimizu and Yamaji, 2003), vibratory alarm behavior (Rosengaus et al., 1999; Myles, 2002) or cannibalism (Rosengaus and Traniello, 2001; Chouvenc et al., 2008) to minimize pathogen load and control the spread of disease in their colony. These behavioral adaptations are referred to as ‘social immunity’, but are best known from the social Hymenoptera (Cremer et al., 2007; Evans and Spivak, 2010). Second, comparative studies between termites and unrelated immune models such as *Anopheles* (Christophides et al., 2004), *Drosophila* (Lemaitre and Hoffmann, 2007) and *Apis* (Evans et al., 2006) suggest that termites retain some principal features of insect innate immune pathways, such as pattern recognition receptors (PRRs), signaling molecules and antimicrobial peptides (AMPs), that are involved in defense against fungi or bacteria (Lamberty et al., 2001; Da Silva et al., 2003; Bulmer and Crozier, 2004, 2006; Gao et al., 2012a; Hussain et al. 2013).
We know from in-depth study of immune models that humoral immune pathways can be differentially stimulated to produce specific antimicrobials in response to infection with different classes of pathogens (Iwanaga and Lee, 2005). For example, the presence of fungi or Gram-positive bacteria can activate the Toll pathway in the fat body of the insect hosts; and the Imd (Immune deficiency) pathway is activated mainly by Gram-negative bacteria (Lemaitre and Hoffmann, 2007). Furthermore, the discovery of Dscam (Down syndrome cell adhesion molecule) in Diptera (Watson et al., 2005; Dong et al., 2006) suggests a potential mechanistic basis of a higher level of pathogen-specific response in insect immunity. However, there is no evidence from termite species to indicate how their immunity response to different pathogens.

In this study, I test to what extent termites are able to mount a pathogen-specific immune response at the humoral level. If termites show a similar level of pathogen-class specific response, then I expect that the expression of immune pathway related genes in response to infectious agents may vary dependent on the type of pathogen. It is not know, however, just how specific the innate immune response can be for any species of termite. I use six species of fungi from three genera (Metarhizium, Beauveria and Aspergillus) to challenge populations of the Eastern subterranean termite Reticulitermes flavipes. I then used quantitative real-time PCR (qRT-PCR) to monitor the level of immune gene expression at six immune loci. If the specificity of the immune-gene response of R. flavipes is limited to a class-level, then I predict a similar expression pattern across all fungi – that is, across all six immune-infection assays regardless of genus or species. If, by contrast, R. flavipes is more finely tuned to specific fungal infections, then I predict a more specific response at the genus or species level.

4.2 Materials and methods

4.2.1 Termite collection and maintenance

I collected multiple geographically isolated colonies of R. flavipes from southern Ontario, Canada in the summer of 2009 and 2010. I trapped termites from free-living colonies along the shores of Lake Erie, and subsequently maintained and perpetuated each colony within plastic containers (34 × 21 × 12 cm) in the laboratory, as described in Raffoul et al. (2011).
Briefly, I maintained colonies of mixed castes (nymphs, workers, soldiers) at population sizes $>10^3$ in subterranean environments that consisted of moist sand and small pieces of wood. For the immune assays, I sampled a total of 35 worker termites from each of three colonies: two from Point Pelee National Park and one from Pelee Island (corresponding to colony codes: NWB, 061 and PI of Scaduto et al., 2012). I then exposed groups of $n = 5$ workers to one of seven alternate treatments, corresponding to a different fungal infection or an untreated control.

### 4.2.2 Fungal infections

To test the effect of different pathogens on the termite immune-gene response, I cultured $n = 6$ entomopathogenic fungal species: *Aspergillus flavus* (Trichocomaceae), *Beauveria bassiana* (Clavicipitaceae), *Metarhizium anisopliae* (Clavicipitaceae), *Metarhizium brunneum* (Clavicipitaceae), *Metarhizium guizhouense* (Clavicipitaceae), and *Metarhizium robertsii* (Clavicipitaceae). *Aspergillus* is a facultative pathogen of many plants and animals, whereas *Metarhizium* and *Beauveria* are obligate entomopathogenic fungi (Boucias and Pendland, 1998; St. Leger et al., 2000). They apply a combination of secreted enzymes and mechanical force to penetrate the host cuticles and access the hemolymph (St. Leger et al., 1993). For each fungus, I cultured conidia on a potato dextrose agar medium at 25 °C, in darkness (Myles, 2002). After sporulation (~14 days), I harvested the fungal conidia to make suspensions of known concentration. First, I used a haemocytometer to estimate the raw spore concentration, then diluted this concentration in sterile 0.02% Tween 80 (Sigma-Aldrich, St. Louis, MO), to achieve a final concentration $1\times10^7$ conidia/mL. I chose this concentration because it is an efficient dose to activate the immune response in various termite species (Hussain et al. 2010; Gao et al., 2012b).

### 4.2.3 Immune inoculation

I infected termites individually by first immersing them into fungal suspensions within a microcentrifuge tube, and then gently swirling for 5 s (as described in Yanagawa and Shimizu, 2007). Following this topical application of conidia, I transferred termites to a VWR grade 415 filter paper (VWR, Radnor, USA) and allowed them to dry for 10 min. For uninfected
(control) termites, by contrast, I handled termites in the same manner but substituted the conidial suspension for a conidia-free Tween 80 solution. After exposure and drying, I returned individual workers to their groups within new Petri dishes (100 × 15 mm) lined with clean filter paper. I maintained treated termites in the same laboratory conditions as above until they were sampled in liquid nitrogen after 48 hrs prior to RNA extraction.

4.2.4 RNA extraction and cDNA synthesis

From each group of termites, I subsampled n = 3 individuals for RNA extraction. I extracted total RNA from whole termite bodies using the TRIzol reagent (Invitrogen, Life technologies, California, USA) with the PureLink RNA Mini Kit (Ambion, Life technologies, California, USA). To minimize spurious variation in gene expression across samples, I first normalized individual extractions within an experimental group to a common concentration (30 ng/µL), and then pooled RNAs together prior to cDNA synthesis. By combining RNA from three individuals per treatment our 3×7 experimental design yielded n = 21 pooled RNA samples (18 treated, 3 control). I reverse transcribed each sample into cDNA using a qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, USA).

4.2.5 Selection of candidate immune genes

To measure the termite immune response, I measured gene expression at four known immune loci, \textit{GNBP1, GNBP2, Relish and Termicin}. In addition, I measured the response at an additional two loci that are strongly implicated in immune function (Gao et al. 2012a). They are: \textit{LRR}, which putatively encodes a leucine-repeat neuronal protein, and \textit{Tc7}, which is a transposable element-derived protein.

4.2.6 Quantitation of immune gene expression

I use Livak and Schmittgen's (2001) $2^{-\Delta\Delta C_T}$ method to estimate differences in gene expression as a function of pathogen treatment. For this analysis, I use $\beta$-\textit{actin} as an endogenously expressed reference gene to normalise experimental variation in gene expression estimates between samples. This gene is stably expressed under our experimental conditions – i.e., has an \textit{M}-value less than one (Hellemans et al., 2007), as calculated using the GENORM utility.
within the QBASEPLUS2.0 software suite (Biogazelle, Zwijnaarde, Belgium). Finally, for qRT-PCR analysis, I used primers that I designed from cDNA sequence (Genbank) for each target locus (Table 4.1). All primers are between 18-25 bp long, melt between 55 °C and 65 °C, and amplify with between 90-105 % efficiency.

