Phenoloxidase Activity in Isoptera: Dynamics Between Life History Traits and Immunocompetence

Thesis presented by:

Jennifer Lindsey Reichheld

To the Department of Biology

In partial fulfillment of the requirements for the degree of

Master in Science

in the field of

Biology

Northeastern University

August, 2010
Phenoloxidase Activity in Isoptera: Dynamics Between Life History Traits and Immunocompetence

by

Jennifer Lindsey Reichheld

ABSTRACT OF THESIS

Submitted in partial fulfillment of the requirements for the degree of Master in Science in the field of Biology in the Graduate School of Arts and Sciences Northeastern University, August 2010
Abstract

Insects are an evolutionarily and ecologically successful taxonomic group. To survive and be competitive, insects must have the ability to defend against the assault of pathogens and parasites. Arthropod immunity is comprised of two major components, the humoral and cellular responses. The latter is heavily dependent on the phenoloxidase cascade, although both components utilize this cascade. Phenoloxidase (PO) is a “versatile” enzyme, responsible for the process of melanization in arthropods. It plays a role in the sclerotization and tanning of newly molted exoskeleton while also being involved in several immune responses such as encapsulation, nodulation and wound healing. The quantification of PO activity has been used as a proxy for estimating the investment in immunity by a variety of species. Relatively few studies have focused on how sociality influences PO activity in social Hymenopterans (i.e. ants, bees and wasps) and fewer published studies exist regarding Isoptera (i.e. termites). Termites are faced with a unique set of challenges while living under significant pathogenic constraints. Their nesting, feeding and foraging ecologies favor exposure to varied microbial pathogenic/parasitic communities and their sociality can exacerbate disease transmission within a colony. Yet, these social insects thrive in microbial rich environments. In this study, PO activity was quantified and used to estimate immune investment as a function of termite phylogeny (and the accompanying nesting, feeding, foraging habits), gender and caste. This work presents evidence that PO activity varies across species and their corresponding specific life history attributes. Taxa that nest in microbial rich environments (i.e. soil) exhibit significantly higher PO levels than arboreal species. In terms of caste membership, those individuals with greater reproductive potential exhibit higher PO investment, although no sex-based differences in PO activity exist in most species tested. The quantification of PO activity during the course of fungal infection by
lethal dosages (2x10^6 and 2x10^8 conidia/ml) of the entomopathogenic fungus *Metarhizium anisopliae* indicates that the termite immune system responds with an up-regulation of PO activity two days after infection relative to controls. Termites provide a unique perspective on the evolution of the immune systems within the eusocial insects. These comparative data indicate for the first time that termite immune investment (measured in terms of PO activity) varies in accordance with predictions made by life history theory.
Acknowledgements

I would like to extend many thanks to my wife, family and friends for their constant support and encouragement. I would like to acknowledge the following people and groups for the role they have played in helping develop my research and writing this thesis.

My advisor, Dr. Rebeca Rosengaus, for introducing me to the extraordinary world of insects, and for providing me with feedback and advice. My committee, Dr. Gwilym Jones and Dr. Wendy Smith, for their advice, support, and teaching. Noah Wilson-Rich, for introducing me to the phenoloxidase assay, helping me develop the assay I used for this research, as well as for his encouragement and enthusiasm. Dr. Veronica Godoy for sharing her lab, her advice, her support, and allowing me unlimited use of the plate reader (Synergy HT-I spectrophotometer). Dr. Mark Bulmer for teaching me multiple techniques and for his unique perspective solving problems.

A special thanks to all of my comrades, graduate and undergraduate students at Northeastern University, who have provided support, advice, encouragement, and laughter. Thank you especially to Marielle Postava-Davignon, Tamara Hartke, and Kelley Schultheis for sharing their ideas, successes, and knowledge, as well as the Rosengaus lab undergraduate students, including Rhamy Zeid, for maintaining termites and spore solutions.

Dr. Shelley Adamo for her expert advice and comments, Dr. Thomas Chouvnec and Elsevier for allowing the use of previously published material in this thesis. To the administrators of the Redwood East Bay Regional Park District for collection of Z. angusticollis permits. The Smithsonian Tropical Research Institute (STRI) in Panama for N. corniger collection.
This research was funded by an NSF CAREER grant (DEB 0447316) awarded to Dr. Rebeca Rosengaus. The Rosengaus lab carries all the required USDA permits for the transportation and maintenance of the termite species used for this research.
# Table of Contents

Abstract .................................................................................................................................................. 3  
Acknowledgements ............................................................................................................................. 5  
Table of Contents ................................................................................................................................ 7  
List of Figures ........................................................................................................................................ 9  
List of Tables ......................................................................................................................................... 12  
Chapter 1: Introduction ........................................................................................................................ 13  
  1.1 Termites as host model systems .................................................................................................... 15  
  1.2 Insect Immune defenses .................................................................................................................. 16  
  The Phenoloxidase Cascade .................................................................................................................. 17  
  1.3 General Methods ............................................................................................................................ 22  
  Termite Collection and Husbandry ....................................................................................................... 22  
  PO Assay ............................................................................................................................................... 22  
  Results .................................................................................................................................................. 27  
  Effect of mass on protein concentration .............................................................................................. 27  
Chapter 2: Influence of nesting, feeding and foraging ecology on the variability of immune investment in the Isoptera .................................................................................................................. 28  
  Abstract ................................................................................................................................................ 28  
  2.1 Introduction ................................................................................................................................... 29  
  2.2 Results .......................................................................................................................................... 33  
  2.3 Discussion ..................................................................................................................................... 39  
    Impact of evolutionary lineage on PO activity ...................................................................................... 39  
    Impact of Colony Size, Demography & Longevity on PO activity ...................................................... 40  
    Impact of cuticle sclerotization on PO activity .................................................................................... 42  
    Impact of nesting, feeding and foraging habits on PO activity ............................................................ 43  
    Tradeoffs and PO activity .................................................................................................................. 45  
Chapter 3: Social organization and phenoloxidase activity: The impact of caste membership on the relative investment in immunocompetence. ................................................................................. 47  
  3.1 Introduction ................................................................................................................................... 48  
    Reproductive Potential ...................................................................................................................... 50  
    Caste Longevity & Task Allocation .................................................................................................... 51  
  3.2 Results .......................................................................................................................................... 54  
    Zootermopsis angusticollis .................................................................................................................. 54  
    Reticulitermes flavipes ....................................................................................................................... 54  
    Nasutitermes corniger ......................................................................................................................... 55  
    Nasutitermes acajutlae .......................................................................................................................... 55  
  3.3 Discussion ..................................................................................................................................... 60  
Chapter 4: Gender and immunocompetence: are termites the exception? ........................................ 66  
  4.1 Introduction ................................................................................................................................... 67  
  4.2 Results .......................................................................................................................................... 70  
    Zootermopsis angusticollis .................................................................................................................. 70
List of Figures

Figure 1a: Overview of the Phenoloxidase Cascade........................................................................19

Figure 1b: Evidence of encapsulation of inert nylon threads inserted into Z. angusticollis. The thread on the top was not inserted while the middle and bottom threads were inserted into the termite’s hemocoel for four days. The melanin deposits on these nylon threads result from the build up of multiple layers of lamellocytes (differentiated blood cells) around the invading pathogen/parasite (Hoffman and Reichart, 1995) and the activation of the prophenoloxidase cascade system in the hemolymph (Wilson et al., 2001). Photograph courtesy of R. Rosengaus.19

Figure 1c: Clear melanotic lesions (arrows) on the ventral and dorsal regions of Zootermopsis angusticollis. The lesions coincided with whether the termite was allowed to walk over infective fungal conidia or was exposed on the dorsum with a droplet of conidia suspension. Photograph courtesy of R. Rosengaus........................................................................................................20

Figure 1d: PO Assay tray after 120 minutes with L-dopa substrate. Rows B, C, F, G have been activated with chymotrypsin thus measuring total PO (proPO and PO), while rows A, D, E, and H have not been activated, thus only measuring active PO. Each individual termites homogenate was split into two wells. The columns in row A and B represent one termite, columns in row C and D represent one termite, columns in row E and F represent one termite and columns in row G and H represent one termite. Wells A1-A6 and B1-B6 contain homogenized Zootermopsis angusticollis soldiers, wells A7-A12 and B7-B12, C1-C8 and D1-D8 contain homogenized Z. angusticollis pseudergates, wells C9-C12 and D9-D12, E1-E12 and F1-F12 contain Z. angusticollis nymphs, wells G2-G9 and H2-H9 contain homogenized Nasutitermes corniger alates, wells G10-G12 and H10-H12 contain control samples................................................................................25

Figure 2a: Figure 2a: Nest ecologies. A, Zootermopsis angusticollis nest, single piece nester. B, Reticulitermes flavipes tunneling, intermediate nester. C, Nasutitermes corniger arboreal nest, multiple site nester. D, Nasutitermes acajutlae arboreal nest, multiple site nester. Photos A, C and D courtesy of M. Postava-Davignon, photo B courtesy of R. Rosengaus.................................31

Figure 2b: Figure 2b: Total (available and stored) median PO activity per gram of Zootermopsis angusticollis as a function of caste. Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and inter-quartile range. Significant difference, P<0.05 is represented with “*”. The outliers, identified by “○”, include cases with values over 1.5 box lengths from the upper and lower edges of the box.................................................................35

Figure 2c: Total median PO activity per gram of Reticulitermes flavipes as a function of caste and colony. Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and interquartile range. Significant difference, P<0.05 is represented with “*”. The outliers, identified by “○”, include cases with values over 1.5 box lengths from the upper edge of the box.................................................................36
Figure 2d: Total median PO activity per gram of *Nasutitermes corniger* as a function of caste, and colony. Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and inter-quartile range. Significant difference, P<0.05 is represented with “*”. The outliers, identified by “°”, include cases with values over 1.5 box lengths from the upper edge of the box.

Figure 2e: Total phenoloxidase activity as a function of caste for four different termite species representing three Isopteran families: Termopsidae, Rhinotermitidae and Termitidae. Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and inter-quartile range. Significant difference in pairwise comparison (at P<0.008, after a Bonferroni correction due to multiple pairwise comparisons) is indicated by different letters. The outliers, identified by “°”, include cases with values over 1.5 box lengths from the upper and lower edges of the box. These data represent the combined PO activity of termites collected from multiple colonies.

Figure 3a: Median PO activity per gram of *Zootermopsis angusticollis* as a function of caste. Sample where treated with chymotrypsin to activate PO. Each boxplot shows the median values and interquartile range. Significant difference in pairwise comparison (P<0.0083 after a Bonferroni correction due to multiple pairwise comparisons) represented by different letters. The outliers, identified by “°”, include cases with values over 1.5 box lengths from the upper edge of the box. These data represent the combined PO activity of termites collected from three colonies.

Figure 3b: Median PO activity per gram of *Reticulitermes flavipes* as a function of caste. Sample where treated with chymotrypsin to activate PO. Each boxplot shows the median values and interquartile range. Significant difference in pairwise comparison (P<0.016 after a Bonferroni correction due to multiple pairwise comparisons) represented by different letters. The outliers, identified by “°”, include cases with values over 1.5 box lengths from the upper edge of the box. These data represent the combined PO activity of termites collected from two colonies.

Figure 3c: Median PO activity per gram of *Nasutitermes corniger* as a function of caste. Samples where treated with chymotrypsin to activate PO. Each boxplot shows the median values and interquartile range. Significant difference in pairwise comparison (P<0.016 after a Bonferroni correction due to multiple pairwise comparisons) represented by different letters. The outliers, identified by “°”, include cases with values over 1.5 box lengths from the upper edge of the box. These data represent the combined PO activity of termites collected from two colonies. Note that the soldier and worker castes have zero reproductive potential.

Figure 3d: Median PO activity per gram of *Nasutitermes acajutlae* as a function of caste. Samples where treated with chymotrypsin to activate PO. Each boxplot shows the median values and interquartile range. Significant difference in pairwise comparison (P<0.016 after a Bonferroni correction due to multiple pairwise comparisons) represented by different letters. The outliers, identified by “°”, include cases with values over 1.5 box lengths from the upper edge of the box. These data represent the combined PO activity of termites collected from one colony. Note that the soldier and worker castes have zero reproductive potential.
Figure 4a: Median PO activity per gram of *Zootermopsis angusticollis* as a function of caste and sex (data combined for multiple colonies). Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and interquartile range. Significant differences were determined using Mann-Whitney U, P<0.05 are represented with “*”. The outliers, identified by “∗∗”, include cases with values over 1.5 box lengths from the upper edge of the box.

Figure 4b: Median PO activity per gram of alate as a function of species and sex. Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and interquartile range. Significant differences were determined using Mann-Whitney U, P<0.05 are represented with “*”. The outliers, identified by “∗∗”, include cases with values over 1.5 box lengths from the upper edge of the box. Note that the significant difference observed for the *Zootermopsis angusticollis* are due to the variation in PO activity between males and females of one of the two colonies.

Figure 5a: Schematized cellular encapsulation process of *Metarhizium anisopliae* in *Reticulitermes flavipes* (A) Conidium attachment to the insect cuticle. (B) Conidium germination. (C) Melanotic reaction of the cuticle and elimination of the fungal elements. (D) Fungal penetration of the cuticle and incipient aggregation of hemocytes. (E) Fungal penetration through the epidermis into the hemocoel and recruitment of hemocytes and incipient humoral melanization. (F) Failed encapsulation and invasion of the hemocoel by the hyphae. *(G, H)* Successful encapsulation and intensification of the melanization. (I) Detachment of the melanized nodule into the hemocoel. (J) Formation of an epidermis-like layer around the melanized nodule. (Reprinted from The Journal of Invertebrate Pathology, 101, Chouvenc et al., Cellular encapsulation in the eastern subterranean termite, *Reticulitermes flavipes* (Isoptera), against infection by the entomopathogenic fungus *Metarhizium anisopliae*, 234-241. 2009 with permission from Elsevier license number 2453771194694.)

Figure 5b – Total PO (stored and active) solid line, active PO dashed line over the course of a seven day period post exposure to *Metarhizium anisopliae*. Colonies (n=4) are combined.

Figure 5c – Total PO (stored and active) for each day post exposure by treatment for each colony. “∗∗” indicate significant differences at P<0.01 when the day post exposure for each treatment, of each colony was compared with the control of the same colony.

Figure A1: Scatter plot of protein content (measured by Bradford assay) and individual mass of *Zootermopsis angusticollis* (no correlation between mass and protein content), *Reticulitermes flavipes* (no correlation after grouping by caste), *Nasutitermes acajutlae* termites(strong positive correlation), and *Nasutitermes corniger* (strong positive correlation).
List of Tables

Table 1a: Life history traits of Zootermopsis angusticollis, Reticulitermes flavipes, Nasutitermes acajutlae, and Nasutitermes corniger. ................................................................. 21

Table 1b: Quantities of PBS buffer used for homogenization of termites. ............................. 26

Table 4a: A compilation of gender-based immune difference studies in solitary and social (*) insects ............................................................................................................................................. 69

Table 5a – Numbers of termites (surving) used in PO assay per treatment for each day post exposure. “*” indicates days in which termites were not exposed, thus the n value is zero .......... 89

Table A1: Number of individuals used during the Bradford assay. Those castes that do not exist for a given species are marked with n/a ................................................................. 105
Chapter 1: Introduction

Social insects are a diverse and ecologically successful taxonomic group (Wilson, 1975), evolving in a world replete with pathogens and parasites that certainly exerted (and continue to exert) considerable selective pressures (Schmid-Hempel, 1998; Fefferman et al., 2007). In particular, ants and termites exploit microbial rich environments. They nest in and/or feed on decayed wood and forage in soil or under the leaf litter, habits which exposes these insects to a variety of microbes, including many pathogenic or parasitic organisms (Sands, 1969; Blackwell and Rossi, 1986; Schmid-Hempel, 1998). In addition, the frequent social interactions among nestmates can exacerbate the impact of parasites/pathogens by increasing the probability of disease transmission within the colony (Rosengaus and Traniello, 1997; Rosengaus et al., 2000b). Previous research however, has demonstrated that social insects evolved behavioral, biochemical and immunological adaptations, which help them cope with pathogens and parasites (Rosengaus et al., 1998a; Starks et al., 2000; Evans and Lopez, 2004; Inagaki et al., 2004; Kaltenpoth et al., 2004; Rosengaus et al., 2004; Fernandez-Marin et al., 2006). More specifically, many of these adaptations can be socially mediated (Traniello et al., 2002; Cremer et al., 2007). The research reported here focuses on quantifying one aspect of the termites’ immune response, the activity of the phenoloxidase enzyme, in an attempt to determine the relative investment in immunocompetence in relation to various life history traits including termite phylogeny, gender and caste. The following hypotheses are addressed by this work:

**Hypothesis 1.** As termites evolved so did their nesting, feeding, and foraging behaviors. The impact of new environments and their concomitant exposure to new disease agents likely influenced their immune systems. It is hypothesized that Z. angusticollis and R. flavipes, the two
termite species that nest in decayed wood and in soil, respectively, should have greater PO activity rates than the more phylogenetically derived arboreal species which are probably under less pathogenic constraints given their arboreal nesting behavior (Chapter 2).