For each amplicon I performed real-time reactions in triplicate using a CFX96 real-time PCR detection system (Bio-Rad). Each single PCR reaction consisted of approximately 52 ng of first strand cDNA template, 0.3 µL of each primer (10 nm), and 5 µL of PerfeCTa SYBR Green FastMix (2×) (Quanta Biosciences) within a final volume 10 µL. I used the following PCR thermal cycle: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 56-62 °C (depending on the locus) for 30 s, and 72 °C for 10 s, plus an additional step of melt curve analysis (from 55 to 95 °C). I performed the melt curve analysis to confirm specificity of primer annealing. I first used Student’s \( t \)-tests to compare the mean expression values at single loci between treated and control groups (one test for each pathogen). I then performed a more inclusive analysis of variance whereby I measured the fixed effects of fungal “pathogen” and “colony” of origin, on gene expression. I performed all statistical analyses using SPSS for Mac (version 18.0) and adopting a \( \alpha \)-value of 0.05.

Finally, I used hierarchical clustering to reveal loci co-expressed in response to a common infection and, likewise, to reveal pathogens that elicit a common transcriptional response. For this analysis, I up-loaded the full pathogen x locus gene expression matrix into CFX Manager software (version 3.0, Bio-Rad) and plotted normalized co-expression data using a clustergram.

4.3 Results

4.3.1 Immune gene expression

I successfully isolated and treated a grand total of \( n = 63 \) individual workers, and measured their gene expression at six target loci (Fig. 4.1). First, I observed a widespread response from our select loci to the infection. A majority of loci (5 of 6) showed significant up-regulation to infection in at least two of the infection trials. The most responsive immune loci were *Relish* and *Termicin*, which are up-regulated against all four *Metarhizium* spp. Another
Table 4.1 Primers used for real-time PCR amplification of one reference gene and six candidate immune genes in *R. flavipes*.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Tm (°C)</th>
<th>Nucleotide sequence (5'-3')</th>
<th>Product size (bp)</th>
<th>GenBank accession No.</th>
<th>PCR efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-actin</strong></td>
<td>59</td>
<td>AGCGGGAAATCGTGCGTGAC</td>
<td>120</td>
<td>DQ206832</td>
<td>90.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAATAGTGATGACCTGGCCGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GNBP1</strong></td>
<td>56</td>
<td>CCCAGGGCTTCAACAGACTC</td>
<td>80</td>
<td>JF683374</td>
<td>93.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCTCCTTCTCCCTTATTCACTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GNBP2</strong></td>
<td>56</td>
<td>CCAAGGGGGCTTATATTT</td>
<td>166</td>
<td>JF683375</td>
<td>94.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAGGACCGTTTGTAGTGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Relish</strong></td>
<td>57</td>
<td>GTATGGGCCATCATACATACTACGC</td>
<td>175</td>
<td>DQ058904</td>
<td>95.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGGTTCATCCTCTTAGCTTCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Termicin</strong></td>
<td>60</td>
<td>CATTGGAGAGGAACTTGTTGC</td>
<td>103</td>
<td>GU906807</td>
<td>93.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACAGTGGCCATAGAGATGATAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LRR</strong></td>
<td>56</td>
<td>TGTGCCTTCTTCCCTCTG</td>
<td>123</td>
<td>JK747883</td>
<td>102.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCCTGGGTTTGTGATTGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tc7</strong></td>
<td>57</td>
<td>GACCTGCTGCCTCAAATG</td>
<td>104</td>
<td>JK747869</td>
<td>103.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGGTTTTTACCCCTTCACC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.1 Relative expression profiles determined by RT-PCR for six candidate immune genes (GNBP1, GNBP2, relish, termicin, and Tc7) corresponding to six fungal treatments (Aspergillus flavus, Beauveria bassiana, Metarhizium anisopliae, Metarhizium robertsii, Metarhizium brunneum, and Metarhizium guizhouense). The expression levels shown as a fold-change relative to an untreated control group (set to 1) and normalized to an endogenously expressed reference gene (β-actin). Bars represent ± SEM, and asterisks indicate significant differences in gene expression between fungus-treated and control groups: * = P < 0.05, ** = P < 0.01, *** = P < 0.001.
two loci, *GNBP1* and *LRR*, are associated in their expression with exposure to three fungi (*M. brunneum*, *M. guizhouense* and *B. bassiana*), while *GNBP2* was only responsive to two (*M. brunneum* and *M. guizhouense*). Finally, the Tc7 locus was not disregulated against any of the six infection trails.

*Metarhizium* *spp.* elicited the strongest response across loci; exposure to *M. anisopliae* induced a major (~9-fold) response at the antimicrobial peptide locus encoding *termicin*, as well as a significant up-regulation at the locus encoding *relish*. Other species of *Metarhizium* yielded a similar pattern in gene response, with *termicin* consistently up-regulated to high degree, with other loci showing smaller but nonetheless significant up-regulation relative to controls. For example, both *M. anisopliae* and *M. robertsii* infections caused an up-regulation at both *termicin* and *relish* loci. Finally, against infection from *M. brunneum* and *M. guizhouense*, five loci were up-regulated following the same expression pattern, with *termicin* showing the highest expression, followed by *Relish, GNBP2, LRR* and *GNBP1* (Fig. 4.1).

The other two fungal genera, represented by *A. flavus* and *B. bassiana*, elicited a weaker response from the termite. Exposure to *B. bassiana* caused two genes, *LRR* and *GNBP1*, to up-regulate to a similar degree (1.52- vs. 1.53-fold). Exposure of termite workers to *A. flavus*, by contrast, had no significant effect among the loci tested. However, the statistical analysis also showed that “colony” itself as an independent factor had a significant effect on gene expression across multiple loci (Table 4.2).

### 4.4.1 Clustering analysis

Of the five genes differentially expressed, some showed similar patterns of expression in response to infection. Figure 4.2 shows the hierarchical clustering of gene-expression information. On one axis, I show that *termicin* and *relish* are relatively similar in their overall response to infection, as are *LRR* and *GNBP1*. On the other axis, pathogens within in the genus *Metarhizium* are more similar with respect to host gene expression pattern than any one species within this genus is to *Aspergillus* or *Beauveria*. In addition, this hierarchical cluster analysis classified six fungal species into two clusters: the four *Metarhizium* species cluster together, while *A. flavus* and *B. bassiana* form a separate cluster.
Table 4.2 Summary of statistical analysis for the effect of “colony” of origin on expression across the six candidate immune loci in *R. flavipes*. Bolded *P*-value with an underline represents a statistically significant difference that is observed between treated and control groups (*P* < 0.05).