**Hypothesis 2.** Termites have a social structure that can include up to five castes (soldier, pseudergate, worker, nymph and alate). Each caste has a unique role in the colony and different reproductive potential. It is hypothesized that future reproductive potential should favor increased immunocompetence (higher PO activity). Those castes that are short lived and sterile are “expendable” and hence should exhibit a reduce immune investment (lower PO activity) while those “indispensable” castes that are responsible for colony reproduction should, relatively speaking, have a greater immune investment, in the form of increased PO activity (Chapter 3).

**Hypothesis 3.** Isopterans do not exhibit the same female biased-sex ratios found in the social Hymenopterans. In the former, both sexes are represented within a colony. Not only are both male and female reproductives required for colony foundation, but also the soldier and worker castes are composed of both sexes, which participate equally in tasks related to colony growth, development and protection. Therefore, contrary to the social Hymenopterans, PO activity in termites is predicted to be similar between the genders within a given species’ caste (Chapter 4).

**Hypothesis 4.** PO is an integral part of the insect immune system. Thus, when under pathogenic assault, changes in PO activity should be expected and PO activity should vary throughout the course of infection. It is hypothesized that non-lethal dosages of the entomopathogenic fungus *Metarhizium anisopliae* trigger the PO cascade causing an increase in PO activity, while lethal dosages of the entomopathogenic fungus disrupt immune function
causing a decrease in PO activity and ultimately death (*Chapter 5*).

### 1.1 Termites as host model systems

Termites live in large highly organized colonies composed of many individuals specialized to perform tasks including reproduction, brood care, nest maintenance and colony defense (Krishna, 1969). Isopterans are found in almost every faunal region (Kambhampati and Eggleton, 2000) and play an important role in their ecosystems by consuming wood and other cellulose-based material, helping recycle nutrients (Krishna, 1969). As termites evolved, so did their nesting habits, progressively moving from a moist, decayed-wood environment to soil and then to arboreal nesting sites, leading to a greater spatial separation between nest and food sources (table 1; Abe, 1987; Noirot and Darlington, 2000). Hence, the evolution of termites is characterized by important changes in nesting, feeding and foraging habits, and presumably by the move from microbial-rich ground-level sites to arboreal locations which support less microbial diversity (Hölldobler and Engel-Siegel, 1984; Tunaz and Stanley, 2009). The order Isoptera, therefore, is a particularly good model system to study the effects that phylogeny, nesting/feeding/foraging ecologies and microbial pressures have had on the evolution of their immune function. Termites live in densely populated colonies and interact frequently with nestmates which can exacerbate the risks of infection within the nest (Rosengaus and Traniello, 1997; Rosengaus et al., 2000b). In spite of living under significant pathogenic constraints, termites have thrived and have become an important constituent of healthy ecosystems. As stated earlier, termites use several, and often simultaneous, mechanisms to reduce the risks of infection. For example, they modify their behavior in the presence of entomopathogenic fungi and nematodes (Rosengaus et al., 1998 a,b; Shimizu and Yamaji, 2003; Wilson-Rich et al., 2007). They also use biochemical secretions with antifungal and antibacterial properties,
(Rosengaus et al., 2000, 2004; Bulmer et al., 2009). Once the anatomical, behavioral and biochemical barriers have been bridged by the invading pathogen or parasite, termites can generate physiological responses at the individual level which include humoral and cellular immune responses (Rosengaus et al., 1999b, 2007). This research quantified PO activity across a range of species, from the primitive one-type nester dampwood termite, *Zootermopsis angusticollis* to the phylogenetically intermediate and soil dwelling termite, *Reticulitermes flavipes* to the most derived arboreal termites, *Nasutitermes corniger* and *Nasutitermes acajutlae*.

### 1.2 Insect Immune defenses

An insect’s immunological response relies on its innate ability to recognize pathogens and parasites as “non-self,” triggering the humoral and/or cellular immune response (Siva-Jothy et al., 2001). Within the humoral branch of the immune response, the recognition of pathogen-associated molecular patterns [PAMP’s such as peptidoglycans, lipopolysaccharides, and β-1,3 glucans (Siva-Jothy et al., 2005)] triggers the Toll and Immune deficiency (IMD) pathways. While the first pathway generates immunological responses against gram-positive bacteria and fungi, the second reacts mainly against gram-negative bacteria (Siva-Jothy et al., 2005). Ultimately, these pathways result in the production of anti-microbial peptides by the fat body, hemocytes and epithelial cells (Siva-Jothy et al., 2005). Recent research has further demonstrated that insect humoral immune responses have a certain degree of specificity (Kurtz and Franz, 2003) and that the immune response can be relatively long-lived (Pham et al., 2007; Haine et al., 2008a). For example, *Tenebrio molitor* injected with lipopolysaccharides to mimic an immune assault, showed increased resistance when later exposed to fungal infection (Moret and Siva-Jothy, 2003). Priming of the immune system has also been demonstrated in bumblebees.
(Sadd and Schmid-Hempel, 2006) and fruit flies (Pham et al., 2007). Furthermore, immune responses can be transgenerational: male bumblebee offspring from immune challenged colonies exhibited increased phenoloxidase activity when compared with controls (Moret and Schmid-Hempel, 2001). Similarly, prior exposure to both killed-bacterial and non-lethal dosages of fungal pathogens elicited a humoral immune response in termites, rendering them less susceptible to a second, otherwise, lethal immune challenge (Rosengaus et al., 1999a). Such responses may include the up-regulation of constitutive proteins as well as the *de novo* production of proteins in the hemolymph (Rosengaus et al., 2007; Bulmer et al., 2009).

Recognition of “non-self” can also result in the induction of the cellular immune responses, specifically phagocytosis, encapsulation and nodule formation. Components of the cellular immune responses can be triggered faster than some of the more specific humoral responses described above (Siva-Jothy et al., 2005; Haine et al., 2008). While phagocytosis appears to be effective against unicellular microorganisms, encapsulation is employed for larger intruders (Siva-Jothy et al., 2005). Both encapsulation and nodule formation involve hemocytes and the activation of the phenoloxidase (PO) cascade.

**The Phenoloxidase Cascade**

The PO enzyme plays a multifaceted role in insect physiology, from sclerotization and hardening of newly molted cuticle to wound healing, nodule formation and encapsulation (Ashida and Hiroko, 1990). Encapsulation is a particularly effective mechanism for coping with invading parasites as it restricts the movement and activities of the parasite within the host’s body cavity. During the process of encapsulation, hemocytes adhere to the foreign surface while recruiting more hemocytes, ultimately encasing the parasite. The cell mass is then melanized
through the activity of the proteolytic PO cascade while generating cytotoxic byproducts (such as quinones and melanin) that may contribute to the death of the invading and now encased parasite (Johansson and Söderhall, 1996; Söderahall and Cerenius, 1998).

The PO cascade has also been found to be an important component in fighting microbial infections. Bacteria, viruses, and fungi can be killed by PO-generated reactive compounds (Shelby and Popham, 2006; Zhao et al., 2007). The cytotoxic byproducts of the PO cascade are a threat to both the parasite as well as the host. Autoreactive melanization of tissue in the areas surrounding an immune insult supports the view that this enzymatic cascade is associated with a cost/damage to the host (Sadd and Siva-Jothy, 2006). Therefore insect PO is synthesized and stored in an inactive form called pro-phenoloxidase (pro-PO). Different forms of this zygogen are found, depending on the insect species, in circulating hemocytes, plasma, the cuticle, mid-gut epithelium and salivary glands (Ashida and Brey, 1997). When parasitic invasion is underway and the host recognizes specific PAMPs, foreign bodies or lysed hemocytes (Ashida and Hiroko, 1990), the serine protease cascade is triggered, resulting in the activation of the pro-prophenoloxidase activating enzyme (Pro-ppA). Pro-ppA is responsible for cleaving pro-phenoloxidase into phenoloxidase (Cerenius and Söderhall, 2004) which in turn catalyses the oxidation of phenols into toxic quinones that are subsequently polymerized into melanin (Söderahall and Cerenius, 1998; figure 1a and 1b). The multiple levels of the PO cascade, as with other enzyme cascades, allow for temporal and spatial control of cytotoxic products (Sadd and Siva-Jothy, 2006). In termites, melanotic lesions are localized to the initial area of fungal invasion (Rosengaus and Traniello 1997; figure 1b,c). Preliminary data in termites have provided evidence that the PO cascade is indeed triggered once foreign objects (such as an inert nylon thread) invade the termite’s hemocoel (Rosengaus and Traniello, 1997). Interestingly,
phenoloxidase may have a dual role, acting not only as an oxygenase as described above, but also as a thermotolerant endonuclease (Sun et al., 2008).

Figure 1a: Overview of the Phenoloxidase Cascade (adapted Cerenius and Söderhall, 2004)

Figure 1b: Evidence of encapsulation of inert nylon threads inserted into Zootermopsis angusticollis. The thread on the top was not inserted while the middle and bottom threads were inserted into the termite’s hemocoel for four days. The melanin deposits on these nylon threads result from the build up of multiple layers of lamellocytes (differentiated blood cells) around the invading pathogen/parasite (Hoffman and Reichart, 1995) and the activation of the prophenoloxidase cascade system in the hemolymph (Wilson et al., 2001). Photograph courtesy of R. Rosengaus.
Figure 1c: Clear melanotic lesions (arrows) on the ventral and dorsal regions of *Zootermopsis angusticollis*. The lesions coincided with whether the termite was allowed to walk over infective fungal conidia or was exposed on the dorsum with a droplet of conidia suspension. Photograph courtesy of R. Rosengaus.
Table 1a: Life history traits of *Zootermopsis angusticollis*, *Reticulitermes flavipes*, *Nasutitermes acajutlae*, and *Nasutitermes corniger*

<table>
<thead>
<tr>
<th>Species</th>
<th><em>Zootermopsis angusticollis</em></th>
<th><em>Reticulitermes flavipes</em></th>
<th><em>Nasutitermes acajutlae</em></th>
<th><em>Nasutitermes corniger</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Distribution</strong></td>
<td>Western North America (Thorne et al., 1993)</td>
<td>Throughout Eastern North America (Vargo and Husseneder, 2009)</td>
<td>Antilles from Puerto Rico to Trinidad, into South America (Thorne et al., 1996)</td>
<td>Mexico to Brazil (Thorne, 1980)</td>
</tr>
<tr>
<td><strong>Nesting Type</strong></td>
<td>Single Site (Shellman-Reeve, 1997)</td>
<td>Multiple Site (Shellman-Reeve, 1997)</td>
<td>Central Site (Shellman-Reeve, 1997)</td>
<td>Central Site (Shellman-Reeve, 1997)</td>
</tr>
<tr>
<td><strong>Foraging behavior</strong></td>
<td>No foraging, consume decaying parts of wood (Lenz, 1994)</td>
<td>Tunnels through soil to locate new food resources, dead decayed wood (Shellman-Reeve, 1997).</td>
<td>Foraging arenas are separate from nest site (Shellman-Reeve, 1997).</td>
<td>Foraging arenas are separate from nest site (Shellman-Reeve, 1997).</td>
</tr>
<tr>
<td><strong>Colony Foundation</strong></td>
<td>Male and female dealate pair (Shellman-Reeve, 1997)</td>
<td>Male and female dealate pair. Budding (Shellman-Reeve, 1997)</td>
<td>Male and female dealate pair; Multiple dealates (Shellman-Reeve, 1997)</td>
<td>Male and female dealate pair; Multiple dealates (Shellman-Reeve, 1997)</td>
</tr>
<tr>
<td><strong>Colony Size</strong></td>
<td>&gt; 8000 (Lenz, 1994)</td>
<td>50,000 - 400,000 (Lenz, 1994)</td>
<td>Millions (Shellman-Reeve, 1997)</td>
<td>Millions (Shellman-Reeve, 1997; Thorne, 1985)</td>
</tr>
<tr>
<td><strong>Spectrum of Eusociality</strong></td>
<td>0-0.25 (Shellman-Reeve, 1997)</td>
<td>0.4-0.75 (Shellman-Reeve, 1997)</td>
<td>0.7-1.0 (Shellman-Reeve, 1997)</td>
<td>0.7-1.0 (Shellman-Reeve, 1997)</td>
</tr>
<tr>
<td><strong>Soldier/Worker Ratio</strong></td>
<td>1-10% soldier (Shellman-Reeve, 1997)</td>
<td>2% Soldier (Long, 2005)</td>
<td>Soldier are 30% of work force (Haverty, 1977)</td>
<td>Soldier are 30% of work force (Haverty, 1977)</td>
</tr>
<tr>
<td><strong>Worker Longevity</strong></td>
<td>4 years before imago age (Shellman-Reeve, 1997)</td>
<td>3-5 years (Shellman-Reeve, 1997)</td>
<td>Up to six 6 months (Shellman-Reeve, 1997)</td>
<td>Up to six 6 months (Shellman-Reeve, 1997)</td>
</tr>
<tr>
<td><strong>Colony Longevity</strong></td>
<td>4-15 years (Shellman-Reeve, 1997)</td>
<td>6-10 years (Vargo and Husseneder, 2009)</td>
<td>20+ years (Shellman-Reeve, 1997)</td>
<td>20+ years (Shellman-Reeve, 1997)</td>
</tr>
<tr>
<td><strong>Task Allocation</strong></td>
<td>No age polyethism, (Rosengaus and Traniello, 2001)</td>
<td>No age polyethism (Long, 2005)</td>
<td>Sex-based polyethism (Waller and La Fage, 1987)</td>
<td>Sex-based polyethism (Waller and La Fage, 1987)</td>
</tr>
</tbody>
</table>
1.3 General Methods

Termite Collection and Husbandry

Four different termite species representing three different families spanning termite phylogeny were chosen for this research: the primitive, dampwood termite *Zootermopsis angusticollis* (n=4 colonies, originally collected in Huddart Park, San Mateo County, California); the phylogenetically intermediate eastern subterranean termite *Reticulitermes flavipes* (n=2 colonies, collected in Middlesex County Massachusetts); the highly derived Neotropical species *Nasutitermes corniger* (n=3 colonies, 2 collected at the Smithsonian Tropical Research Institute in Panama and 1 in Ft. Lauderdale, Florida); and *Nasutitermes acajutlae* (n=1 colony, collected in the Island of St. John, Virgin Islands). Entire nests were collected from the wild and transported back to the USDA inspected containment facility (Northeastern University). All species were fed birch as a food source and provided with unlimited water. *Reticulitermes flavipes, N. corniger* and *N. acajutlae* were maintained at 28°C and 80% RH while *Z. angusticollis* colonies were maintained at 25°C and approximately 80% RH.

PO Assay

Termites were flash-frozen in liquid nitrogen and stored at -80°C. Freezing samples facilitates the breakdown of cells and emptying of cell contents (including the intracellular PO and other molecules involved in the PO cascade; Barns and Siva-Jothy, 2000) into the insect’s body cavity. By processing the entire animal rather than only the hemolymph, we quantified the entire stock of PO of each individual (Contreras-Garduno et al., 2007). While other studies have measured and inferred, relative investment in immunocompetence by only quantifying PO
activity directly from hemolymph samples (Adamo, 2004a; Armitage and Siva-Jothy, 2005; Pomfret and Knell, 2006), the small size and limited amounts of hemolymph that can be drawn from *Reticulitermes* and *Nasutitermes* precluded using this technique. Thus, given that the assay included tissue other than hemolymph, it is possible that the results may overestimate PO activity as it relates to immune function. On the other hand, measuring total body PO activity (including that located in the cuticle and other tissues) may provide a reliable relative measure of total immunocompetence as the cuticle is not just inert armor but has shown active involvement in immune defenses as well (Brey et al., 1993; Chouvenc et al., 2009a). The phenomenon of density dependent prophylaxis has also been linked to darkening of the cuticle in several gregarious insect species (Barnes and Siva-Jothy, 2000; Wilson et al., 2002). In fact, it has been found that the darker cuticle color in *T. molitor* corresponds with an increase in hemocyte density and phenoloxidase activity (Armitage and Siva-Jothy, 2005). Furthermore, PO found in the cuticle of *Bombyx mori* has been shown to be transported there from the hemolymph (Asano and Ashida, 2001). Hence, we are confident that estimating PO activity by processing the entire individual rather than only a small volume of hemolymph, should provide a reliable measure of relative immune investment.

Before processing, each termite was sexed and weighed individually and subsequently crushed with a pellet pestle in a microcentrifuge tube with PBS buffer. The volume of PBS buffer varied according to the termite species given their large differences in size and mass (table 1b). Samples were centrifuged at 4°C for 8 minutes at a speed of 10,000Xg. Of the supernatant, 110µl were removed and placed into a 0.6ml microcentrifuge tube. Samples were then divided into two 50µl aliquots and loaded into separate wells of a 96 well plate. The first of these aliquots was treated with 70µl α-chymotrypsin (from bovine pancreas, 2mg per 1.5ml, Sigma
Chymotrypsin cleaves pro-PO into PO, thus allowing for the total quantification of both the inactive zymogen (stored PO) and the active enzyme. The second aliquot was treated with 70 µl PBS buffer and was assumed to reflect the amount of active PO potentially available for immediate immune response at the time the insect was frozen. The 96 well plate (figure 1d) was incubated at room temperature for 20 minutes. In a darkened room, 150 µl of 5mM L-dopa (Acros Organics) was added to each well just prior to the first absorbance measurement. L-dopa served as a substrate on which PO acts to form melanin. As the proteolytic cascade progressed, the production of melanin increased. The accumulation of melanin pigment in the well can therefore be measured by quantifying absorbance of the sample. Absorbance was read at 492 nm using the Synergy HT-I spectrophotometer (kindly provided by V. Godoy, Northeastern University) every two minutes for two hours. Both the rates of enzymatic activity and amounts of melanin produced represent indirect measures of the relative investment in cellular immunity. PO activity rates were calculated by first determining the slope of the linear phase of the reaction (Adamo, 2004a). The slope was then divided by the mass of each individual to control for size differences. By comparing PO activity rates across species, across colonies from the same species, between males and females within colonies, among different castes and among individuals during the progression of fungal infection, insights can be shed on 1) the dynamics among this component of the immune response and different life-history traits and 2) the influence that sociality and disease have had on termite immunocompetence.
Figure 1d: PO Assay tray after 120 minutes with L-dopa substrate. Rows B, C, F, G have been activated with chymotrypsin thus measuring total PO (proPO and PO), while rows A, D, E, and H have not been activated, thus only measuring active PO. Each individual termites homogenate was split into two wells. The columns in row A and B represent one termite, columns in row C and D represent one termite, columns in row E and F represent one termite and columns in row G and H represent one termite. Wells A1-A6 and B1-B6 contain homogenized Zootermopsis angusticollis soldiers, wells A7-A12 and B7-B12, C1-C8 and D1-D8 contain homogenized Z. angusticollis pseudergates, wells C9-C12 and D9-D12, E1-E12 and F1-F12 contain Z. angusticollis nymphs, wells G2-G9 and H2-H9 contain homogenized Nasutitermes corniger alates, wells G10-G12 and H10-H12 contain control samples.
Table 1b: Quantities of PBS buffer used for homogenization of termites

<table>
<thead>
<tr>
<th>Species</th>
<th>Caste</th>
<th>PBS used to homogenize</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Zootermopsis angusticollis</em></td>
<td>Alate</td>
<td>145μl</td>
</tr>
<tr>
<td></td>
<td>Soldier</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nymph</td>
<td>140μl</td>
</tr>
<tr>
<td></td>
<td>Pseudergate</td>
<td></td>
</tr>
<tr>
<td><em>Reticulitermes flavipes</em></td>
<td>Alate</td>
<td>145μl</td>
</tr>
<tr>
<td></td>
<td>Soldier</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pseudergate</td>
<td></td>
</tr>
<tr>
<td><em>Nasutitermes corniger</em></td>
<td>Worker</td>
<td>122μl</td>
</tr>
<tr>
<td></td>
<td>Soldier</td>
<td></td>
</tr>
<tr>
<td><em>Nasutitermes acajutlae</em></td>
<td>Worker</td>
<td>122μl</td>
</tr>
<tr>
<td></td>
<td>Soldier</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alate</td>
<td>130μl</td>
</tr>
</tbody>
</table>

*Bradford Assay Method*

The Bradford Assay was used to determine protein concentration (mg protein/ml protein homogenate) for *Z. angusticollis, R. flavipes, N. acajutlae, and N. corniger*. The Quick Start Bradford Protein Assay (BioRad) was performed using previously collected and frozen termites. Termites were sexed, weighed and homogenized in varying amounts of PBS buffer dependent on the species. These volumes are consistent with those of the PO Assay (table 1b). Samples were centrifuged at 4°C for 8 minutes at a speed of 10,000Xg. Subsequently 50μl volumes of sample were transferred to each well of a 96 well plate, and 100μl PBS buffer was added in order to have a sufficient volume. Pre-made protein standards, 150μl, were pipetted into the assay tray, 150μl of dye was added to each of the standards and termite sample using a multi-channel micropipetter which was subsequently used to mix the samples. Protein standards were run in
triplicate. Absorbance was read at 595nm using the Synergy HT-I spectrophotometer courtesy of Dr. Veronica Godoy.

Results

Effect of mass on protein concentration

The four tested species are vastly different in their size and mass (see table A1 for sample sizes). For this reason, we attempted to normalize PO activity levels across species by standardizing the immune measure as a function of total protein content. After performing a Bradford assay on a subset of individuals of each species, we concluded that standardizing as a function of total protein content was not appropriate (figure A1). In two of the species (Z. angusticollis and R. flavipes), total protein content did not correlate with mass ($P=0.292$, $P=0.01$, respectively). A significant positive correlation between total protein and mass was observed for both Nasutitermes species ($P<0.001$). Given that the differences between total protein content across species were as great as the differences seen in both their mass and their PO activity, and that not all species exhibited a consistent positive correlation between the immune parameter and their protein content, we felt that mass was a more reliable factor to use in our standardization methods than protein content. All PO data is therefore presented as PO activity/gram of insect. (See Appendix A for more detailed results)
Chapter 2: Influence of nesting, feeding and foraging ecology on the variability of immune investment in the Isoptera

Abstract

Termites colonize a diverse number of ecosystems throughout much of the world and play an ecologically critical role in nutrient recycling. This study investigates the impact that termite phylogeny and its accompanying nesting, feeding and foraging habits, have on phenoloxidase activity (PO), a critical component of the insect immune arsenal involved in wound healing, encapsulation and nodule formation as well as the production of toxic quinones known to damage invading pathogens. Through a comparative approach, we estimated relative PO levels across four different species that span the termite phylogeny, from the basal single-piece nester *Zootermopsis angusticollis* to the intermediate subterranean termite *Reticulitermes flavipes* to the most derived arboreal species *Nasutitermes corniger* and *Nasutitermes acajutlae*. Our results indicate that PO levels are independent of phylogeny per se. Instead, investment in PO function correlates with the nesting and foraging habits of each species and their assumed underlying pathogenic risks. On average, the subterranean termite *R. flavipes* had seven times the PO levels than those of *Z. angusticollis*, which in turn, had 80 times more PO activity than either of the two *Nasutitermes* species, which exhibited negligible PO activity. Relative to arboreal nesting, ground and soil dwelling behavior likely result in increased exposure risks to pathogenic microorganisms. Such constraints may have favored the evolution of higher levels of PO investment in subterranean species. These comparative results indicate for the first time that PO activity in termites may be significantly influenced by species-specific life history attributes.
2.1 Introduction

Isoptera contains seven recognized families comprised of over 2600 species (Inward et al., 2007). Although all extant species are eusocial, the degree of social complexity as well as other life history traits varies considerably within this order (table 1a). Particularly, vast differences exist with respect to reproductive potential, caste differentiation, colony size and demography, nesting architecture, feeding habits and foraging strategies (Abe, 1987; Shellman-Reeve, 1997; Thorne, 1997; table 1a). This order has been divided into two general groups. The phylogenetically primitive or “lower” termites, which include six families (Mastotermitidae, Kalotermitidae, Termopsidae, Hodotermitidae, Rhinotermitidae and Serrititermitidae), are all closely associated with hindgut microbiota consisting of bacterial and cellulolytic protozoan symbionts (Shellman-Reeve, 1997; Brune, 2006). The most derived, “higher” termites (Termitidae) contain only bacterial hindgut symbionts (Abe, 1987).

Other striking differences exist among termite species particularly with respect to the exploitation of food resources and nesting sites. For example, Abe (1987) first described three general types of nests within Isoptera: one-piece-, intermediate- and separate-type nesters (figure 2a). The former occupy a single piece of dead decaying wood; the nests functions as both a nest site and a food source. One-piece nesters do not forage for food and thus, the wood they inhabit represents a single limiting resource that can ultimately be completely consumed leading to food shortages and perhaps colony death (Shellman-Reeve, 1997). Intermediate-type nesters also nest in dead decaying pieces of wood. However, they construct foraging paths through the soil to locate and exploit new food resources. Relative to the one-piece nesters, this strategy creates greater resource availability, resulting in more stable, larger colonies that may persist for many years (Abe, 1987; Shellman-Reeve, 1997; table 1a). Finally, central site nesters construct nests
that are distinctly separate from their foraging arenas. Upon locating new food sources, foragers return to the central nest to recruit nestmates to further exploit the resources. Many of their nests are built above ground in the form of large conspicuous epigeous mounds and arboreal carton-like nests (Abe, 1987; Shellman-Reeve, 1997). Separate piece nesters are characterized by complex and densely populated colonies that are relatively long lived (Abe, 1987; Shellman-Reeve, 1997; table 2a, figure 2a).

The diversity of such nesting, feeding and foraging tactics surely impacts many of their other life history traits (table 1a), including investment in immune function. It is hypothesized that variation in nesting, feeding and foraging habits among termites place these social insects under different pathogenic constraints. Because immune defenses are considered to be energetically costly (Sheldon and Verhulst, 1996; Freitak et al., 2003; Tyler et al., 2006; Valtonen et al., 2009), investment in immune resistance should be tailored according to exposure risk. Previous research has shown that soil-dwelling insects are exposed to a myriad of microbes, many of which are potentially entomopathogenic (Holt, 1996; Cruse, 1998; Rosengaus et al., 2003; Kurihara et al., 2008; Tunaz and Stanley, 2009; Postava-Davignon, 2010). The finding that soil-inhabiting insects have significantly higher number of nodules present in the hemocoel relative to that of insects exploiting other non-soil resources is consistent with the hypothesis that these insects live under more intense pathogenic constraints (Tunaz and Stanley, 2009). Cruse (1998) found that the basal species *M. darwiniensis*, a multiple site nester occurring in the soil, like *R. flavipes*, had ten times the cuticular microbial load of the central site nesters (*Coptotermes lacteus* and *Nasutitermes exitiosus*). Similarly, Rosengaus et al. (2003) found that termites nesting in moist wood the *Z. angusticollis*, had higher nest and cuticular microbial loads.
than the drywood species *Incisitermes minor, I. schwarzi,* and *Cryptotermes cavifrons.* Hence, high microbial loads are likely correlated with high degrees of humidity and levels of decomposition of nesting and feeding resources. These conditions may help explain why the evolutionary transition of nesting and feeding habits in termites were accompanied by significant diversification of immune related genes (likely under positive selection; Bulmer and Crozier, 2004).

Arboreal insects, on the other hand, may be under less stringent pathogenic selection pressures. This is perhaps one reason why arboreal weaver ants have atrophied metapleural glands (Hölldobler and Engel-Siegel, 1984). Although such glands produce potent antimicrobial secretions in most other ant species, their loss suggests that arboreal ants are exposed to fewer microbes than their terrestrial counterparts (Hölldobler and Engel-Siegel, 1984). Taken together, the cited literature supports the view that microbial exposure varies with nesting, feeding and foraging strategies and that insects with a greater risk of exposure should invest more heavily in immune function.

To test if immune investment varies according to nesting, feeding and foraging habits, levels of PO activity were determined for four different termite species: *Zootermopsis angusticollis, Reticulitermes flavipes, Nasutitermes acajutlae* and *Nasutitermes corniger.* This comparative approach provides, for the first time, a relative measure of immune function within the Isoptera.

*Zootermopsis angusticollis* and *R. flavipes,* the two termite species that nest in decayed wood and in soil, respectively, should have higher immune investment, measured as greater PO activity rates, than the more derived arboreal species. Understanding the relationship between microbial pressures, nesting, feeding, and foraging strategies and their immune investment will
help recognize the role that pathogens and parasites may have played in the evolution of their sociality.

2.2 Results

A linear regression model including the variables gender (beta coefficient=0.128, P=0.001), caste (beta coefficient=0.291, P=0.001), species (beta coefficient=1.210, P=0.001), and colony of origin (beta coefficient=-0.665, P=0.001) revealed an $R^2$ value of 0.468. All variables were significant and independent predictors of PO activity (SPSS 13). The overall model indicated that 46.8% of the variability seen in the PO activity of termites was explained by the variables listed above.

Closer inspection revealed that caste-specific patterns of PO activity across colonies were inconsistent (figure 2b, 2c, 2d). For example, while pseudergates and nymphs of *Z. angusticollis* originating from colony 2 had lower PO levels than their counterparts of colony 3 or 4, alates of colony 2 had significantly higher levels of PO activity than alates from colony 3 (figure 2b). The only *Z. angusticollis* caste that did not vary significantly in PO activity amongst colonies was the soldier caste (figure 2b). For *R. flavipes*, the soldier and pseudergate caste of Colony 9 had consistently higher PO activity levels per gram of termite than the corresponding castes of colony 10, except for the alates, which had similar median PO activity levels (figure 2c). In the case of *N. corniger*, soldiers of colony 5 had significantly higher PO activity levels/gram of termite compared to colony 6 soldiers. However, workers of the two colonies exhibited no significant differences in their PO levels (figure 2d). Unfortunately, *N. corniger* alates were not available for inter-colony comparisons of their PO activity levels and only one *N. acajutlae* colony was available for this study. The data indicate that termite species, colony of origin and
caste (to be discussed in Chapter 3) all appear to be significant factors influencing PO activity in this social insect order.

In spite of these inter-colony differences, the magnitude of PO levels relative to other species was indeed consistent. Regardless of colony of origin, *R. flavipes* always demonstrated higher PO levels than any of the tested *Z. angusticollis* colonies, which in turn consistently exhibited higher PO levels than any of the *N. corniger* or *N. acajutlae* colonies (figure 2b). Given the similar intra-specific pattern across colonies, their data were pooled for further analyses.

Although the total phenoloxidase activity (active PO and stored PO) varied significantly across the representative termite species, such variation was independent of phylogeny since *R. flavipes*, a member of a phylogenetically intermediate termite family, had on average seven times more PO activity than the basal *Z. angusticollis*. The latter had 80 times the PO levels than the most derived *N. acajutlae* and *N. corniger*, which had negligible levels of PO activity (figure 2e).
Figure 2b: Total (available and stored) median PO activity per gram of Zootermopsis angusticollis as a function of caste. Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and inter-quartile range. Significant difference, P<0.05 is represented with “*”. The outliers, identified by “°”, include cases with values over 1.5 box lengths from the upper and lower edges of the box.
Figure 2c: Total median PO activity per gram of *Reticulitermes flavipes* as a function of caste and colony. Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and interquartile range. Significant difference, $P<0.05$ is represented with “*”. The outliers, identified by “°”, include cases with values over 1.5 box lengths from the upper edge of the box.
Figure 2d: Total median PO activity per gram of *Nasutitermes corniger* as a function of caste, and colony. Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and inter-quartile range. Significant difference, P<0.05 is represented with “*”. The outliers, identified by “○”, include cases with values over 1.5 box lengths from the upper edge of the box.
Figure 2e: Total phenoloxidase activity as a function of caste for four different termite species representing three Isoptera families: Termopsidae, Rhinotermitidae and Termitidae. Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and inter-quartile range. Significant difference in pairwise comparison (at P<0.008, after a Bonferroni correction due to multiple pairwise comparisons) is indicated by different letters. The outliers, identified by "°", include cases with values over 1.5 box lengths from the upper and lower edges of the box. These data represent the combined PO activity of termites collected from multiple colonies.
2.3 Discussion

In termites, investment in immune function, measured as PO activity, could have been shaped by multiple life history attributes. Factors such as evolutionary lineage, species, colony of origin, colony size, caste, gender, longevity, individual reproductive potential, nesting architecture, feeding habits and foraging strategies and their concomitant pathogenic pressures, could have all played important roles in influencing PO levels. This research is the first to attempt to elucidate the relative importance of several of these life history attributes in the degree of immunocompetence exhibited within the Isoptera. Below several factors are discussed that could conceivably influence PO levels in this social insect order. Based on the following circumstantial evidence, pathogenic risks associated with the nesting, feeding and foraging behavior is one variable that explains levels of PO investment in this insect order.