<table>
<thead>
<tr>
<th></th>
<th><strong>GNBP1</strong></th>
<th></th>
<th></th>
<th><strong>GNBP2</strong></th>
<th></th>
<th></th>
<th><strong>Relish</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>F</em></td>
<td><em>df</em></td>
<td><em>P</em></td>
<td><em>F</em></td>
<td><em>df</em></td>
<td><em>P</em></td>
<td><em>F</em></td>
</tr>
<tr>
<td><em>A. flavus</em></td>
<td>2.429</td>
<td>2</td>
<td>0.130</td>
<td>19.252</td>
<td>2</td>
<td><strong>&lt;0.001</strong></td>
<td>2.382</td>
</tr>
<tr>
<td><em>B. bassiana</em></td>
<td>0.102</td>
<td>2</td>
<td>0.904</td>
<td>29.152</td>
<td>2</td>
<td><strong>&lt;0.001</strong></td>
<td>6.407</td>
</tr>
<tr>
<td><em>M. anisopliae</em></td>
<td>5.362</td>
<td>2</td>
<td><strong>0.022</strong></td>
<td>12.595</td>
<td>2</td>
<td><strong>0.001</strong></td>
<td>4.017</td>
</tr>
<tr>
<td><em>M. robertsii</em></td>
<td>7.107</td>
<td>2</td>
<td><strong>0.009</strong></td>
<td>12.394</td>
<td>2</td>
<td><strong>0.001</strong></td>
<td>13.65</td>
</tr>
<tr>
<td><em>M. brunneum</em></td>
<td>1.511</td>
<td>2</td>
<td>0.260</td>
<td>0.852</td>
<td>2</td>
<td>0.451</td>
<td>0.460</td>
</tr>
<tr>
<td><em>M. guizhouense</em></td>
<td>0.370</td>
<td>2</td>
<td>0.699</td>
<td>0.555</td>
<td>2</td>
<td>0.588</td>
<td>0.442</td>
</tr>
<tr>
<td></td>
<td>Termicin</td>
<td></td>
<td>LRR</td>
<td></td>
<td>Tc7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
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<td>---</td>
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<td>F</td>
<td>df</td>
<td>P</td>
<td>F</td>
<td>df</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td>A. flavus</td>
<td>11.096</td>
<td>2</td>
<td><strong>0.002</strong></td>
<td>9.769</td>
<td>2</td>
<td><strong>0.003</strong></td>
<td>1.186</td>
</tr>
<tr>
<td>B. bassiana</td>
<td>8.053</td>
<td>2</td>
<td><strong>0.006</strong></td>
<td>5.665</td>
<td>2</td>
<td><strong>0.019</strong></td>
<td>3.630</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>1.884</td>
<td>2</td>
<td>0.194</td>
<td>1.956</td>
<td>2</td>
<td>0.184</td>
<td>1.826</td>
</tr>
<tr>
<td>M. robertsii</td>
<td>2.954</td>
<td>2</td>
<td>0.091</td>
<td>1.848</td>
<td>2</td>
<td>0.200</td>
<td>2.417</td>
</tr>
<tr>
<td>M. brunneum</td>
<td>0.934</td>
<td>2</td>
<td>0.420</td>
<td>3.498</td>
<td>2</td>
<td>0.064</td>
<td>0.593</td>
</tr>
<tr>
<td>M. guizhouense</td>
<td>0.591</td>
<td>2</td>
<td>0.569</td>
<td>3.993</td>
<td>2</td>
<td><strong>0.047</strong></td>
<td>1.571</td>
</tr>
</tbody>
</table>
Figure 4.2 Hierarchical cluster analysis of five differentially expressed candidate immune genes. Each gene is represented by a single row of colored boxes; each fungal treatment is represented as a single column of colored boxes. Red color represents up-regulation, green color represents down-regulation, and black color represents no change on gene expression. The lighter the shade color, the greater the relative expression difference. The expression scale is represented as a gradation of color ranging from 9.5 fold induced genes indicated by saturated red to 0.5 fold repressed genes indicated by saturated green.
4.5 Discussion

Compared to the adaptive immune system of vertebrates, invertebrates are limited to an innate response and as such are less specific in their response to infection (Little et al., 2005; Rowley and Powell, 2007). However, invertebrates are nonetheless capable of pathogen-specific responses (Kurtz, 2005; Little et al., 2005; Rowley and Powell, 2007). Using an established immune-infection assay, and quantitative PCR, I showed that a series of putative termite immune genes are differentially expressed in response to different fungal pathogens. Specially, I found that some Canadian populations of the Eastern subterranean termite were able to adjust the amount of immune gene expression of recognition (GNBP1, GNBP2, LRR), signalling (Relish) and effector (termicin) genes in response to infections caused by different genera or even different species of entomopathogenic fungi. I therefore demonstrated that at least one pest species of termite has some capacity to adjust its immune gene response according to the nature of the pathogen – in this case, to the generic and even species level (Figure 1; Table 2). Termite innate immune responses are not only subject to broad class of pathogen types – for example, Gram-negative bacteria vs. Gram-positive bacteria vs. fungi – but can recognize and adjust this response to genus- or species-level.

4.5.1 Immunological specificity in *R. flavipes*

The entomopathogenic fungi selected in this study have been shown to be pathogenic to many termite species (Lenz, 2009; Chouvenc et al., 2010). For instance, the conidia of *M. anisopliae* caused a higher mortality to *Coptotermes formosanus* workers than *B. bassiana* did (Hussain et al., 2010). *Aspergillus* species show lower virulence against some termite species than *Beauveria* or *Metarhizium* (Chouvenc et al., 2008).

According to our expression profiles (Fig. 4.1), *R. flavipes* workers exhibit a high degree of specificity in their immune gene expression in response to *Aspergillus*, *Beauveria* and *Metarhizium* species. *Aspergillus flavus* showed the lowest virulence against *R. flavipes*, since none of the candidate immune genes displayed a significant change at transcript level. *Beauveria bassiana* had a slightly higher virulence than *A. flavus*, as the expression of *GNBP1* and *LRR* are up-regulated in the *B. bassiana* infected group. Although four *Metarhizium*...
species are arranged into a single cluster (Fig. 2), they still varied in their pathogenicity for *R. flavipes*. The fungi *M. brunneum* and *M. guizhouense* activated more immune genes to be expressed, and these fungi may be more virulent to *R. flavipes* than *M. anisopliae* and *M. robertsii*. The quantitative difference in the amount of up-regulation between immune genes from the six fungal species suggests that the termite’s immunological specificity is indeed at the species-level. However, the potential mechanism underlining this immunological specificity has yet to be fully resolved.

4.5.2 Hypothetical immune pathway in *R. flavipes*

According to what is known from the innate insect immune response of *Anopheles* (Christophides et al., 2004) and *Drosophila* (Lemaitre and Hoffmann, 2007), I can predict that our candidate immune genes in *R. flavipes* would be evolutionarily conserved to show similar expression trends in, and be functionally related to, an antifungal signaling pathway. Hypothetically, in the hemolymph GNBP1 functions as a pattern recognition receptor and binds to β-1,3-glucans in fungal cell walls to initiate the first immune signal that indicates the presence of the fungal pathogen (Bulmer et al., 2006). Then, LRR with its transmembrane domain may function as a signal transfer station to receive this “recognition of infection” signal, and trigger an intracellular antifungal singling pathway. Relish, as the last transcription factor, is transported into the nucleus of the immune cell where it activates the expression of the antimicrobial gene termicin (Bulmer et al., 2006). Termicin can then respond to the infection by breaking down the fungal cell membrane (Lamberty et al., 2001; Da Silva et al., 2003). In addition, GNBP2 may play a role as a supplement to directly attack highly virulent fungal pathogens (Bulmer et al., 2009), such as *M. brunneum* or *M. guizhouense*.

4.5.3 Colony of origin

In this study, I observed high variations in the expression pattern of candidate immune genes among three tested colonies (Table 2). Statistical analyses further indicated that, besides fungal “pathogen”, “colony” of origin is another factor that can affect the expression of some immune genes such as *GNBP2, Relish*, and *Termicin* in the presence of certain fungal species. It must be pointed out that this observation may be purely coincidental, and is not in our
original expectations. By increasing the group size or introducing more *R. flavipes* colonies, I should be able to further prove the authenticity of this finding in future. However, previous termite studies show that colony of origin could be a significant and independent factor of termite survival and immune function (Rosengaus et al. 1998; Calleri et al. 2006; Wilson-Rich et al. 2007; Fuller et al., 2011). Furthermore, these Canadian populations of the Eastern subterranean termite are genetically variable (Scaduto et al., 2012), which may also contribute to the plasticity of immune response in populations spread across different habitats with distinct selective pressures.