Impact of evolutionary lineage on PO activity

The results clearly demonstrate that evolutionary lineage alone is not responsible for the inter-specific differences in PO activity. The highest PO activity levels were exhibited by *R. flavipes*, a phylogenetically intermediate species nested between the basal *Z. angusticollis* and most derived *N. corniger* and *N. acajutlae*. Such striking differences in PO levels suggest that positive selection acting on *R. flavipes* likely resulted in significant investment in immune defenses. Although microbial loads were not determined in this study, there is a large body of literature showing that soil environments are more heavily colonized by abundant and more diverse microbial communities than above ground sites (Evans, 1982; Wcislo, 1996; Cruse, 1998; Schmid-Hempel, 1998; Tunaz and Stanley, 2009; Postava-Davignon, 2010).

Given its high PO activity, it is not surprising that *R. flavipes* is an ecologically
successful species with a widespread distribution throughout eastern North America (Vargo and Husseneder, 2009). The high PO activity may also help explain why the use of pathogens as agents of biological control against this well known urban pest have been unsuccessful (Chouvenc et al., 2008). Chouvenc et al., (2008) and Rosengaus (pers. communication) found that $R.\ flavipes$ can cope with extremely high dosages of fungal conidia that would otherwise quickly and effectively kill other termite species. Such resistance could be attributable, in part, to their high PO levels.

**Impact of Colony Size, Demography & Longevity on PO activity**

Although not specifically tested here, it appears that PO levels are not correlated with colony size, colony demography, caste composition or colony longevity. Mature $Z.\ angusticollis$ colonies are fairly small, ranging in size from 600 to 8,000 individuals and surviving from 4 to 15 years, and are limited by the wood they inhabit (Shellman-Reeve, 1997). Mature colonies of $R.\ flavipes$, on the other hand, range in size from 50,000 to 900,000 individuals (Shellman-Reeve, 1997) although they can be as large as several million individuals (Thorne et al., 1999) and survive up to 10 years (Vargo and Husseneder, 2009). Mature $Nasutitermes$ colonies can reach up to five million individuals and colonies can persist for more than 20 years (Shellman-Reeve, 1997). Thus, $R.\ flavipes$, with its intermediate colony size and colony longevity still exhibited the highest immune investment relative to $Z.\ angusticollis$ and $Nasutitermes$ sp.

Increased group size has been considered an important factor favoring density dependent prophylaxis, a phenomenon of heightened immune investment in gregarious insects (Barnes and Siva-Jothy, 2000; Stow et al., 2007; Ruiz-González et al., 2009). $Tenebrio\ molitor$ raised under high-density conditions exhibited increased investment in PO activity (Barnes and Siva-Jothy,
Cotter et al., (2004) also found Egyptian cotton leafworms raised in high densities had higher PO activity and encapsulation rates, but lower antibacterial rates. As expected, some social insects also exhibit density dependent prophylaxis. Adult bumblebees, for example, have increased PO activity when grouped compared to solitary individuals (Ruiz-González et al., 2009). The production of progressively stronger antimicrobial agents in bees is also correlated with increased group size (Stow et al., 2007). However, comparative data on termites does not support the link between group density and immunocompetence. If colony density were to be positively correlated with immune function, then Nasutitermes, not Reticulitermes, would have exhibited the highest PO activity levels. Previously, Pie et al., (2005) reported a lack of density dependent prophylaxis in Z. angusticollis and suggested that the maintenance of heightened immunity in densely populated social insect colonies could be too costly, particularly when these insects can cope with potential pathogens through relatively energetically inexpensive behaviors such as mutual grooming and nest hygiene (Rosengaus et al., 2010).

These results also provide no evidence that colony longevity is necessarily correlated with high PO levels as Nasutitermes sp. have the longest lived colonies (Shellman-Reeve, 1997) and yet have the lowest PO values. Individual-level, rather than colony-level longevity, may be more important in fostering higher immune function. Worker longevity in both Zootermopsis sp. and Reticulitermes sp. ranges between 3-5 years, while workers of Nasutitermes apparently only live for up to 6 months (Shellman-Reeve, 1997). Keller and Genoud (1997) provide evidence that insect eusociality is associated with a 100 fold increase in lifespan of queens. Therefore, individual life-span may be a more significant predictor of individual immunity than colony-level longevity. The possibility exists that investment in immunity may be more critical to species containing long-lived workers since these individuals are more likely to re-encounter
pathogens throughout their lives relative to short-lived organisms (Dunn, 1990). Unfortunately, individual longevity in termites may be coupled with reproductive potential (Thorne et al., 2002) and it is impossible to separate the independent effects of each of these confounded variables.

Caste development and reproductive opportunities in lower termites (including Zootermopsis sp. and Reticulitermes sp.) are highly flexible relative to the developmentally restricted Nasutitermes (Thorne, 1997). Shorter individual life-spans and no potential for future direct reproduction may explain the low investment in PO activity recorded for Nasutitermes. More in-depth discussion on the effect of reproductive potential on immunocompetence is presented in chapter 4.

**Impact of cuticle sclerotization on PO activity**

Differences in anatomical characteristics may also influence the degree of immune investment in the Isoptera. The tested species vary significantly in both size and degree of cuticular sclerotization. Given that PO levels were standardized as a function of mass unit (i.e. per gram of termite), size differences cannot account for our significant results. Unfortunately, the degree of cuticular sclerotization could not be controlled. The cuticle is a complex chitonous matrix that is not only the first line of physical defense against a pathogen acting as a mechanical barrier (Marmaras et al., 1996), but also plays an active role in immune reactions (Brey et al., 1993). For example, in *T. molitor*, cuticular melanization corresponds with increased disease resistance (Barnes and Siva-Jothy, 2000; Armitage and Siva-Jothy, 2005). Darker cuticles were correlated with higher hemocyte counts and greater PO activity levels relative to lighter colored insects. Although Wilson et al. (2001) found a positive correlation between cuticular melanization and phenoloxidase activity in *Spodoptera exempta*, our results do not support this observation in termites. *Reticulitermes flavipes* is by far the lightest colored species included in the study, yet it has significantly higher PO activity levels than the more sclerotized
Nasutitermes sp. The possibility exists that instead of investing in darkening their cuticle with the deposition of melanin, which is the final byproduct of the same PO immune related cascade, R. flavipes holds most of its PO as inactivated pro-PO in order to combat pathogens only when they have invaded the insect. In this way, R. flavipes likely stores large quantities of this versatile enzyme while minimizing the negative side effects triggered by the active circulating PO (Sadd and Siva-Jothy, 2006). The degree of sclerotization neither correlates with PO activity in the worker and soldier castes of the three studied species, nor explain the higher PO levels in the alate caste of R. flavipes relative to the alates of Zootermopsis and Nasutitermes (figure 2d) since, at least visually, all species have heavily pigmented imagoes.

Impact of nesting, feeding and foraging habits on PO activity

Differences in the nesting, feeding and foraging habits of termites, together with their respective risks of infection by microorganisms, may help explain the relative higher investment on PO activity for R. flavipes, followed by Z. angusticollis which in turn is higher than the low investment in PO activity exhibited by Nasutitermes.

The most primitive species, Z. angusticollis, is a single piece nester (Shellman-Reeve, 1997; Thorne, 1997). By consuming its own nest, this species reduces the risk of predation and disease while limiting the food resource available to the colony over time. However, nesting in rotting wood may expose individuals to a large array of microbes, some of which could be potential pathogens (Sands, 1969; Rosengaus et al., 2003, 2004). Thus, Z. angusticollis is potentially under greater pathogen pressure than arboreal nesting termites but a lower risk of infection than the soil dwelling R. flavipes. The assumed intermediate risks of infection to Z. angusticollis corresponds with its intermediate PO activity levels.
*Reticulitermes flavipes* is a subterranean species that tunnels through soil to locate new food resources consisting of dead decayed wood (Shellman-Reeve, 1997). Although no estimation of cuticular and environmental microbial loads is available in the literature for *R. flavipes*, previous reports indicate that soil dwelling insects are under higher pathogenic pressures than other insects exploiting different environments (Cruse, 1998; Holt, 1996; Kurihara et al., 2008; Tunaz and Stanley, 2009; Postava-Davignon, 2010). In this study, the subterranean termite *R. flavipes* had the highest PO activity levels, presumably due to its continuous use of microbial rich soils during foraging.

Both species of *Nasutitermes* used here are arboreal nesters, constructing their nests out of cartonous material that is colonized by lower microbial loads than the surrounding areas (Postava-Davignon, 2010). Other research indicates arboreal nesting insects are under less pathogenic pressure than soil dwelling insects (Hölldobler and Engel -Siegel, 1984; Wcislo, 1996). Thus, the lowest PO activity level recorded for *Nasutitermes* corresponds with a lower risk of infection.

Given that immunological responses are energetically costly (Sheldon and Verhulst, 1996; Siva-Jothy et al., 1998), animals living under reduced pathogenic constraints should forgo investing in immune function, including PO activity, particularly if behavioral and/or biochemical secretions are effective against pathogenic microorganisms (Rosengaus et al., 1998a; Rosengaus et al., 2000a, 2004; Chapuisat et al., 2007; Simone et al., 2009). In fact, low-cost behavioral interactions amongst nestmates and/or antimicrobial glandular secretions (like the terpenoids produced by *Nasutitermes* sp.; Rosengaus et al., 2000a), may help explain the reduced number of immune-related genes detected in the honey bee where as many as one-third fewer immune genes have been identified relative to other insects (Evans et al., 2006).
Tradeoffs and PO activity

Evolution of eusociality in termites has been driven by several forces, including feeding and nesting ecology (Shellman-Reeve, 1997) as well as disease (Rosengaus and Traniello, 1993; Schmid-Hempel, 2003; Thorne and Traniello, 2003). Because immunity is costly and resources are limiting (Sheldon and Verhulst, 1996), it has been assumed that immune defense represents a trade-off with other fitness parameters (Schmid-Hempel, 2003). The costs of increased disease resistance are often associated with changes in an organism’s physiology (Schmid-Hempel, 2003) including reduced fertility (Calleri II et al., 2006; McKeen et al., 2008), growth, reproductive success (Yang et al., 2007), or metabolic rate (Freitak et al., 2003). Our results indicate that different termite species vary significantly in their levels of PO activity and point to the possibility that nesting, feeding and foraging habits are one of the most important factors influencing such differential immune investment. It is important to remember that this study focused on measuring a single component of the immune system across termite species. Other analyses on immunity, such as hemocyte and fat quantification (Wilson-Rich et al., 2008), as well as the production of antimicrobial peptides via humoral responses (Rosengaus et al., 1999b, 2007) are needed before any generalizations about overall immune function can be developed. Ideally, analyses on multiple measures of immunity, together with in vivo susceptibility assays (preferably using a variety of ecologically relevant pathogens) will provide a more complete picture of how and which life history traits may have affected the evolution of immune function.

This study raises questions on how the unique feeding, nesting and foraging ecologies together with other life history traits can influence the evolution of immunity in social insects. Invariably, investment in immunocompetence, encompassing behavioral, biochemical and physiological adaptations to resist disease, is critical to survival of organisms living in large,
densely populated communities.
Chapter 3: Social organization and phenoloxidase activity: The impact of caste membership on the relative investment in immunocompetence.

Abstract

Termites exhibit a complex caste system in which specialization in particular tasks ultimately increases colony fitness. Most individuals in a colony are sterile and engage in colony maintenance, foraging, defense and brood care, while a few individuals undertake reproductive duties. The degree of task specialization varies across termite species and therefore, comparative studies are capable of teasing the impact that caste membership has on an individual’s immune function. Four termite species ranging from the phylogenetically primitive to the most derived were used for the quantification of relative phenoloxidase activity (PO), an enzyme involved in immunological responses. The phylogenetically primitive termite, Zootermopsis angusticollis, exhibits a flexible caste system in which members have the potential for attaining reproductive status. In the phylogenetically intermediate Reticulitermes flavipes, most members of the society maintain reproductive plasticity with the exception of the soldier caste. Finally, the most derived species, Nasutitermes acajutlae and Nasutitermes corniger, have completely sterile worker and soldier castes. Our results indicate that PO activity varies significantly as a function of reproductive potential. In three of the four species tested, reproductive potential was positively correlated with PO activity. Reticulitermes flavipes was the exception with all castes having similar PO levels, regardless of reproductive potential. The subterranean life-style of R. flavipes and concomitant high risks of pathogen exposure during foraging may explain the heightened PO levels in the worker and soldier castes relative to that of the reproductive line. This work represents the first comparative study of termite PO levels as a function of species and caste
membership. Although additional species should be studied in order to gain a greater
understanding of the impact caste has on immune investment, we propose that reproductive
potential is one determinant factor in driving the levels of immunocompetence in eusocial
ingsects.

3.1 Introduction

Eusociality is exhibited in a restricted number of insect species, mostly within the
Hymenoptera (ants, bees and wasps) and the Isoptera (termites). Their societies are
characterized by individuals from different generations living together while participating in
cooperative brood care of younger nestmates and where reproduction is restricted to only a few
individuals (Wilson, 1975). Group living has significant advantages over a solitary lifestyle. For
example, organized groups of individuals can out compete solitary organisms for resources
(Hölldobler and Wilson, 2009), have higher foraging efficiency, better predator defense and
reproduction (Rosengaus et al., 1998b). More recently, it has been suggested that group living in
social insects facilitates disease resistance (Rosengaus et al., 1998b; Traniello et al., 2002;
Cremer et al., 2007).

Although the Hymenoptera has converged with Isoptera in many attributes of their social
organization, there are distinct differences between these two social insect orders.
Hymenopterans are holometabolus insects, and thus, the young are mostly dependent upon and
cared for by adults. In sharp contrast, termites undergo hemimetabolus development; the young
can participate in colony labor at a much earlier age (Rosengaus and Traniello, 1993; Crossland
and Traniello, 1997; Thorne and Traniello, 2003). While in Hymenoptera there is a distinct
female-biased sex ratio, the work force in Isoptera is comprised of both males and females,
although some of the higher termites exhibit a sexual dimorphism (Roisin, 2000). The role of male and female reproductives differs between these two orders as well. In the Isoptera, both the king and queen participate in the reproductive and parental duties of the young society while in most Hymenoptera, the kings are short lived and the queens alone are responsible for reproduction and ultimately, colony growth (Lin and Michener, 1972; see chapter 4 for sex related differences in immunity).

Additionally, the degree of complexity in the social organization within the Hymenoptera and Isoptera varies among species. In termites, five specialized castes exist: larva, nymph, worker, soldier and the reproductive (Wilson, 1971). Larvae are immature individuals that lack wing buds and soldier characteristics. By undergoing progressive molts, larvae may become either soldiers or nymphs (individuals with external wing buds) and ultimately proceed to the imago line (fully winged individuals that disperse from their natal colony to initiate a new colony). Pseudergates are found primarily in the lower termites and are “false” workers because they still retain the capability of reproducing. In other more derived termite species, the workers have completely given up the ability to reproduce, becoming a true sterile caste. Soldier morphology varies greatly among the Isoptera but generally includes highly sclerotized heads with either mechanical or chemical defensive adaptations. Across this order, the majority of soldiers are sterile, although in the lower termites instances of soldiers attaining reproductive status exist (Thorne, 1997). In the lower termites, there are two types of reproductives: those individuals that are derived from winged individuals (primary reproductives) and those reproductives that differentiate within their natal colony and replace the primary reproductives in a colony if and when the founding reproductives die (secondary reproductives). Each caste plays a unique role in the colony and thus, it is reasonable to assume that each caste invests differently
in immunity according to the risk inherent to each specialized task, their longevity and/or their reproductive potential.