### 4.6 Acknowledgments

I thank Dr. Michael Bidochka for the gift of fungal strains, Dr. R Greg Thorn for expert advice on fungal culturing. I also thank Alison Camiletti and Emma Mullen from the Social Biology Group at Western University for help and discussion throughout all aspects of this project. This work was funded by an NSERC (Canada) Discovery Grant to G.J.T.

### 4.7 References


Chapter 5

5 Social context affects immune gene expression in a subterranean termite

A version of this chapter has been submitted to *Insectes Sociaux*.

5.1 Introduction

Immunity is fundamental to all life but social life adds a new level to models of infection and resistance (Schmid-Hempel, 2011). Eusocial insects that live in dense groups are vulnerable to socially transmitted disease, but may likewise have evolved socially-enabled defences that increase individual resistance to disease. Termites (Isoptera) are one example of a social insect with the potential for ‘social immunity’ – a phenomenon known best from the social Hymenoptera whereby individuals mitigate each other’s susceptibility to infection (Traniello et al., 2002; Cotter and Kilner, 2010; Rosengaus et al., 2011; Babayan and Schneider, 2012).

Like all insects, termites have an individualistic (innate) immune response that is generally characterized by the expression of antimicrobial molecules, and the activation of cell-mediated reactions that include cellular encapsulation, phagocytosis and nodulation against microbial invaders (Iwanaga and Lee, 2005; Ferrandon et al., 2007). Though termites are less well-studied than insect immune models such as *Anopheles* (Christophides et al., 2002), *Drosophila* (Ferrandon et al., 2007) or *Bombyx* (Tanaka et al., 2008), or even other social insects (e.g., Evans et al., 2006; Schluns and Crozier, 2009), we do know that certain species of termites can mount an immune response by releasing antimicrobial molecules (Lamberty et al., 2001; Da Silva et al., 2003; Bulmer and Crozier, 2004, 2006; Thompson et al., 2003; Rosengaus et al., 2007; Gao et al., 2012a) or via cellular encapsulation within their hemolymph (Chouvenc et al., 2009).

Above the level of the individual, certain termite species may complement their innate immunity by expressing a type of social immunity (Rosengaus et al., 2011) – that is, they take
advantage of their persistent and repeated interactions with kindred to mutually bolster immunity or mutually reduce susceptibility to disease. Termites can, for example, groom nestmates of fungal spores (Yanagawa and Shimizu, 2007), respond to and communicate information about hygienic breaches to the colony (Rosengaus et al., 1999; Staples and Milner, 2000; Myles, 2002a), limit the spread of disease by sequestering or cannibalizing infected nestmates (Rosengaus and Traniello, 2001; Chouvenc et al., 2008; Mburu et al., 2009), produce antifungal feces and other body exudates for release within the nest (Rosengaus et al., 1998), and may even be able to transfer immunity via a mechanism dubbed ‘social vaccination’ (Traniello et al., 2002). We do not know, however, how these or any social response to infection might impact the innate response of individual termites.

In this study I use Canadian populations of the Eastern subterranean termite (*Reticulitermes flavipes*) to test for social effects on the innate immune response of a social insect. I first challenge termites with a fungal pathogen and then measure immune gene expression as a function of social context. I predict that termites exposed to fungal infection will either ramp-up immune gene expression within groups owing to the socially amplified potential for pathogen transmission, or individual termites will temper innate immune genes expression in response to a socially mitigated pathogen load.

5.2 Materials and methods

5.2.1 Termite collections, experimental design and treatment with fungus

I trapped large numbers of termites from multiple unrelated free-living colonies in southern Ontario (Canada). I subsequently maintained and perpetuated these collections within the laboratory, as described in Raffoul et al. (2011). Briefly, I maintained colonies of mixed castes (nymphs, workers, soldiers) at population sizes of at least 1,000 individuals within plastic containers (34 x 21 x 12 cm) replete with a standard mix of soil, moist sand and small pieces of wood.

To test for social effect on immunity, I simply measured immune gene expression as a function of infection and social context (Fig. 5.1). Specifically, I compared gene expression at six immune-related loci between healthy (i.e., non-infected control) and infected termites.
<table>
<thead>
<tr>
<th></th>
<th>Infected</th>
<th>No infection control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single</strong></td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td><strong>Grouped</strong></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>24 hrs</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>48 hrs</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 5.1** Experimental design showing how termites are infected in social isolation (single termite) or in a social group of five (grouped termites), and how immune gene response was recorded after a relatively short (24 hrs) or long (48 hrs) time frame post infection. In total, I replicated this design across three unrelated termite colonies.
that were treated in social isolation or within small social groups. I measured the multi-locus transcriptional response at 24-hrs or 48-hrs post infection.

I cultured the fungus *Metarhizium anisopliae* on potato dextrose agar at 25 °C in darkness until sporulation (~14 days) (Myles, 2002b). I then harvested the conidia to suspend in sterile 0.02% Tween 80 solution (Sigma-Aldrich, St. Louis, MO), to a final concentration $1 \times 10^7$ conidia per mL. I have previously shown this dose to be suitable for inducing a non-lethal immune response in *R. flavipes* (Gao et al., 2012b). Prior to treatment, I sorted termites from their natal colonies into Petri dishes (100 × 15 mm). I then infected termites individually by immersing workers into the fungal spore suspension and gently swirling for 5 seconds (after Yanagawa and Shimizu, 2007). Non-treated termites were handled similarly but using a spore-free solution of Tween 80. I then removed excess fluid from each termite by resting it on filter paper (grade 415, VWR International, Radnor, PA) for 10 minutes. Following exposure, I gently returned workers to Petri dishes lined with fresh filter paper. After 24 or 48 hrs, I flash-froze all termites into liquid nitrogen prior to immune gene expression analysis.

### 5.2.2 RNA extraction, cDNA synthesis and qRT-PCR

I extracted total RNA from whole termites using a combined TRIzol (Life Technologies, Carlsbad, CA) and PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA) protocol. For grouped samples (Fig. 5.1) I attempted to minimize variation arising from the extraction procedure itself by normalizing RNA concentrations among individuals (to 30 ng/µL) prior to pooling them into a single extract. I synthesized cDNA from all RNA samples using a qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD).

To measure the immune response I selected six loci previously implicated as important to fungal infection in termites. Four of these loci are *GNBP1* and *GNBP2* (gram-negative binding proteins) (Bulmer and Crozier, 2006), *relish* (Bulmer and Crozier, 2006) and *termicin* (Lamberty et al., 2001; Da Silva et al., 2003). The remaining two loci, *LRR* (leucine-repeat neuronal protein) and *Tc7* (tigger transposable element-derived protein 7-like), are up-regulated upon fungal infection in *R. flavipes* (Gao et al., 2012a) and are therefore inferred to be immune-responsive. For each of these six loci, I designed amplicon primers from cDNA sequences using PRIMER3 software (v0.4.0; Table 5.1).
For each amplicon, I performed real-time polymerase chain reactions (qRT-PCR) in triplicate using a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA). I primed each reaction with 52 ng of first strand cDNA template, 0.3 µL of each primer (10 nm), and 5 µL of PerfeC Ta SYBR Green FastMix (2×) (Quanta Biosciences, Gaithersburg, MD), in a final volume of 10 µL. I used the following PCR thermal cycle: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 56-62 °C (depending on the locus) for 30 s, and 72 °C for 10 s. Finally, to guard against non-specific priming I programmed a post-PCR melt (from 55 to 95 °C) to allow for a melt curve analysis (Pfaffl, 2001).

I first performed an inclusive analysis that simultaneously specified treatment and social context as fixed factors (MANOVA). I then used a Student’s t-test for differences in mean expression between treated and control reactions at single loci and under a given social context. I performed all statistical analyses using SPSS for Mac (version 18.0). Finally, to quantify gene expression I applied the 2−∆∆C_T method of Livak and Schmittgen (2001), and chose β-actin as an endogenously expressed reference gene to normalize experimental variation in gene expression estimates between samples. β-actin was stably expressed under our experimental conditions – i.e., had an M-value less than ‘1’ (Hellemans et al., 2007), as evaluated using the GENORM utility within QBASEPLUS2.0 software (Biogazelle, Zwijnaarde, Belgium).

5.3 Results

Topical exposure of termites with fungal spores induced a strong immune gene response. This response was evident at 24 hours ($F = 545.38$, $P < 0.001$) and 48 hours ($F = 68.24$, $P = 0.001$) post infection (Table 5.2). Likewise, social context alone affected immune gene expression at 24 hours ($F = 25.53$, $P = 0.004$) and 48 hours ($F = 15.78$, $P = 0.010$) post infection. Finally, there is a significant interaction between infection and social context whereby grouped termites become less responsive to infection. This interaction is, however, time-sensitive; it is
Table 5.1 Primers used for real-time PCR amplification of one reference gene and six candidate immune genes in *R. flavipes*.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Tm (°C)</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Product size (bp)</th>
<th>GenBank accession No.</th>
<th>PCR efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>59</td>
<td>AGCGGGAAATCGTGCGTGAC</td>
<td>120</td>
<td>DQ206832</td>
<td>90.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAATAGTGATGACCTGGCCGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GNBPI</td>
<td>56</td>
<td>CCCAGGCTTCAACAGACTC</td>
<td>80</td>
<td>JF683374</td>
<td>93.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCTCCTTCTCCTTATTCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GNBPII</td>
<td>56</td>
<td>CCAGGGGGCTTATTATCTTT</td>
<td>166</td>
<td>JF683375</td>
<td>94.9</td>
</tr>
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<td></td>
<td></td>
<td>GAGGGACGTTTGGATGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relish</td>
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<td>175</td>
<td>DQ058904</td>
<td>95.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGGGTCATCCTCTTAGCTTCAG</td>
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</tr>
<tr>
<td>Termicin</td>
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<td>CATTTCAGAGAACTTGTGTC</td>
<td>103</td>
<td>GU906807</td>
<td>93.4</td>
</tr>
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<td></td>
<td></td>
<td>ACAGTGCGATAGAGATGATAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LRR</td>
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<td>JK747883</td>
<td>102.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCCTTGGTTTGTGATTGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tc7</td>
<td>57</td>
<td>GGACCTGGCTGCTCAATATG</td>
<td>104</td>
<td>JK747869</td>
<td>103.7</td>
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<tr>
<td></td>
<td></td>
<td>GGGTTTTTACCCCTTTCACC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.2 Factors affecting immune gene expression at two time points post infection (24 and 48 hrs), as evaluated from a multi factorial analysis of variance (MANOVA) in which fungal infection and social context are coded as fixed factors. In addition I report factor-dependent sources of variation via their interaction terms. Significant values are shown in bold letters.

<table>
<thead>
<tr>
<th>Multivariate tests</th>
<th>24 hrs</th>
<th>48 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wilks’ lambda</td>
<td>F</td>
</tr>
<tr>
<td>Fungal infection</td>
<td>0.001</td>
<td>545.39</td>
</tr>
<tr>
<td>Social context</td>
<td>0.030</td>
<td>25.53</td>
</tr>
<tr>
<td>Fungal infection × Social context</td>
<td>0.153</td>
<td>4.44</td>
</tr>
</tbody>
</table>
weak at 24 hrs ($F = 4.44, P = 0.087$) but becomes significant at 48 hrs ($F = 112.66, P < 0.001$).

All but one locus (all but $Tc7$) responded to infection with $M. anisopliae$, as evidenced by the up-regulation of the corresponding transcript in infected versus control termites at 24- or 48-hrs after infection (Fig. 5.2). The innate immune response was most evident after 48 hrs, where 5 of 6 loci showed significant up-regulation relative to non-infected controls. For some loci, this immune response varied as a function of social context. That is, $GNBP1$, $GNBP2$ and $LRR$ were up-regulated in isolated but not in grouped termites (48 hrs; Fig. 5.2b), a pattern that is captured by a significant infection $\times$ context interaction effect at each of these three loci ($GNBP1$: $F = 9.16, P = 0.016$; $GNBP2$: $F = 12.15, P = 0.008$; $LRR$: $F = 16.42, P = 0.004$; Table 5.3). A fourth locus also shows a social effect, but in the opposite direction; termicin is more responsive within social groups (Fig. 5.3b), as indicated by a significant infection $\times$ context effect after 48 hrs ($F = 12.94, P = 0.007$; Table 5.3).

5.4 Discussion

This study tests a central prediction from sociobiological theory – namely, that individual phenotypes may become interdependent within a social context. This superorganismal concept has its origins in kin theory, and can potentially explain how seemingly group-level adaptations can evolve at the level of the individual or gene (Hölldobler and Wilson 2009; Bourke 2011). Innate immunity is one phenotype that may be particularly responsive to social context – especially given the costs associated with individual immunity (McKean et al., 2008; Cotter et al., 2010) and the potential to reduce this cost via social interaction whereby individuals mitigate each other’s susceptibility to disease (Cremer et al., 2007; Cotter and Kilner, 2010; Babayan and Schneider, 2012). In this study, I test for social effects on the individual immune response of subterranean termites – a taxon (Rhinotermitidae) known for its complex social structure (Vargo and Husseneder, 2009) and for its susceptibility to individual (Chouvenc et al., 2009; Gao et al., 2012a; Hussain et al., 2013) as well as group-level (Myles, 2002a; Yanagawa and Shimizu, 2007; Chouvenc et al., 2008) infection. Using Canadian populations of $Reticulitermes flavipes$ as a model, I show that levels of
Figure 5.2 Gene expression patterns at six immune-related loci in *R. flavipes* following exposure to the fungus *M. anisopliae*. The upper panel (a) shows the normalised –fold difference in gene expression after 24 hrs, whereas the lower panel (b) shows the pattern after 48 hrs. Bars represent ± SEM, and asterisks indicate significant differences in gene expression between fungus-treated (grey bars) and untreated control termites: * = $P < 0.05$, ** = $P < 0.01$. 
Table 5.3 Factors affecting immune gene expression on a locus-by-locus basis at two time points post infection (24 and 48 hrs), as evaluated by a one-way analyses of variance in which fungal infection and social context are coded as fixed factors. Significant values are shown in bold.