**Reproductive Potential**

Within the termites, the degree of sociality falls on a spectrum based on reproductive skew, that ranges in value from 0 to 1 (Shellman-Reeve, 1997). Sherman et al. (1995) used lifetime reproductive success to quantify the degree of eusociality. The results were used to create an index of reproductive skew. Biologically speaking, the lower the reproductive skew index of a species, the more likely individuals are to become reproductive during their lifetime. On the other hand, the higher the reproductive skew, the less likely they are to attain reproductive status. Not surprisingly, species with higher reproductive skew also tend to have higher levels of altruism and a higher prevalence of sterile worker and soldier castes (Shellman-Reeve, 1997). The reproductive skew index is therefore useful when trying to predict the interaction between different life history traits and reproductive potential. Of the species included in this study, *Zootermopsis angusticollis* has the lowest reproductive skew falling below 0.25. *Reticulitermes flavipes* falls in the mid-range of the scale between 0.4 and 0.75 while both species of *Nasutitermes* fall above 0.70 on the scale (Shellman-Reeve, 1997).

Eusociality is not only correlated with reproductive skew, but also appears to be significantly associated with increased longevity (Keller and Genoud, 1997). Queens of highly eusocial species exhibit low rates of aging when compared to solitary insect adults (Keller and Genoud, 1997). In separate studies, longevity has also been correlated with better immunocompetence (Dunn, 1990). Although the present research did not examine directly the interactions among lifespan of an insect, its reproductive potential and its levels of altruism and
immunity, it is possible that those castes with the greatest reproductive potential also have the longest lifespan because they have been selected to invest more heavily in immunological defenses.

Therefore, it is hypothesized that future reproductive potential should influence the investment in immune function. The higher the reproductive potential of an individual within the colony, the more “valuable” (in relation to personal and colony fitness) and thus, the greater its investment in immunocompetence should be (i.e. increased PO activity). As described earlier, nestmates in the phylogenetically primitive termites have, in general, high reproductive potential (regardless of their caste; Shellman-Reeve, 1997): workers and even soldiers can attain reproductive status in the colony in the absence of the established queen and king (Miller, 1969). In sharp contrast, higher termites, have a more restricted and pre-determined caste development; soldiers and workers are entirely sterile and have no reproductive potential relative to the reproductive line (Noirot, 1969). Therefore, we expect PO activity (a proxy for measuring immune investment) to be highest in the castes that can attain reproductive status within a colony.

Caste Longevity & Task Allocation

Although reproductive potential likely influences investment in immune function, the possibility exists that levels of immunocompetence in the sterile castes are also impacted by the risk of infection associated with task specialization. There are two potentially contradictory hypotheses to explain differential immune investment amongst neuter (sterile) castes. The first hypothesis suggests that castes with a higher risk of exposure to disease should invest more heavily in their immune system (Bocher et al., 2007), irrespective of reproductive potential
and/or longevity. Foragers of the ant *Cataglyphis velox*, for example, have been reported as having increased PO activity when compared with workers that remain within the confines of the nest presumably because foragers venturing outside the colony to locate new food resources are exposed to more pathogenic microorganisms (Bocher et al., 2007). Unfortunately, studies focusing on the pathogenic exposure risk hypothesis have produced inconsistent results. In *Acronymex echinatia* and *Atta colombica*, workers associated with higher risk tasks (like foraging and waste management) have lower immune investment relative to those individuals whose tasks require them to remain in the nest (Brown et al., 2006; Poulsen et al., 2006). Such divergent results point to the need for further studies on what factors affect immune function and how the existence of significant trade-offs between metabolic activity and immune investment influence disease susceptibility (Köning and Schmid-Hempel, 1995). Different tasks likely vary in their metabolic requirements and specialized behaviors may influence the resources available for immune investment (Doums and Schmid-Hempel, 2000; Brown et al., 2006; Poulsen et al., 2006; Remolina and Hughes, 2008). To the best of our knowledge, there are no studies examining the immunocompetence of the different castes of termites in relation to task-related pathogenic risks.

In addition, investment in immunocompetence may also be influenced by aging. The alternative hypothesis of senescence predicts a lower immunity as individuals age due to the natural deterioration of the immune system (Bocher et al., 2007). There is empirical evidence to support the immuno-senescence hypothesis in some eusocial insects (Doums et al., 2002; Woyciechowski and Moroń, 2009). There is also evidence that supports the view that ongoing changes in the immune system of insects are not necessarily a deterioration of the overall immune system but rather a restructuring of investment into different immune compartments.
such as for example, cellular vs. humoral immunity (Rolff, 2001b; Schmid et al., 2008; Wilson-Rich et al., 2008). A case in point is the decline in hemocyte counts in *Apis mellifera* as bees age, while the encapsulation response remains stable resulting in a more vigorous immune system (Wilson-Rich et al., 2008). Rosengaus and Traniello (2001) found that homogenous groups of nymphs (the oldest individuals before turning into reproductives) were less affected by *M. anisopliae* than younger instar groups, pointing to a positive correlation between age and immunocompetence. These latest results do not support the immuno-senescence hypothesis in termites. Interestingly, individual termites vary in longevity as a function of species: the more derived central nesting workers of the genus *Nasutitermes* have much shorter life-spans than the functional workers of both *Z. angusticollis* or *R. flavipes* (Shellman-Reeve, 1997; see life history table 1a for more information). Based on the immuno-senescence hypothesis, it is predicted that shorter-lived castes, such as the workers and soldiers, will exhibit decreased investment in PO, regardless of risk of exposure. Investment in immune function is energetically expensive (Sheldon and Verhulst, 1996) and therefore a short-lived and/or sterile caste member (an “expendable” cohort) should exhibit lower PO activity than the long-lived and “indispensable” members of the reproductive caste.

As a result of a multitude of studies focusing on the factors influencing immunocompetence, we can only ascertain that overall immune investment in insects may ultimately be impacted by the combined effects of age, reproductive potential, high risk of infection and/or longevity. Given that many of these variables are confounded, establishing the relative and independent significance of each of these factors will likely remain elusive.
3.2 Results

Results indicate that within each species, total PO activity (active PO and stored PO) varied significantly between castes (figures 3a, 3b, 3c and 3d). With the exception of *R. flavipes*, the reproductive caste in all other tested species had consistently the highest total PO levels relative to the other castes. Although total PO activity was significantly different between all three castes of *R. flavipes*, the total PO median values were all within a very small range (3.7 to 4.3 activity/g termite; figure 3b). In contrast, total PO activity ranges for *Z. angusticollis* (0.59 to 2.3 activity/g termite; figure 3a), *N. corniger* (0.05 to 1.1 activity/g termite, figure 3c) and *N. acajutlae* (0.07 to 0.7 activity/g termite, figure 3d) were larger than those of *R. flavipes*. Detailed analyses of total PO levels for each species are presented in turn, below.

*Zootermopsis angusticollis*

*Zootermopsis angusticollis* PO activity differed significantly among the four castes. As predicted by the proposed reproductive potential hypothesis, alates (the future reproductive caste, n=67) exhibited almost twice the median total PO activity of nymphs (n=106). Pseudergates (n=73) and soldiers (n=26) exhibited significantly lower total PO activity than alates and nymphs (figure 3a.) In this species, there is a clear positive association between reproductive potential and PO activity.

*Reticulitermes flavipes*

Pairwise comparison of three available castes of *R. flavipes* indicate a significant difference in total PO activity between soldiers (n=44) and each of the other two castes, alate (n=81) and pseudergate (n=45, table 3a and figure 3b, respectively). Unlike the other species
studied, the highest total PO activity was found in the soldier caste (1.3 times higher than alate PO activity)

*Nasutitermes corniger*

Pairwise comparisons of the total PO activity of *N. corniger* indicate a significant difference between alates (n=36) and the other two castes, worker (n=46) and soldier (n=42, figure 3c). The alate caste had a twenty-fold difference in total PO activity when compared with the negligible activity of the workers and soldiers. Workers had 5.7 times the total PO activity of soldiers.

*Nasutitermes acajutlae*

Pairwise comparisons of *N. acajutlae* indicate a significant difference across all three castes. Similarly to *N. corniger*, *N. acajutlae* alates (n=38) had a ten fold higher total PO activity than that of the soldiers (n=21) and workers (n=23; figure 3d). Soldiers had 0.1 fold increase in total PO activity compared to that of workers, although this difference is statistically significant it likely has not biological significance.
Figure 3a: Median PO activity per gram of *Zootermopsis angusticollis* as a function of caste. Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and interquartile range. Significant difference in pairwise comparison (P<0.0083 after a Bonferroni correction due to multiple pairwise comparisons) represented by different letters. The outliers, identified by “°”, include cases with values over 1.5 box lengths from the upper edge of the box. These data represent the combined PO activity of termites collected from three colonies.
Figure 3b: Median PO activity per gram of *Reticulitermes flavipes* as a function of caste. Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and interquartile range. Significant difference in pairwise comparison (P<0.016 after a Bonferroni correction due to multiple pairwise comparisons) represented by different letters. The outliers, identified by “°”, include cases with values over 1.5 box lengths from the upper edge of the box. These data represent the combined PO activity of termites collected from two colonies.
Figure 3c: Median PO activity per gram of *Nasutitermes corniger* as a function of caste. Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and interquartile range. Significant difference in pairwise comparison (P<0.016 after a Bonferroni correction due to multiple pairwise comparisons) represented by different letters. The outliers, identified by “°”, include cases with values over 1.5 box lengths from the upper edge of the box. These data represent the combined PO activity of termites collected from two colonies. Note that the soldier and worker castes have zero reproductive potential.
Figure 3d: Median PO activity per gram of *Nasutitermes acajutlae* as a function of caste. Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and interquartile range. Significant difference in pairwise comparison (P<0.016 after a Bonferroni correction due to multiple pairwise comparisons) represented by different letters. The outliers, identified by “°”, include cases with values over 1.5 box lengths from the upper edge of the box. These data represent the combined PO activity of termites collected from one colony. Note that the soldier and worker castes have zero reproductive potential.
3.3 Discussion

These results show for the first time that termite caste influences immune investment, at least as measured by total PO activity levels. The comparative approach used here allows for the determination of relative investment as a function of caste and perhaps its underlying reproductive potential. Each caste contributes differently to the success of the colony; workers provide food and shelter and soldiers afford protection, while the reproductive caste specializes on the production of new colony members. Previous research provides evidence that both the age of an insect and the pathogenic risks associated with the task an individual performs may also impact relative investment in the immune system (Bocher et al., 2007). These results point to a third, not necessarily independent factor that influences immune function: individuals who retain some semblance of reproductive potential appear to invest more in at least one aspect of their immune system. This differential investment in immunocompetence may translate into higher longevity and thus higher individual direct fitness for the reproductive caste and higher indirect fitness for the sterile castes (workers and soldiers).

The reproductive caste of three of the four investigated species consistently exhibited the highest investment in PO relative to the other castes. Although trade-offs between fecundity and immunity have been identified in insects (Rolff and Siva-Jothy, 2002), including termites (Callei II et al., 2006, 2007), the adults tested here were not yet reproducing. This suggests that the last molt in Z. angusticollis is accompanied by significant readjustments in the levels of PO activity and that these adjustments are independent of the endocrinological and physiological changes after copulation. It is unknown however, whether such increases in PO activity are related to the increased melanization and sclerotization of the alate cuticle relative to that of the preceding nymphal stage. Nevertheless, the higher PO levels in alates, whether due to immune
investment or darker integument, likely represent an important strategy in ensuring lower susceptibility to infection by pathogens and parasites as well as reduced risk of cuticular injuries during the critical periods of dispersal, tandem running and colony establishment. It is also important to bear in mind that the integument plays a critical role in disease resistance; it is a mechanical barrier to pathogens (Dunn, 1990; Brey et al., 1993), and both Rosengaus and Traniello (1997) and Chouvnec et al., 2009a) have shown that cuticular melanization takes place as *M. anisopliae* attempts to penetrate this first line of defense. Furthermore, the darker integument may not be of significant importance in total PO activity as the lightest color species *R. flavipes* has the greatest PO activity rates while the darkest colored species of the genus *Nasutitermes* has the lowest PO activity rates.

PO activity among the castes of *Z. angusticollis*, a species with high reproductive plasticity (Shellman-Reeve, 1997), mirrors their reproductive potential. Alates have the highest reproductive potential and highest PO level activity, followed by nymphs, which in turn are followed by the functional worker caste. Finally, soldiers are least likely to become secondary reproductives and exhibit the lowest PO levels even though there are instances where soldiers have been found to differentiate into reproductive soldiers (Thorne, 1997; Thorne et al., 2003). Unfortunately, relatively little is known about the physiological immune defenses of termites and few studies have compared immunocompetence as a function of caste membership. Constitutive proteins of *Z. angusticollis* soldiers have been found to be less fungistatic than those of the functional worker caste (Rosengaus et al., 2007). The present results using PO as an indirect measure of one component of the overall immune function of *Z. angusticollis* soldiers are consistent with the lower fungistatic activity of their humorally produced peptides.
Contrary to expectation, *R. flavipes* soldiers exhibited significantly higher PO activity than workers or alates, although the PO rates across all castes were less variable and significantly higher than those of the other tested species. These results do not support the progressive deterioration of an individual’s immune response with age (senescence hypothesis) nor the proposed reproductive potential effects on immune investment. It is likely that in *R. flavipes*, the similar levels of PO activity across castes reflects the high risk of exposure to pathogens given their subterranean lifestyle. A recent study found that soil dwelling insects exhibit significantly higher nodule formation, melanotic “scars”, than insects found in above-ground plant material (Tunaz and Stanley, 2009), suggesting that subterranean insects come in contact with a higher pathogenic load and increased immune assault. This is consistent with the PO levels found in *R. flavipes*, a soil dwelling termite compared to the single piece (no foraging) wood nesting *Z. angusticollis* and the arboreal nesting *Nasutitermes* sp (see chapter 2).

Although *R. flavipes* workers exhibit reproductive plasticity and can differentiate into neotenic reproductives, soldiers can not become reproductive (Miller, 1969; Noirot, 1986). Thus *R. flavipes* PO activity is not congruous with the reproductive potential hypothesis presented above. The “high risk” hypothesis may better explain the results obtained for this species. The soldier caste is responsible for colony defense and thus is equipped with relatively large mandibles and an enlarged highly sclerotized head. Both of these morphological traits are also evident in *Z. angusticollis* soldiers. Yet, the defensive behavior of the soldier castes of these two species differs substantially: *R. flavipes* soldiers are much more aggressive than *Z. angusticollis* soldiers. The latter are reclusive and tend to protect the colony while hiding at the same time (personal observation). *Reticulitermes flavipes*, on the other hand, soldiers lunge forward snapping their enlarged sclerotized mandibles toward the intruder. If these behavioral
differences are indeed evident under natural conditions, then we would expect the potential for wounding to be higher in the *R. flavipes* soldier caste than in *Z. angusticollis*. Because PO plays an important role in wound healing (Ashida and Hiroko, 1990), the amount of PO activity may reflect the increased risk of injury faced by the soldier caste in a way similar to that of the foraging workers of *Cataglyphis velox* which have greater PO activity than those workers that remain in the safety of the nest (Bocher et al., 2007).

There are a number of opposing studies in which insects that leave and work outside the nest actually have lower immune investment than those colony members that reside within the boundaries of the nest (König and Schmid-Hempel, 1995; Doums and Schmid-Hempel, 2000; Brown et al., 2006). One such study found that smaller leaf-cutting ants within the nest had higher survival rates when challenged with pathogenic fungus (Poulsen et al., 2006). The defensive tasks associated with *R. flavipes* soldier places these individuals at greater risk to injury and ultimately infection. The role of protection may have lead to increased immunocompetence. Further evidence examining behavior and termite immunocompetence is necessary to solidify this hypothesis.