<table>
<thead>
<tr>
<th>Univariate tests</th>
<th>Gene</th>
<th>24 hrs</th>
<th></th>
<th>48 hrs</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>d.f.</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td>Fungal infection</td>
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<td>0.001</td>
<td>13.07</td>
</tr>
<tr>
<td></td>
<td>Termicin</td>
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<td>&lt;0.001</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
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</tr>
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<tr>
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<tr>
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<td>0.156</td>
<td>9.16</td>
</tr>
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<tr>
<td></td>
<td>GNBp2</td>
<td>1.10</td>
<td>1</td>
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</tr>
<tr>
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<td>12.94</td>
</tr>
<tr>
<td></td>
<td>Tc7</td>
<td>1.61</td>
<td>1</td>
<td>0.240</td>
<td>1.36</td>
</tr>
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</table>
transcription at select immune loci vary as a function of infection and social context (Fig. 5.2). In addition to these two primary effects, I demonstrate a functional interaction between personal immunity and social context in a termite, suggesting that individual termites can adjust their immune response to the immediate social context.

Our assay showed a strong immune-gene response to fungal infection in *R. flavipes* (Table 5.2). Entomopathogenic fungi are often deployed in the context of termite pest control (Rath, 2000). *Metarhizium anisopliae* used in this study, as well as other species of fungi (e.g. *Beauveria bassiana*), can reliably be administered to induce an immune response, most often via topical application. For subterranean termites, this immune response to fungal infection is typically measured as a function of survivorship (Gao et al., 2012b), defensive behaviour (Myles, 2002a; Yanagawa et al., 2007), cellular encapsulation (Chouvenc et al., 2009), or up-regulation of immune transcripts (Gao et al., 2012a; Hussain et al., 2013). In general, it is clear that subterranean termites respond to *M. anisopliae*, to which they are naturally vulnerable (Zoberi, 1995). The present study builds on previous work to profile immune gene expression of *R. flavipes* across multiple loci (Gao et al., 2012a); following exposure to a fungal spore solution, the five loci sampled are not uniform in their response, with some loci more responsive than others. I likewise show that this response to infection varies with sampling period, with some loci significantly disregulated at 24 hrs, and others only after 48 hrs.

Our assay also showed a strong immune-gene response to social context (Table 5.2). Previous studies have shown that social context can affect measures of immunity, even in the absence of infection. For example, bees (Stow et al., 2007), thrips (Turnbull et al., 2011) and wasps (Hoggard et al., 2011) secrete cuticular antimicrobials in relation to social group size. In addition, bumblebees appear to differentially regulate their immune response when alone or in groups, independent of infection (Ruiz-Gonzalez et al., 2009; Richter et al., 2012). In termites, Gao et al. (2012b) showed the survivorship of *R. flavipes* following infection increased with small increases to social group size, and likewise showed how survivorship can vary when groups of fixed size are composed of different social castes. In the context of these studies, our observations suggest that social context on its own is an important predictor of termite and social insect immune function.
Finally, our assay shows how an individual’s response to infection varies with social context. Two *gram-negative binding protein* genes (*GNBP1, GNBP2*) and a *leucine-rich repeat* immune gene (*LRR*) showed a 1.5 to 3.5-fold increase in gene expression after 48 hrs when termites were maintained in social isolation. By contrast, these three loci were not up-regulated upon infection within small social groups (Fig. 5.2b). While previous works has clearly demonstrated the potential for infection or social context to independently influence termite health, few studies have manipulated both variables within a single, multi-factorial assay. Gao et al (2012b) were the first to show that individual Eastern subterranean termites modulated their immune response as a function of social context: termites grouped by caste varied in survivorship upon infection, demonstrating that the nature of social interaction can impact an individual’s resistance to infection. In this study I extend our understanding of how social context can impact termite health by showing that group size can likewise affect an individual’s response to infection, in this case measured by immune gene transcription. In so far as our assay reflects variation in group size (1 versus 5), I do detect a social group-size effect on the termite immune response. This group-size effect has previously been implicated as evidence for social immunity in subterranean termites (Yanagawa and Shimizu, 2007; Gao et al., 2012b). The apparent suppression of the individual immune response within a group context is consistent with the notion of ‘herd immunity’ in which group membership confers some advantage that mitigates the spread of disease (Cotter and Kilner, 2010; Babayan and Schneider, 2012). For highly social insects, this advantage may stem from cooperation, caste specialization, interaction, spatial structure, or some combination of these factors.

The social effect on immune-gene response to infection was, however, not always in the direction expected for herd immunity. The antimicrobial peptide termicin did show a context-dependent response to infection, but in this case was reduced among singletons and over-expressed within infected groups (at 48 hrs, but not at 24 hrs; Table 5.3). *Termicin*, like *GNBP1, GNBP2* and *LRR*, therefore does show a socially-modulated response to infection, but this response is in the ‘opposite’ direction. This contrasting pattern – here, with an apparent amplification of immune-gene response within social groups – suggests ‘herd susceptibility’ (Cotter and Kilner, 2010) in which group membership amplifies the threat of infection. Regardless, the social effect on expression of all four of these genes (*GNBP1,
indicates that social context can affect an individual’s response to infection, and can do so on a locus-by-locus basis. The specific pattern showing GNBP1, GNBP2 and LRR as less responsive, and termicin as more responsive, to infection within groups suggest that group living can impose costs or benefits, and these are not uniformly distributed across the immune genome. These two distinct scenarios may be indicative of the physiological functions of these immune genes. Termicin is secreted via the cuticle (Hamilton and Bulmer, 2012) and may inhibit M. anisopliae on the surface (Hamilton et al., 2011). Its up-regulation in infected groups may therefore represent a mutually beneficial response. The up-regulation of other three genes (GNBP1, GNBP2 and LRR) in singletons, by contrast, may represent some unknown in vivo immune pathway that is activated when grouped defence is not available. For obligatory social animals like termites, group membership is in itself important to individual health. Our study builds on this understanding to show that infection and social context are not just independent predictors of termite health, but rather interact to explain variation in the immune response that would not otherwise be evident.

5.5 Acknowledgments

I thank Michael Bidochka for the gift of fungal strains, and Greg Thorn for advice on fungal culturing. I also thank Gordana Rasic, Alison Camiletti and Emma Mullen from the Social Biology Group at Western University for helpful discussion. This work was funded by a Natural Sciences and Engineering Research Council (NSERC) of Canada Discovery Grant to G.J.T.

5.6 References


Chapter 6

6 General discussion

Immunity is fundamental to all life but social life in particular imposes a large burden on the immune system by increasing susceptibility to socially transmitted disease. Termites are among most typical and representative of all social insects. To resist infection and control disease transmission in large, densely populated colonies, termites have evolved a complex defense mechanism that includes a series of immune responses at the physical, cellular, humoral, and group levels. At the group level, many inducible cooperative behaviors, such as allo-grooming, can powerfully limit the impact of microbial invaders (Rosengaus et al., 1998a,b; Rosengaus et al., 1999; Myles, 2002; Traniello et al., 2002; Shimizu and Yamaji, 2003; Chouvenc et al., 2008; Chouvenc and Su, 2010; Rosengaus et al., 2011). Meanwhile, at the individual level, innate immune response can release both humoral effectors and cellular components into hemolymph to protect against the infections (Lamberty et al., 2001; Da Silva et al., 2003; Thompson et al., 2003; Bulmer and Crozier, 2004, 2006; Rosengaus et al., 2007; Chouvenc et al., 2009; Avulova and Rosengaus, 2011). However, the knowledge of how these immune mechanisms connect, influence and cause-and-effect each other is very limited. To this end, I studied the immune responses in individuals and groups of the Eastern subterranean termite, *R. flavipes*, in response to a series of entomopathogenic fungi at the level of both individual and group with the aim of understanding the proximate and ultimate factors affecting interactions between innate immune response and social immunity in termites.