Although overall, reproductive potential explains the variation in the present PO activity data, it is important to discuss the potential effect of age on immunocompetence. Age has been found to correspond with immunocompetence in some situations. The tasks accomplished by the work force of *Apis mellifera* are distributed by age. As worker bees age and become foragers, their investment in PO increases while their hemocyte count decreases (Schmid et al., 2008; Wilson-Rich et al., 2008). Unfortunately, honey bee age polyethism confounds two of the variables we are interested in testing as independent predictors of immune investment: aging and task allocation risk. None of the termite species studied here exhibit age polyethism (Dunn,
1990; Rosengaus and Traniello, 2001; Long, 2005). Therefore, the confounding effects of age and risk associated with caste-specific tasks can be separated in the termites.

Studies have shown that as individuals age their investment in immunity changes. This could be related to aging or change in the risk associated with their assigned task (Rolff, 2001b; Doums et al., 2002; Schmid et al., 2008; Wilson-Rich et al., 2008). *Zootermopsis angusticollis* susceptibility to fungal disease is dependent on instar, older termites had a significant advantage in resisting infection (Rosengaus and Traniello, 2001). This change in immune investment could be due to increased exposure to a different set of pathogens as an insect’s role in the colony shifts or that the immune system matures with age. Although individuals of different age were not tested here, specialized labor of each caste leads to different physiological demands as well as different potential disease risk. One caste’s investment in immunocompetence may not only be of a different magnitude, but also be different between the two physiological compartments (humoral versus cellular), or type of strategy for resisting disease (from behavioral to biochemical to immunological). For example, *Nasutitermes* soldiers have cephalic glands containing several terpenoids, which are used both as anti-predator and anti-fungal defenses (Rosengaus et al., 2000a; Fuller, 2007). Investment in such biochemical dual protection may be less energetically costly than the upregulation of PO activity or other immune responses (Rosengaus et al., 2010) and thus, selection for high immune investment may have been low for this caste. Similar conclusions have been made for ants (Chapuisat et al., 2007; Castella et al., 2008;) and bees (Simone et al., 2009) where the incorporation of resins into their nest or the increased antimicrobial activity of resin extracts are negatively correlated with physiological immune measures. Most striking is the reduced number of immune genes in honey bees relative to other insects (Evans et al., 2006), a result that has been attributed to the fact that behavioral
responses may be faster, cheaper and extremely effective, thus honeybees have been "released" from requiring an ample array of immune-related genes.

It appears that reproductive potential, aging, longevity and pathogenic risks related to task allocation may have all played a significant role in shaping immune investment across the Isoptera. Three of the four termite species tested, *Z. angusticollis*, *N. corniger* and *N. acajutlae* all have alates with significantly higher PO activity than their neuter worker and soldier castes. This observation is consistent with the hypothesis that an open reproductive potential and/or aging increases immunocompetence. On the other hand, PO data on *R. flavipes* does not support the reproductive potential/aging predictions. Instead, this species points more towards the significance of high risks of injury and infection while exploiting microbially rich subterranean niches (Holt, 1996; Cruse, 1998; Tunaz and Stanley, 2009) in shaping PO activity levels. It is difficult to separate the relative contribution of each of these factors on immune investment since all three factors: age, reproductive potential and task allocation are usually confounded variables. Further studies are required to assess which variable, or combination of variables, are molding immune investment in relation to each individual's life history characteristics. Moreover, multiple assessments of the varied immunological responses (cellular and humoral) and interactions/trade-offs with other behavioral and biochemical modes of disease resistance are needed to pull apart the relative contribution of each of these factors on the evolution of immune function in Isoptera. Expanding these initial comparative approaches would greatly benefit from including not only other termite and social Hymenopteran species, but also measures of immune investment in roaches such as *Cryptocercus punctulatus* (the proposed prototermite ancestor), which would allow some inferences about the evolution of immune investment as eusociality was established in the termites.
Chapter 4: Gender and immunocompetence: are termites the exception?

Abstract

Animals often exhibit sex-biased immunity where females are usually more immunocompetent than males. This dichotomy is also commonly found in insects. Studies of social Hymenopterans appear to conform to the expectation that females generate a more efficient immune response than males. However, their genetics and several of their life history attributes may render this taxonomic group unsuitable for the study of sex-based immunocompetence. For example, bees, wasps and ants have female-biased sex ratios with females undertaking the critical tasks within the colony. Ultimately, tasks performed by the female-worker force translate into higher colony-level fitness. Males (drones), on the other hand, do not play a major role in colony life and die soon after copulation with the future queen(s). Such gender differences in longevity could explain why females are more immunocompetent than males. Termites are also social insects, but their colonies do not exhibit the same sex-ratio biases seen in social Hymenopterans. In termites, both male and female reproductives are similarly long-lived and both participate in colony foundation. For the most part, male and female workers and soldiers engage in daily colony maintenance, brood care and defense, respectively. The combination of both sexes contributing to colony work in the Isoptera, along with similar male-female longevity and pathogen-exposure risks suggest that both sexes should have been selected for equivalent immune investment. This study investigates the differences in relative phenoloxidase (PO) activity, an enzyme that plays a multifaceted role in the immune system, between males and females of four phylogenetically diverse termite species. Consistent with life history theory, the results indicate that, with the exception of Zootermopsis
angusticollis alates, PO activity does not differ significantly between males and females.

4.1 Introduction

Males and females appear to respond differently to immune challenges. In general, females are thought to be more immunocompetent than males. Examples of this sex-based divergence in immune function are found throughout the animal kingdom, including both invertebrates and vertebrates (Zuk and McKean, 1996; Rolff, 2001a, b; Restif and Amos, 2010). One potential explanation for the gender-biased immune responses in vertebrates involves the presence of testosterone. Although high levels of testosterone lead to expression of secondary sexual traits in males and perhaps higher overall fitness, this hormone also acts as an immuno-suppressor (Zuk et al., 1996; Zuk and McKean, 1996; Rolff, 2001a). This so called "immunocompetence handicap" hypothesis however, is not applicable to insects since insects lack testosterone (Braune and Rolff, 2001). Yet, the gender-based immunocompetence dichotomy is also evident in this taxonomic group (Rheins and Karp, 1985; Zuk and McKean, 1996; Kurtz and Sauer, 1999; Baer et al., 2005). What factors are then responsible for the stronger immune system of female insects? Why have female insects been selected to generate more effective responses against parasites and pathogens than males? One possible reason is that differences in male and female life history attributes influence their degree of immune investment (Fedorka et al., 2004; Jacot et al., 2004; Vainio et al., 2004; Zuk et al., 2004; Schmid-Hempel, 2006; Restif and Amos, 2010). Factors such as longevity, age, reproductive potential as well as differences in reproductive interests and strategies between the sexes may drive immunocompetence (see table 1a; chapter 1). This is particularly true for social insects, where intense social interactions and high within-colony genetic homogeneity may further promote infection amongst nestmates (Rosengaus and Traniello, 1997). Theoretical and empirical studies
on several social Hymenoptera species have indicated a strong female-biased
immunocompetence (O'Donnell and Beshers, 2004; Vainio et al., 2004; Baer et al., 2005; Baer
and Schmid-Hempel, 2006), although inconsistent results have also been reported (Ruiz-
González and Brown, 2006). Social Hymenopterans, however, may not be the best test group to
identify gender differences in immune function given their haplo-diplod genetics and that several
of their life history traits are sex-linked. For example, haploid males have half the immune genes
of females, most females in a nest have no reproductive potential (sterile workers) while males
(i.e drones) reproduce. Moreover, within the reproductive caste, queens are long-lived while
males die soon after mating (Wilson, 1971). Such gender-coupled life history traits in the social
Hymenopterans preclude disentangling the independent role that gender plays on immunity.

Termites, on the other hand, offer a unique opportunity to study gender-based differences
in immunity without the confounded traits of the social Hymenoptera. Both male and female
termites are diploid; the sterile worker and defensive castes are for the most part comprised of
members of both sexes. Both male and female reproductives are necessary for colony foundation
and both genders are equally long-lived. Moreover, labor and paternal/maternal care to young
during the initial stages of colony foundation is essential for the successful establishment of a
colony (Rosengaus and Traniello, 1991; Shellman-Reeve, 1997; Thorne, 1997). This study
tested, for the first time, whether male and female termites differ in their relative phenoloxidase
(PO) activity, an enzyme that plays a multifaceted role in the immune system, across four
phylogenetically diverse termite species.
Table 4a: A compilation of gender-based immune difference studies in solitary and social (*) insects.

<table>
<thead>
<tr>
<th>Order</th>
<th>Species</th>
<th>Difference between sexes, measure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coleoptera</td>
<td>*Euoniticellus intermedius</td>
<td>Females had higher encapsulation rates than males.</td>
<td>(Pomfret and Knell, 2006)</td>
</tr>
<tr>
<td>Diptera</td>
<td>*Scathophaga stercoraria</td>
<td>Sexually mature females had greater hemolymph PO activity than males. No difference was observed for non-mature flies.</td>
<td>(Schwarzenbach et al., 2005)</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td>*Acromyrmex echinatork</td>
<td>Female workers had approximately 4 times higher encapsulation rates compared with reproductive males.</td>
<td>(Baer et al., 2005)</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td>*Bombus terrestris</td>
<td>Female workers had higher encapsulation response compared with the reproductive males.</td>
<td>(Baer and Schmid-Hempel, 2006)</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td>*Bombus terrestris</td>
<td>Female workers had higher encapsulation rates than both haploid &amp; diploid males.</td>
<td>(Gerloff et al., 2003)</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td>*Bombus terrestris</td>
<td>Female workers did not differ in susceptibility to <em>Crithidia bombi</em> when compared with haploid males.</td>
<td>(Ruiz-González and Brown, 2006)</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td>*Formica exsecta</td>
<td>Queens had increased encapsulation rates compared to males.</td>
<td>(Vainio et al., 2004)</td>
</tr>
<tr>
<td>Isoptera</td>
<td>*Zootermopsis angusticollis</td>
<td>No difference in the effect of eluted proteins on the viability of <em>M. anisopliae</em> conidia.</td>
<td>(Rosengaus et al., 2007)</td>
</tr>
<tr>
<td>Isoptera</td>
<td>*Zootermopsis angusticollis</td>
<td>Paired female primary reproductives had lower encapsulation rate than paired males.</td>
<td>(Calleri II et al., 2007)</td>
</tr>
<tr>
<td>Isoptera</td>
<td>*Zootermopsis angusticollis</td>
<td>Sex of dealates was not a significant predictor of termite survival after exposure to <em>M. anisopliae</em>.</td>
<td>(Rosengaus et al., 2000b)</td>
</tr>
<tr>
<td>Mecoptera</td>
<td>*Panorpa vulgaris</td>
<td>Females had increased hemolymph lysozome like activity and superior phagocytoc capacity than males.</td>
<td>(Kurtz et al., 2000)</td>
</tr>
<tr>
<td>Mecoptera</td>
<td>*Panorpa vulgaris</td>
<td>Females had increased PO activity in hemocytes where males have none</td>
<td>(Kurtz and Sauer, 2001)</td>
</tr>
<tr>
<td>Odonata</td>
<td>*Lestes viridis</td>
<td>Females had higher PO activity than males</td>
<td>(Rolff, 2001b)</td>
</tr>
<tr>
<td>Odonata</td>
<td>*Mnais costalis</td>
<td>Females had higher hemocyte PO levels than males</td>
<td>(Siva-Jothy et al., 2001)</td>
</tr>
<tr>
<td>Odonata</td>
<td>*Lestes forcipatus</td>
<td>Females showed greater melanization of bead implants. No gender difference in the rates of parasitism by mites.</td>
<td>(Yourth et al., 2002)</td>
</tr>
</tbody>
</table>
4.2 Results

A linear regression model revealed that gender (beta coefficient = 0.128, P=0.001), caste (beta coefficient =0.291, P=0.001), species (beta coefficient =1.210, P=0.001), and colony of origin (beta coefficient =-0.665, P=0.001) (SPSS 13) were all significant variables influencing PO activity. However, when PO levels were examined within a species, PO activity did not vary significantly between males and females of the same caste, with one exception.

Zootermopsis angusticollis

Male and female nymphs and pseudergates did not differ significantly in their PO activity levels (figure 4a). Unfortunately the majority of soldiers assayed in Z. angusticollis were female. Male soldier sample size was too low to reliably test for gender differences. In contrast to the nymphs and false workers, alate PO activity was significantly different between males and females (Mann-WhitneyU=362, P<0.0001). Such significance was mainly driven by inter-sexual
differences between alates originating from colony 3, with males having significantly higher PO activity than females (WhitneyU=60, P<0.0001). Colony 2, on the other hand, did not exhibit a significant difference in PO activity between the males and females alates (Mann-WhitneyU=72, P=0.5). Given the inter-colony variation in PO levels of male and female alates, it is premature to conclude whether this caste exhibits gender-biased immunocopetence.

_Reticulitermes flavipes_

PO activity between males and females of _R. flavipes_ was only analyzed for alates because male and female _R. flavipes_ workers and soldiers are morphologically indistinguishable unless dissections are performed making the potential differences between gender impossible to distinguish (Howard and Haverty, 1980). The results indicate a lack of gender-based immunocompetence (measured as PO activity) in _R. flavipes_ alates (Mann-Whitney U=130, P=0.3 for colony 9, while Mann-Whitney U=249, P=1.0 for colony 10, figure 4b).

_Nasutitermes acajutlae_

_Nasutitermes_ sp. exhibit sex-based polyethism making it impossible to distinguish the independent effect of gender and caste on PO activity (see chapter 3 for caste differences). Unfortunately we only had access to alates from a single colony of _N. acajutlae_, however no significant difference in PO activity was found between males and females (Mann-Whitney U=153, P=0.6, figure 4b).

_Nasutitermes Corniger_

_N. corniger_ alates did not exhibit significant difference in PO activity rates between females and males (Mann-WhitneyU=95, P=0.06, figure 4b).
Figure 4a: Median PO activity per gram of *Zootermopsis angusticollis* as a function of caste and sex (data combined for multiple colonies). Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and interquartile range. Significant differences were determined using Mann-Whitney U, *P < 0.05* are represented with ‘*’. The outliers, identified by ‘°’, include cases with values over 1.5 box lengths from the upper edge of the box.
Figure 4b: Median PO activity per gram of alate as a function of species and sex. Samples were treated with chymotrypsin to activate PO. Each boxplot shows the median values and interquartile range. Significant difference were determined using Mann-Whitney U, P<0.05 are represented with "*". The outliers, identified by "°", include cases with values over 1.5 box lengths from the upper edge of the box. Note that the significant difference observed for the *Z. angusticollis* are due to the variation in PO activity between males and females of one of the two colonies.
4.3 Discussion

Gender differences in immunocompetence exist throughout the insect kingdom (see table 4a). Bateman (1948) suggested females and males differ in their life history traits and strategies in order to maximize their reproductive success. Females gain fitness by investing in energetically expensive gametes, being choosy and increasing their longevity, while males gain fitness by producing energetically “cheap” sperm and mating with multiple partners. Given that an effective immune system likely lengthens survival and that the reproductive interests and strategies of both genders are not necessarily aligned, it is reasonable to expect differences in immunocompetence, particularly when both reproduction and immunity rely on a limited pool of resources. Yet studies on insect gender-biased immunocompetence are inconsistent, ranging from those supporting a female-biased immune function, to those where no perceptible differences in immunocompetence are evident between the sexes. Moreover, a few examples exist where male-biased immune investment has been reported (see table 4a). Such diverse results suggest that immunocompetence is not intrinsically linked to gender, but rather that differences in life history traits between males and females may be the important driving force in the evolution of immune function.

A case in point is the differential encapsulation rates between genders of two closely related species of ground cricket, Telogyrillus commodus and Telogyllus oceanicus. In T. commodus, a seasonal egg layer, males had significantly higher encapsulation rates than females (Zuk et al., 2004). The authors suggested this was due to the high-energy demands of egg production over a short period of time and the trade offs between limited resources and immune investment. In contrast, T. oceanicus, a year-round egg-layer in which resources are allocated over a longer reproduction time frame, males and females exhibited equivalent encapsulation
response (Zuk et al., 2004). This research exemplifies how immune investment can be affected by different life history attributes, in this case breeding schedule, even between closely related species. In yet another cricket species, Gryllus texensis, both sexes have similar immunocompetence before the onset of sexual maturity, but once individuals begin to display reproductive behaviors (like mate calling), male immunocompetence decreases while female immune activity increases, suggesting that males trade-off immunity while females do not (Adamo et al., 2001).

Few studies have examined this phenomenon in the social insects. Moreover, the majority of these studies utilize the social Hymenoptera and compare reproductive males with non-reproductive females. For example, in Acromyrmex echinatior, female workers, which are sterile, have four times the encapsulation rate of males which are reproductive (Baer et al., 2005), indicating that sterile females have a higher immune investment than the drones. Because reproductive potential may be a critical life history trait influencing immune investment (Callei II et al., 2006; Yang et al., 2007; McKean et al., 2008; discussed in Chapter 3), these comparisons, in reality, provide little information given the striking differences in reproductive potential between these two castes. To control for reproductive potential, the appropriate comparison should be between reproductive females and reproductive males (drones). At least one such study exists. Vainio et al., (2004) found the encapsulation rates of the wood ant Formica exsecta to be greater in reproductive females than in reproductive males, which die soon after copulation. However, even in this situation the life history differences between longevity of males and females preclude us from applying this finding to the Isoptera.

Given the confounding effect that haplo-diploid genetics may have on studies of gender-bias immune function, it is imperative that research on this topic be carried out on diploid social
insects. By controlling for differences in allelic number between the two sexes, the existence of immunological sexual dimorphism can be assessed as a function of life history attributes independently of number of alleles. In this respect, the diploid termites appear to be a better taxonomic social group than the social Hymenoptera to examine gender-based immune differences. The present work using termites represents a more controlled look at how immunocompetence varies between the sexes without the added confounding effects of genetics and gender-specific life history traits of other social insects.

Although the reproductive life history of males and females may explain a number of reported gender-based immune biases (Adamo et. al., 2001; Rolff, 2001b, Siva-Jothy et al., 2001; Gerloff et al., 2003; Vainio et al., 2004; Baer et. al., 2005; Pomfret and Knell, 2006), it can not fully explain instances in which both sexes play an equal role in offspring survival. Unlike many social insects, bi-parental care is the norm in termites, particularly during the early stages of colony foundation (Wilson, 1971; Rosengaus and Traniello, 1991; Shellman-Reeve, 1997; Thorne, 1997). The reproductive caste tested in this study (alate form) was not functionally reproductive at the time of PO quantification. Instead, they were preparing to fly from the nest in search of a mate and a suitable nesting site. Hence, it is predicted that PO activity at this stage should be similar between male and female, as the females are not yet facing significant trade-offs between costly reproduction and immunocompetence. Furthermore, because both sexes are long-lived and both contribute equally to colony foundation (Rosengaus and Traniello, 1991; Shellman-Reeve, 1997; Thorne, 1997), both genders should be under comparable selective pressures and hence are expected to invest similarly in PO responses even after they have successfully established a new colony.

These results are mostly congruent with such expectations, with the exception of one Z.
angusticollis colony, in which statistically significant differences between the sexes in the alate forms were recorded for only one colony. In his case, the magnitude of PO investment was biased towards males rather than females, contradicting the expectation that females are more immunocompetent. Whether these statistical differences translate into biological significance remains to be determined. Obviously, a larger number of colonies and a greater sample size will be needed to assess whether male and female Z. angusticollis alates consistently differ in PO investment. Although inconclusive, the fact that alate males of one Z. angusticollis colony had higher PO than their female counterpart is consistent with greater encapsulation rates exhibited by mated male termites (Calleri II et al., 2007) and could be indicative of resource trade-offs in preparation for reproduction.

Resource trade-offs have been reported in Z. angusticollis where primary reproductives exposed to M. anisopliae subsequently produced fewer eggs (Calleri II et al., 2006). The onset of mating and reproduction could alter the immune investment of both males and females, as is the case in other insect species (Adamo et al., 2001; Schwarzenbach et al., 2005). However, the variation in PO activity seen here does not translate directly into lower survival rates. Rosengaus et al., (2000b) found that the sex of de-alates was not a significant predictor of termite survival when exposed to M. anisopliae. Thus the sex-based difference we found in one colony may not have any biological significance. PO activity is just one weapon in an arsenal against parasites and pathogens, and in this case may be involved in resource trade-off. It was expected that in species where castes are of mixed sex without a gender work role distinction, like in Z. angusticollis, no difference in PO activity would be found. As predicted, there was no gender bias found in the PO activity of either the pseudergate or the nymphal castes of Z. angusticollis, suggesting that immune investment is equal among the sexes for the non-reproductive castes and
likely as well for the reproductive caste. This is congruous with Rosengaus et al., (2007) study, which found that eluted proteins from male and female pseudergates did not vary in their effect on the viability of *M. anisopliae* conidia.

As expected the *R. flavipes* and *Nasutitermes* sp. did not exhibit a gender bias in PO activity within the alate caste. However, the sexes of the worker and soldier castes of *R. flavipes* could not be established because sex cannot be determined using morphological, non-destructive means (Howard and Haverty, 1980). In the more derived *Nasutitermes* sp., soldiers which represent approximately 30% of the work force in a colony, are male (Haverty, 1977) while workers are female (Noirot, 1969). These species exhibit sex-based polyethism where caste is coupled with gender (Waller and La Fage, 1987). Therefore it is impossible to separate the independent impacts that gender and reproductive potential have on PO activity. However, based on the significant caste differences in PO activity found within *Z. angusticollis* and *R. flavipes*, it is likely that immune differences are better explained by life history traits associated with caste membership, and that sex is irrelevant as these individuals are all sterile (see chapter 3). Thus PO activity would not be expected to vary by sex but instead by caste.

A larger number of termite species must be tested in order to make generalizations about gender-biased immunocompetence in the order Isoptera. It is important to keep in mind that none of the insects tested in this study were actively reproducing. In fact, PO measurements were obtained from adult reproductives that had not yet disperesed. Nevertheless, our results show that even before dispersal, adult termites of at least four species spanning the phylogenetic range of the Isoptera do not exhibit gender biases in PO activity. Similarly, no observable differences in PO activity were recorded between the sexes of the sterile castes.
Chapter 5: Mycosis and phenoloxidase activity in the primitive dampwood termite *Zootermopsis angusticollis*.

Abstract

Little is known about the immunological response of insects through the course of infection. Here we examine how the activity of the phenoloxidase (PO) enzyme changes as the progression of fungal infection on the dampwood termite *Zootermopsis angusticollis* takes place. PO is a critical component in multiple immune reactions including nodule formation and encapsulation, responses that are common when insects fight disease. Termites were first exposed to varying concentrations of the entomopathogenic fungus *Metarhizium anisopliae* and subsequently, levels of PO activity were determined on day one through day seven post-exposure. Termites from each of the four tested colonies exhibit a dynamic temporal response. Three of four colonies treated with $2 \times 10^6$ and $2 \times 10^8$ conidia/ml, had significant increases in PO activity on day two relative to their respective controls, followed by a decline in activity on day three post-exposure and thereafter. The increase in PO levels corresponds with the time at which the fungus penetrates the insect’s epidermis and invades the hemocoel. This study is the first to focus on how PO levels change with the progression of fungal infection by an ecologically relevant pathogen instead of using only immune elicitors. Phenoloxidase is an enzyme that plays an important role in insect physiology and its quantification can be a valuable tool for predicting immune function in relation to life history traits, fitness, and resistance to fungal infection.
5.1 Introduction

Termites nest and forage in areas where diverse fungal communities thrive (Sands, 1969; Blackwell and Rossi, 1986). Hence, it is reasonable to assume that termites have evolved adaptations to fight-off fungal disease. *Metarhizium anisopliae* is a ubiquitous fungal pathogen, found in many termite nesting and feeding sites (Zoberi and Grace, 1990; Milner et al., 1998). *M. anisopliae* can be lethal to insects (Wang and St. Leger, 2006) including *Zootermopsis angusticollis* (Rosengaus and Traniello, 1997; Rosengaus et al., 1998b) and it has been considered as a possible biological control agent against termite pests (Chouvenc et al., 2009b).

Social insects utilize a number of different strategies to combat disease (Schmid-Hempel, 1998; Rosengaus et al., 2010; Schlüns and Crozier 2010). For example, honey bees have been found to raise the temperature of their hive in the presence of certain disease (Starks et al., 2000).

Termites, ants and bees often incorporate materials that contain antimicrobial properties into their nests (Rosengaus et al., 2000a; 2004; Chapuisat et al., 2007; Bulmer et al., 2009; Simone et al., 2009) and dead or sick termites are often cannibalized (Rosengaus and Traniello, 2001; Wilson-Rich et al., 2007). When exposed to *M. anisopliae*, *Z. angusticollis* immediately responds to the pathogen by increasing allogrooming (Rosengaus et al., 1998a; 2000b) and engaging in a vibratory display that alerts nestmates to the presence of lethal dosages of conidia (Rosengaus et al., 1999a; Myles, 2002). In *M. anisopliae*-exposed *R. flavipes*, the majority of the conidia attached to the insect’s cuticle are removed by mutual grooming; those conidia that remain tend to be in the cuticular folds or other inaccessible areas which then can cause infection (Chouvenc et al., 2009b). Once the biochemical, behavioral and anatomical barriers have been breached by the fungus, termites rely on an individual’s physiological responses to fend-off invading pathogens. The conidia that remain attached to the insect start producing cuticle
degrading enzymes that debilitate the exoskeleton so that the germ tube finally penetrates into the hemocoel. The differentiation into hyphal bodies and the production of destruxins follow, ultimately paralyzing and killing the host (Vilcinskas et al., 1997; Wang and St. Leger, 2006; Chouvenc et al., 2008; see fig. 5a from Chouvenc et al., 2009a). The invasion process triggers the insect’s innate immune system, both at the cellular and humoral levels (Söderahall and Cerenius, 1998; Siva-Jothy et al., 2005; Rosengaus et al. 1999, 2007; Chouvenc et al., 2009a; Avulova and Rosengaus, submitted). Previous research has shown that insect hemocytes recognize and phagocytize conidia (Gillespie et al., 2000a). However, this process is particularly compromised as the hyphal bodies of M. anisopliae disrupt the insect’s immune function by producing an anti-adhesive protective coat that masks the presence of β-1,3-glucans. In this way, the fungal pathogen becomes “invisible” to the insect’s immune defenses (Wang and St. Leger, 2006). Although ample research exists as to the susceptibility of insects to M. anisopliae (Vilcinskas et al., 1997; Kershaw et al., 1999; Chouvenc et al., 2009b), little is known about the physiological responses elicited in the host as they respond to a fungal infection (Rosengaus et al., 1999b, 2007; Wang and St. Leger, 2006; Calleri II et al., 2007; Avulova and Rosengaus, submitted). Therefore, the dynamics between mycosis and PO activity in termites merits further study.
Figure 5a: Schematized cellular encapsulation process of *Metarhizium anisopliae* in *Reticulitermes flavipes*.

(A) Conidium attachment to the insect cuticle. (B) Conidium germination. (C) Melanotic reaction of the cuticle and elimination of the fungal elements. (D) Fungal penetration of the cuticle and incipient aggregation of hemocytes. (E) Fungal penetration through the epidermis into the hemocoel and recruitment of hemocytes and incipient humoral melanization. (F) Failed encapsulation and invasion of the hemocoel by the hyphae. *(G, H) Successful encapsulation and intensification of the melanization. (I) Detachment of the melanized nodule into the hemocoel. (J) Formation of an epidermis-like layer around the melanized nodule.* (Reprinted from The Journal of Invertebrate Pathology, 101, Chouvenc et al., Cellular encapsulation in the eastern subterranean termite, *Reticulitermes flavipes* (Isoptera), against infection by the entomopathogenic fungus *Metarhizium anisopliae*, 234-241. 2009 with permission from Elsevier license number 2453771194694.)
The phenoloxidase cascade is triggered by a variety of pathogen-associated molecular pattern (PAMPS), including β-1, 3-glucans, which are typically the main component of fungal cell walls (Ashida and Hiroko, 1990; Cerenius and Söderhall, 2004; Wang and St. Leger, 2006). Rosengaus and Traniello (1997) and Chouvenc et al. (2009a) found melanotic lesions and nodule formation at the point of *M. anisopliae* germ tube invasion in *Z. angusticollis* and *R. flavipes*, respectively. Since the PO cascade plays an important role in melanization and nodule formation, it is reasonable to expect PO activity to vary through the course of fungal infection. Yet, few studies have addressed this issue (Korner and Schmid-Hempel, 2004), and none have used termites as a host animal.

Data presented here show how PO levels of *Z. angusticollis* change as a function of the progression of mycosis. It was hypothesized that non-lethal dosages of the fungus *M. anisopliae* should cause an increase in PO activity followed by a return to normal PO levels as the fungal infection is cleared. Lethal dosages of the entomopathogenic fungus, on the other hand, should disrupt the termite’s immune system and lower PO levels should be evident as mycosis progresses.

### 5.2 Methods

#### Fungal Conidia Preparation

*Metarhizium anisopliae* was harvested from dead sporulating *Z. angusticollis* and placed in a 0.1% suspension of sterile Tween 80, following the protocols of Rosengaus and Traniello (1997). Conidia viability was determined by plating 10µl of conidia solution on a microscope slide coated with a thin layer of solidified potato dextrose agar (Rosengaus and Traniello, 1997).
Germination rate was recorded after 18 hours of incubation at 25 °C and conidia viability was always greater than 90%. Conidia concentration was estimated using a hemocytometer at X 400 magnification. Through serial dilutions, conidia dosages were prepared and contained 2 x 10^8, 2 x 10^6, and 2 x 10^3 conidia/ml (see Rosengaus and Traniello 1997 for a detailed description of the methodology).

**Direct Dorsum Exposure Method**

To test whether PO activity is influenced by the progression of fungal infection, termites of *Z. angusticollis* were cold anesthetized and placed on their dorsum over a 3µl of a particular conidia concentration or a control Tween 80 suspension lacking conidia for one hour at 4°C following the protocols of Traniello et al., (2002). Direct exposure on the dorsum of each individual allowed for equivalent dosages of fungal conidia inoculae. Termite nymphs were then placed in groups of 4-5 individuals of the same sex in Petri dishes lined with filter paper (Whatman # 1) moistened with 300µl of distilled sterile water. To maintain high humidity, the Petri-dishes were stacked and stored in a plastic box lined with a moist paper towel. Termites were maintained undisturbed in these groups and were flash frozen in liquid nitrogen beginning 24 hours after initial exposure and then every 24 hour thereafter for a total of seven consecutive days.

**5.3 Results**

Although termites were exposed and collected through day seven post-exposure, the mortality rate after day five was such that the sample size was reduced to levels that made statistical analyses unreliable. Therefore, only data for the first five days were included (See
Total PO activity (i.e. stored + active) was substantially greater than the levels of active PO. The former also exhibited greater fluctuations when compared with active PO (Figure 5b). The regression model, which included the variables: colony of origin, treatment, and days post exposure explained only 26% of the variation in total PO activity. Linear regression analysis revealed that the total PO activity was highly correlated with colony of origin (colony 1, beta coefficient=-0.348, P=0.001; colony 2, beta coefficient=-0.494, P=0.001; colony 3, beta coefficient =-0.245, P=0.001; colony 4, predictor, P=0.001), day two (beta coefficient =0.186, P=0.001) and day 4 (beta coefficient =0.242, P=0.001) post exposure. Surprisingly, given the high level intra- and inter-colony variation in PO activity, treatment was not a significant predictor of total PO activity (Tween 80, P=0.955; 2x10^3 conidia/ml, P=0.927, 2x10^8 conidia/ml, P=0.482). In order to elucidate the source of PO variation, additional detailed analyses were carried out by comparing total PO levels for each conidia dosage for, each day post exposure and for each colony to the its corresponding control. This analysis showed that total PO activity when termites were exposed to 2x10^6 and 2x10^8 conidia/ml suspensions increased significantly their PO levels on day two relative to their respective controls (Mann-Whitney U test, 2x10^6 treatment for Colony 1, n=8, Mann-Whitney U=0.0, P<0.001; Colony 3, n=8, Mann-Whitney U= 0.0, P<0.001; Colony 4 n=10, Mann-Whitney U =5.0, P<0.001; 2x 10^8 treatment for Colony 1 n=7, Mann-Whitney U=3.0, P=0.006; Colony 3, n=7, Mann-Whitney U=5.0, P<0.01; Colony 4, n=10, Mann-Whitney U=5.0, P<0.001; figure 5c).
Table 5a – Numbers of termites (surviving) used in PO assay per treatment for each day post exposure. “*” indicates days in which termites were not exposed, thus the n value is zero.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Number of individuals exposed/day</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80 (Control)</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>0*</td>
</tr>
<tr>
<td>2x10^3 conidia/ml</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>11</td>
<td>8</td>
<td>0*</td>
</tr>
<tr>
<td>2x10^6 conidia/ml</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>0*</td>
</tr>
<tr>
<td>2x10^8 conidia/ml</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>0*</td>
</tr>
<tr>
<td>Tween 80 (Control)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>0*</td>
</tr>
<tr>
<td>2x10^3 conidia/ml</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>0*</td>
</tr>
<tr>
<td>2x10^6 conidia/ml</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>2x10^8 conidia/ml</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Tween 80 (Control)</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>2x10^3 conidia/ml</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>5</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>2x10^6 conidia/ml</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>6</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>2x10^8 conidia/ml</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Tween 80 (Control)</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2x10^3 conidia/ml</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2x10^6 conidia/ml</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2x10^8 conidia/ml</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>11</td>
</tr>
</tbody>
</table>
Figure 5b – Total PO (stored and active) and active PO over the course of a seven day period post exposure to *M. anisopliae*. Colonies (n=4) are combined.
Figure 5c – Total PO (stored and active) for each day post exposure by treatment for each colony. "*" indicate significant differences at P<0.01 when the day post exposure for each treatment, of each colony was compared with the control of the same colony. Colony 1 and 2 were not exposed for day 5 for the control (tween 80) and 2x10^3 thus comparing 2x10^6 and 2x10^8 were not possible for this day.