6.1 Innate immune response in *R. flavipes*

6.1.1 Discovery of immune-related genes

One of the most intriguing questions in the evolution of insect immunity is why some species, whatever solitary or social, especially from Hymenoptera, show significant evolutionary plasticity in their immune gene diversity. Some of them lose a considerable number of immune genes in their genomes (Evans et al., 2006; Bonasio et al., 2010; Smith et al., 2011). For example, the sequenced *Apis mellifera* genome possesses only one-third as many immune
genes when compared to the genomes of *Drosophila* and *Anopheles* (Evans et al., 2006). Others, like some ants, maintain an expansion in the number of immune genes in their genomes (Simola et al., 2013). For the first scenario, insect innate immunity may afford to lose a certain number of immune genes if some alternative defensive strategies are available, for example: social immunity; otherwise, if the reserved immune genes play roles in multiple innate immune responses (Vilcinskas, 2013). For the second scenario, the environmental factors, such as living condition, appear to play a significant role in facilitating an expansion in the number of immune genes or in certain immune gene families in some insect species (Simola et al., 2013; Vilcinskas, 2013).

In order to test the evolutionary plasticity of immunity in non-hymenopteran eusocial insect (Chapter 3), I used SSH to construct a normalized subtracted cDNA library of genes excessively expressed upon the infection of *M. anisopliae* in *R. flavipes*. This technology has been successfully used to discover many immune-related genes from a few insect species, including firebrat, drone fly, red flour beetle, and weevil (Altincicek and Vilcinskas, 2007a,b; Altincicek et al., 2008; Anselme et al., 2008). Gerardo et al. (2010) used a similar experiment to show that pea aphids are lacking many molecular elements of the antibacterial pathway (Imd pathway).

By introducing a single fungal treatment to *R. flavipes*, I was able to test the diversity of termite immune genes within a certain antifungal pathway (i.e. Toll pathway) at the early stage of the innate immune response. Toll pathway is widely conserved in insects (Christophides et al., 2004; Evans et al., 2006; Lemaitre and Hoffmann, 2007; Zou et al., 2007; Tanaka et al., 2008), but the number and type of immune genes associated with it different widely across species (Kafatos et a., 2009). My screen captured 19 different transcripts highly expressed in response to the fungal pathogen (Table 3.2). The majority of the ESTs are functionally defined as long noncoding RNAs (IncRNAs). Another two transcripts are homologous to the genes encoding leucine-rich repeat neuronal protein 2-like (abbr. LRR) and tigger transposable element-derived protein 7-like (abbr. Tc7) (Fig. 3.1), and were further studied in Chapter 4 and Chapter 5. Since none of 19 transcripts are homologous to the known immune genes from other insect models, therefore, it is very challenging to predict the immune gene diversity with limited molecular information.
However, other factors may cause the same scenario in my cDNA library. In the presence of fungal conidia, a series of immune responses can be activated in termites, which includes defensive behaviors (Rosengaus et al., 1999; Myles, 2002; Shimizu and Yamaji, 2003; Chouvenc et al., 2008; Yanagawa et al., 2008), cellular encapsulation (Chouvenc et al., 2009), or up-regulation of immune transcripts (Thompson et al., 2003; Rosengaus et al., 2007; Hussain et al., 2013). These immune responses occur at multiple levels and may cooperate with or compensate each other. Therefore, some of them may not need to be fully activated.

So far, it remains unclear, what kind of immune responses (behavioural, cellular, humoral) would be activated to which degree (non-specific, semi-specific, or specific) in termite immunity to combat this specific fungal strain. This consideration raised the research question for Chapter 4.

6.1.2 Pathogen-specific immune response

Insects lack the adaptive immune response of vertebrates, but their innate immune response is nonetheless pathogen-specific (Lemaitre and Hoffmann, 2007; Pham and Schneider, 2008). For example, the humoral immune pathways are differentially attuned to different classes of pathogens (Fig. 1.1), with Toll pathways being primarily responsive to fungi and Gram-positive bacteria, and the Imd pathway yielding antimicrobials only in response to Gram-negative bacteria (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007).

In Chapter 4, I tried to extend our current knowledge of this semi-specific framework in insect immunity to *R. flavipes* precisely, and demonstrated how specific the certain immune pathway responded can be to variation from within a single class of pathogens. Using qRT-PCR to monitor six immune genes response to infection from different pathogenic fungi, I found that *R. flavipes* expressed specific combinations of immune genes following exposure to fungal spores. Some previous studies have investigated the susceptibility of termites to different fungal infections. Hussain and Wen (2012) showed that *M. anisopliae* caused a higher mortality in *Coptotermes formosanus* workers than did *B. bassiana*. Compared to *M. anisopliae* and *B. bassiana*, *Aspergillus* sp. was less virulent to termites (Chouvene et al., 2008). In my study, *Metarhazium* spp. consistently induced more immune genes to be over-expressed at a higher level than *Beauveria* sp. and *Aspergillus* sp. (Fig. 4.2). In addition, *R.*
flavipes immunity appeared to be more susceptible to *M. brunnneum* and *M. guizhouense* infections than *M. anisopliae* and *M. robertsii* infections. By up-regulating a specific combination of immune genes, termite innate immune responses could specifically respond to different fungal infections. These pathogen-specific expression patterns suggest a high degree of immunological specificity does exist in *R. flavipes* innate immunity, and the degree of this specificity is subject to species-level.

The observations of the highly specific immune response are not rare in insects. One potential mechanism underlying the cellular-level has been detected in some species (Watson et al., 2005; Dong et al. 2006). Over 18,000 cell-surface receptors can be formed by Dscam, a hypervariable receptor, for pathogen-specific recognition, and eventually activate phagocytosis (Watson et al., 2005; Dong et al. 2006). In my assay, the specific combinations of immune gene expression suggest a plausible mechanism for a highly specific immune response at the humoral-level.

Effectively mounting an immune system is costly to individual survival (Rolff and Reynold, 2009). With a high degree of specificity in immunity, termites may able to keep a relatively low energy investment to withstand varied infections. Compared to my study in Chapter 3, the cDNA library did not capture many immune-related molecules at the early stage of immunity perhaps due to a pathogen species-specific response. To protect against *M. anisopliae*, a limited number of immune-related genes may need to be up-regulated via the humoral immune response.

### 6.2 Social immunity in *R. flavipes*

In eusocial society, maintaining relatively constant density (number of individuals) and caste composition (ratio) is the essential basis for the performance of the colony-level tasks, such as foraging, parental care, nest maintenance, and defensive behaviors (Schmid-Hempel, 1998; Cremer and Sixt 2009; Lenz, 2009). However, an over crowded living environment or a caste imbalanced society could also contribute to the contact-mediated disease transmission (Boomsma et al., 2005; Schmid-Hempel, 2005; Fefferman et al., 2007; Naug, 2008).
Although my arena test does not directly record what kind of specified social behaviors that the infected individuals performed, I did observe that allo-grooming and alarming behavior appeared immediately and lasted for the whole period of exposure in all fungal infected groups. The emergence of these social defensive behaviors upon infection is consistent with previous observations with termites (Rosengaus et al., 1999; Myles, 2002; Yanagawa and Shimizu, 2007). In my arena tests, without opportunity for termites to modify their environment, direct social interactions may be initially effective in dealing with acute infection.

6.2.1 The number of social interactions

The evidences from a few termite studies indicate that individuals reared within groups are more resistant to infection than are solitaires (Rosengaus and Traniello 1997; Rosengaus et al., 2000; Shimizu and Yamaji, 2003; Yanagawa and Shimizu, 2007). However, without comparison to healthy controls, we do not know if this group size effect is directly related to disease resistance.

By comparing the survivorships between healthy and *M. anisopliae* infected groups of *R. flavipes* (Chapter 2), I found that initial increases to group size appeared to improve the survivorship of *R. flavipes*, but this effect had nothing to do with disease resistance (Table 2.1). This study, as the first one, to treat termites over a broad range of group size classes (n=5, 10, 20, 40), indicates that individual immunity of *R. flavipes* is affected by the variation in the number of social interactions, and that the predominant mechanism for mutually conferred resistance is density independent. In other words, *R. flavipes* individuals from groups with different sizes maintain the same immunocompetence to withstand infection, and the communal defences may not be a matter of the number of social interactions. However, since the tested group sizes are much less than the natural colony size of *R. flavipes* (Lepage and Darlington, 2000), I cannot deny the possibility of the existence of a density-dependent immunity in *R. flavipes* colonies.
6.2.2 The nature of social interactions

By manipulating the group composition (soldier, worker, nymph) in a given group size (n=10), I found that the variation in caste ratio affected survivorship of fungal-infected *R. flavipes* (Chapter 2). And the nature of social interaction effect is strongly related to disease resistance (Table 2.1). Disease resistance of *R. flavipes* groups varied in a complex way with caste ratio (soldier: worker: nymph), such that groups slightly deficient in workers (4:2:4) best avoided infection, while those enriched for the worker caste (1:8:1) were most susceptible (Fig. 2.2).

Soldiers are unable to perform grooming due to their specialized mandibles (Roisin, 2000; Eggleton, 2011). They may become a burden to the worker castes when they combat the invasion of microorganisms via communal defensive behavior. However, groups enriched for soldiers survived relatively better in my test. It is possible that soldiers send out warning vibrations upon infection (Noirot and Darlington, 2000), or otherwise excite colonymates into a defensive mode. Soldiers may also be well equipped to remove or guard infected individuals away from the colony (Milner et al., 1998). Nymphs resemble workers and are probably more versatile than soldiers with a greater repertoire of behavioural tasks (Wilson, 1971; Noirot and Darlington, 2000). The co-variance between soldier and nymph numbers in my experiment may have contributed to group enabled immunity in the worker-less groups. Regardless, my results suggest that soldiers and nymphs in combination are important to the colony in pathogen defence, and that pathogen-mediated selection for particular caste ratios, or even instar ratios within castes (Rosengaus and Traniello, 2011), may therefore be an important aspect of termite social evolution.

A very recent genomic study of lower termite species, *Zootermopsis nevadensis*, further proved that social castes could invest differently in immune defence (Terrapon et al., 2014). In *Z. nevadensis*, reproductive females maintained a significantly higher level of immune gene expression compared to workers, nymphs, soldiers and even reproductive males. This observation provides a molecular-level reference to my prediction that social immunity in termites might also be subject to divisions in labour.
6.3 Effect of social immunity on innate immune response in *R. flavipes*

There is no doubt that being a member of social groups can provide many benefits from the groupmates via communal communications, such as a better disease resistance (Cremer et al., 2007; Cotter and Kilner, 2010). However, it remains unclear whether those behaviors that contribute to individual survival in infection can directly affect the immune responses at the molecular-level.

In Chapter 5, I extended our understanding of the effect of social living on individual immunity to the molecular-level. By measuring the expression of six immune genes (same as Chapter 4) to fungal infection, I found that social context significantly suppressed the expression of three immune genes (*GNBP1*, *GNBP2* and *LRR*), but the expression of *termicin* tended to be more responsive within infected groups, compared to the response of singly infected individuals (Fig. 5.2).

In termites, *GNBP1* and *GNBP2* are the known pattern recognition receptors (PRRs) which can recognize the β-1,3-glucans in fungal cell walls. In my previous study (Chapter 3), *LRR* is described as a plausible Toll-like receptor. The up-regulation of these receptors may indicate that some unknown antifungal pathway is activated. *Termicin* is a termite-unique antifungal peptide, and constitutively secreted via salivary gland (Lamberty et al., 2001; Da Silva et al., 2003; Hamilton et al., 2011; Hamilton and Bulmer, 2012). Termites can spread *termicin* onto their cuticles by grooming. This process can be considered as a social immune response if *termicin* spread by allo-grooming (Cotter and Killner, 2010; Bulmer et al., 2012). In my study, *termicin* significantly up-regulated in infected groups indicate that this humoral effector also play a significant role in achieving social immunity in *R. flavipes*. By consistently secreting *termicin*, the grouped individuals benefit a social-level protection via allo-grooming from each other. On the contrary, the singly infected *R. flavipes* is unable to obtain any social-level benefits, other innate immune response musts be activated (up-regulation of *GNBP1*, *GNBP2*, or *LRR*), and take over (suppress the up-regulation of *termicin*) to prevent the infected situation from deteriorating even further (Fig. 5.2b). This is a
convincing evidence of a direct trade-off between individual immunity and social immunity in \textit{R. flavipes}.

6.4 Antifungal immune pathway

In Chapter 4, I proposed a hypothetical antifungal pathway for \textit{R. flavipes} immunity. The expression patterns from Chapter 5 further proved the strong correlation in expression between \textit{GNBP1} and \textit{LRR, relish} and \textit{termicin} (Fig. 4.2).

As I predicted (Fig. 6.1), GNBP1 circulates in the hemolymph to recognize the specific pattern (β-1,3-glucans) in fungal cell walls. Then, LRR with a transmembrane domain functions as the Toll-like receptor to receive the “recognition”, and triggers an intracellular single. GNBP2 appears branch out from this pathway, and directly jump into combat. The final transcription factor, relish is released into nucleus to activate the final effector production of this antifungal pathway. Termicin is released back into hemolymph to break down the fungal cell membrane (Da Silva et al., 2003).

6.5 Conclusions and future directions

In this thesis, I have demonstrated the interaction between the group-level immunity (social immunity), and the individual-level immunity (innate immunity) in one termite species. By manipulating different social variables, I found group caste composition and social context are important to \textit{R. flavipes} to perform an effective social level protection from infection. I also found a convincing evidence of a direct trade-off between social immunity and innate immune response, which further indicates the social immunity among individuals can significantly affect innate immune response within individuals.

Due to the large number of \textit{R. flavipes} required, I did not control for age and sex, and manipulated test group with small size. However, age and sex of \textit{R. flavipes} may also be important as natural factors at the group-level to individual immunity. The small group size used does not to mimic the real social environment (population size) for \textit{R. flavipes} in nature. In the future, we can introduce more social parameters into the experimental design, such as a larger group size, social castes with different sex, or even different developmental stages (i.e.
larval instars). Since the first genome of a lower termite species, *Z. nevadensis*, was published, this genomic information can offer us a better insight into termite innate immunity. The aforesaid series of studies will eventually help us to solve mysteries behind the evolution of immunity in these social insects.
Figure 6.1 Hypothetical antifungal pathway in *R. flavipes*.
6.6 References


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Robert Brucker, Ph. D.

Biological Sciences

Vanderbilt University
Appendix B: Chapter 2 supplement material

Figure A. Cause-of-death analysis. The pooled number of cadavers retrieved from replicate (n = 3) treated and control termite populations over a 15-day census period. We retrieved significantly more cadavers from the treated group, suggesting that topical exposure to *M. anisopliae* was a significant cause of death. All cadavers were cultured on PDA medium to stimulate germination of fungal mycelia. Of the 312 cultured cadavers that germinated mycelia (picture), all (100%) were from the treated group.
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# Curriculum Vitae

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<thead>
<tr>
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