- Colony 1
- Colony 2
- Colony 3
- Colony 4
5.4 Discussion

The present study determined the temporal pattern of PO as a function of fungal infection in *Z. angusticollis*. The results appear to indicate that PO activity is affected through the course of fungal infection. Rosengaus et al., (1998b) and Rosengaus and Traniello (2001) have shown that colony of origin is a known predictor of termite survival rates when exposed to *M. anisopliae*. The results here confirm that colony of origin is a significant predictor of total PO activity. According to the regression model, colony of origin, day two and day four were the most significant predictors of PO activity. Figure 5b provides a global overview of PO levels for both active PO and total PO (both stored proPO and activated PO) in relation to conidia dosage and time post exposure. These data indicate first that all colonies exhibited total PO (stored and available) levels that are approximately twice those of the available enzyme. Second, the ranges of active PO levels per mg termite in the controls fall between 0.0 and 0.69 while total PO activity per mg termite falls between 0.36 and 2.97 (Tween 80 treatment; figure 5b). These ranges are similar between colonies, suggesting that such levels may be representative of the species as a whole. Finally, levels of active PO do not seem to fluctuate as much as those of total PO levels. Active PO has the potential to cause cytotoxic damage (Sadd and Siva-Jothy, 2006), and thus, it may be adaptive to store PO in the inactive zymogen form. The consistent levels of active PO are likely tolerable levels for the size of the species. Colony 2 exhibited slow and small scale changes in total PO activity with no major PO peaks evident throughout the entire time course of infection (figure 5c). In contrast, colonies 1, 3 and 4 followed similar trends.

Relative to their respective controls, these three colonies exhibit an increase in total PO activity over the course of the first two days post conidia-exposure, followed by a decline (Figure 5c). The peak on day two for colonies 1, 3, and 4 was significantly different for $2\times10^6$ and $2\times10^8$.
conidia/ml exposures when compared with total PO activity in the control treatment. According to the linear regression model day four post-exposure was also a significant predictor of total PO activity. Days three to five, depending on the colony, were characterized by another increase in total PO activity (Colonies 1 and 2 did not have controls to compare to on day five, figure 5c). Although this second increase in PO activity within conidia-exposed termites may indicate recovery of depleted PO resources, we doubt this is actually the case. Given that the second rise in PO activity for termites exposed to the $2 \times 10^8$ and $2 \times 10^6$ conidia/ml suspension coincided with the only PO peak exhibited by termites in the control treatment, it can not be concluded that the second peak is related to an immunological response against the fungus. For both conidia-exposed and control insects, the increase in total PO activity by day four likely represents a delayed response to the cold temperatures during the exposure procedure and/or stress following the removal from their parental colony. Stress can have an immunosuppressive response in insects (Adamo, 2004b; Seppälä and Jokela, 2010). In particular heat has been associated with immune suppression in *Lymnaea stagnalis*, where PO activity and antimicrobial concentrations decreased as water temperatures were increased from 15°C to 30°C (Seppälä and Jokela, 2010). Adamo and Parsons (2004) found a number of stressors including increased heat and restraint caused increased disease susceptibility. However, they did not find that a decrease in temperature impacted survival when crickets were exposed to *Serratia marcescens*. Given the studies on temperature and increased susceptibility it appears unlikely that the exposure to cold temperatures during conidia exposure led to the increase in PO activity seen on days three and four. It is likely that the manipulations related to the removal from their nest followed by the determination of each individual’s gender may have impacted PO levels. Recently Adamo (2010) found octopamine, a stress induced hormone, caused an increased number of hemocytes
in the hemolymph. It is plausible that all of these actions induced a stress response that heightened the immune reaction in both controls as well as conidia-exposed termites and that such a stress-induced boost in PO levels was evident on day four post manipulation.

The results can be interpreted from the perspective of each partner in this pathogenic association. From the perspective of the termite host, the increase in total PO activity by the second day post-exposure may indeed reflect an immunological response in an attempt to control further invasion by the entomopathogen. This increase in PO activity coincides exactly with the time at which the germ tube is invading the termite’s hemocoel (figure 5a) and thus it is reasonable to assume a link between PO induction and fungal invasion. The subsequent decline of PO on the third day post-exposure in termites exposed to $2 \times 10^6$ conidia/ml and $2 \times 10^8$ conidia/ml can be attributed to depletion of circulating PO while the termite is coping with the infection. Alternatively, from perspective of the fungus, the sharp decline of PO levels by the $2 \times 10^8$ exposed termites seen on day three relative to the control treatment may represent an inability of the host to produce more PO because the hyphal bodies disrupted the hosts immune system (Wang and St. Leger, 2006).

Other species of insects exhibit a dynamic temporal immune response, which varies as a function of species, pathogen, and immune measure (Gillespie et al., 2000b; Korner and Schmid-Hempel, 2004; Ryder, 2007; Haine et al., 2008b). In the present study, the magnitudes of observed PO fluctuations as well as the temporal patterns in which the fluctuations took place differ between colonies through the course of the five day trial. Three out of the four colonies tested (colonies 1, 3 and 4) all exhibit significant increases in PO activity on day two post exposure to a $2 \times 10^6$ and $2 \times 10^8$ conidia. The germ tube penetrates the cuticle by 36 hours post infection and then proceeds to breach the epidermis by 48 hours post exposure (Chouvenc et al.,
2009b; see figure 5a). The increase in PO activity observed on day two is thus consistent with the germ tubes entering the hemocoel at approximately the same time frame. Several other studies have examined the dynamics of PO activity (Korner and Schmid-Hempel, 2004; Haine et al., 2008b), however only one of these uses an actual pathogen (Gillespie et al., 2000b). The other studies used molecules or killed pathogens to induce an immune response. Haine et al. (2008) found no clear pattern in PO activity when testing a wide variety of microbial immune elicitors on Tenebrio molitor. Unfortunately, this study used only immune elicitors associated with bacterial pathogens and did not test those associated with fungi, therefore her results cannot be directly compared with the results described here. Gillespie et al. (2000b) found PO activity decreased by day two post exposure to M. anisopliae in the desert locust, however the locusts used were injected with the pathogen while the fungus used in this study had to breach the exoskeleton of the termite before entering the hemocoel. Injecting the pathogen allows immediate access to the hemocoel and thus speeds up the process by forgoing the two days it would require to breach the cuticle bypassing the first lines of immune defense. Korner and Schmid-Hempel (2004) also found a complex temporal pattern after injecting bumble bees with immune elicitors including laminarin, a β-1,3-glucan typically found on the surface of the fungal cell wall. Laminarin caused a steady increase in PO activity from 8 hours to 96 hours post injection, but in this study, the researchers also used a direct delivery method by injecting the immune elicitor into the hemocoel. Ryder (2007) performed a similar temporal study by examining the encapsulation rate in crickets over the course of five days. The most rapid encapsulation period was six hours after implantation of a nylon filament. Since encapsulation requires the use of PO, it is reasonable to assume that an increase in PO activity would accompany the rapid encapsulation rate. Again, the time course of PO activity in Ryder’s
experiments (Ryder, 2007) is not contradictory to what is presented here since the insertion of an implant bypasses the initial period required by the conidia to break through the external barriers to access the insect’s hemocoel. When undergoing a more natural mode of infection (through the attachment, degradation and penetration of the exoskeleton into the hemocoel) rather than an injection, day two is the critical period for the termite to mount an immune defense. Rosengaus and Traniello (1997) established that mortality due to *M. anisopliae* infection was dosage dependent. In fact survivorship and PO activity for the lowest conidia concentration after three days post-exposure did not differ from controls in any of the colonies. This corresponds with Chouvenc’s (2009a) description of the encapsulation process of *M. anisopliae* in *R. flavipes* where by day three post-exposure the encapsulation of germ tubes is well underway, successfully controlling fungal infection.

In order to provide a realistic estimate of how the overall immune responses are affected by the progression of fungal infection, we need to measure the immune response on a variety of levels, including amongst others, PO activity, antimicrobial protein induction and upregulation, and hematopoietic responses while using both PAMPS (such as lipopolyssacharides, peptidoglycangs, glucans) as well as live pathogens. This is the first examination of changes in termite PO physiology over the course of an infection caused by an ecologically relevant pathogen instead of relying on immune elicitors. Although the use of such compounds allow us to study properties of the host’s immune function without the confounding effects of illness on the host’s immunocompetence, the use of active pathogens permits studying the co-evolutionary interactions between the hosts and their pathogens. Phenoloxidase plays an important role in insect physiology and its quantification can be a valuable tool for predicting immune function in relation to life history traits, reproductive fitness, and fungal infection. The technique developed
here can be used to continue investigating the role that PO plays in termite ecological immunity.
Chapter 6: Overall Conclusion

Life history theory seeks to understand and explain how various variables associated with an organism’s life cycle influence the success of that organism when living under specific circumstances (Stearns, 1976; Nylin and Gotthard, 1998). In order to increase fitness, an organism must survive long enough to reproduce and pass its genetic makeup into the next generation. To achieve this, animals are required to allocate their limited amount of energy into somatic growth and repair, cell maintenance, homeostasis, immune defenses as well as reproduction and depending on the species, parental care. Given that energy for these vital processes is limiting, the conflicting energetic demands set the stage for potential trade-offs. Trade-offs among these various life history traits allow a certain degree of flexibility to organisms by permitting them to re-direct energy to the most imminent need. This is particularly beneficial in situations where organisms face changing hardship such as resisting invasion by parasites and/or pathogens. In these situations, the immune system becomes activated, diverting energy away from other important biological processes (Freitak et al., 2003; Calleri II et al., 2007; McKean et al., 2008). The research presented here shows how different intrinsic and extrinsic life history traits can influence immune investment in termites.

Insects are an extremely diverse and successful taxon found to inhabit almost all corners of Earth. In particular, social insects play a significant ecological role in the habitats they exploit (Abe, 1987). Because social insects nests are densely populated and constructed in microbially rich environments (such as decayed wood and/or soil; Rosengaus et al, 1998; 2003), they are prone to contract and spread infectious diseases. Parasites and pathogens are known to be significant agents of selection and can influence greatly the investment in immunity (Schmid-Hempel, 1998). Termites, as well as other social insects, have evolved multiple response levels
against disease, including behavioral, biochemical, immunological and societal adaptations that reduce disease susceptibility (Schmid-Hempel 1998; Cremer et al., 2007; Schlüns and Crozier, 2009; Rosengaus et al., 2010).

The present work, falls within the relatively recent discipline of “ecological immunology”, an area that has flourished within the last 15 years. It seeks to explain the costs and benefits associated with immune investment and identify potential trade-offs incurred by hosts when dealing with invading microorganisms (Sheldon and Verhulst, 1996; Rolff and Siva-Jothy, 2003; French et al., 2009). Insects in general, are useful test organisms in ecological immunology as they have a relatively simple immune system when compared with vertebrates. Moreover, insects are easy to maintain in the laboratory and their environmental conditions can be easily manipulated. Social insects, in particular, represent an excellent taxonomic groups to study the interaction between immune investment, sociality and trade-offs. Recent research indicates that social immunity and certain aspects of herd immunity can render nestmates more resistant to disease (Traniello et al., 2002; Cremer et al., 2007; Rosengaus et al., 2010).

Within the social insects, the Isoptera serve as an out-group to the social Hymenoptera (ants, bees and wasps) given some striking life-history differences between these two insect orders which merit further attention, particularly as they relate to their impact on immune function. Individuals within Hymenopteran societies have relatively higher coefficient of relatedness that those of termite societies (Wilson, 1971). Termite colonies are composed of diploid males and females most of which are offspring of a founding monogamous pair, while the Hymenopteran societies are composed mainly of diploid females and few haploid males. In termites both sexes play equally important long lasting roles in colony life while in the social Hymenoptera, only females contribute significantly to colony growth and development (Wilson,
Comparing and contrasting immune investment as a function of these striking differences may help elucidate the role that these intrinsic life history traits play on fashioning immune investment.

The series of comparative analyses in this thesis examined how one component of the immune system, phenoloxidase, varies across termite species, caste membership and its underlying reproductive potential, gender, as well as through the progression of fungal infection. Furthermore, these studies allow us to look at which extrinsic factors impact immune investment across phylogenetically related organisms with unique life history traits. By choosing species with diverse nesting and foraging habits (from nesting entirely within wood to subterranean and arboreal nesters, Shillman-Reeve, 1997), we can infer whether varying levels of microbial exposure correlate with immune investment. By analyzing PO levels as a function of these unique life history attributes (table 1a) we have now established, for the first time that, PO investment in termites is shaped in accordance with expected predictions from life history theory. Based on this initial information, we can start addressing the evolutionary impact that each of these life history traits has on the development of termite immunocompetence.

Nesting Ecology

The impact of new environments and their concomitant exposure to new disease agents appear to have influenced termite immune systems (Bulmer and Crozier, 2004; 2006). It was hypothesized that Z. angusticollis and R. flavipes, the two termite species that nest in decayed wood and in soil, respectively, should have greater PO activity rates than the more phylogenetically derived species, which are likely under less pathogenic constraints because of their arboreal nesting behavior (Hölldobler and Engel-Siegel, 1984; Holt, 1996; Weislo, 1996;
Cruse, 1998; Rosengaus et al., 2003; Kurihara et al., 2008; Tunaz and Stanley, 2009; Postava-Davignon, 2010). In this study R. flavipes, a subterranean nester and forager, had the highest PO activity of all species tested, while the arboreal nesting Nasutitermes sp. had the lowest levels of PO activity. These results suggest that termite immune systems vary as a function of each species nesting and feeding ecology, regardless of their phylogenetic position. Although estimates of nest, soil and cuticular microbial loads were not performed on any of these termite species, there is ample empirical evidence that soil-dwelling organisms live under higher pathogenic constraints than arboreal organisms (Hölldobler and Engel-Siegel, 1984; Holt, 1996; Wcislo, 1996; Cruse, 1998; Rosengaus et al., 2003; Kurihara et al., 2008; Tunaz and Stanley, 2009; Postava-Davignon, 2010). Hence, the feeding and nesting ecologies of social insects appear to heavily influence the evolution of at least one component of their immune investment.

Reproductive Potential

Each caste in termite society has a unique role in the colony, and therefore, a different underlying reproductive potential. While workers and soldiers are generally sterile, the kings and queens are responsible for colony reproduction. It is reasonable to assume that future reproductive potential should favor increased immunocompetence. A higher investment in immune function likely results in an increased survival, which in turn increases an individual’s fitness potential. This analysis indicates a positive relationship between reproductive potential and PO activity for three of the four species tested (Z. angusticollis, N. acajutlae, and N. corniger). In sharp contrast, R. flavipes does not exhibit a positive association between reproductive potential and PO activity levels. Contrary to expectation, the sterile soldiers of R.
*flavipes* had higher total PO activity than those of alates. This surprising result suggests that the higher risk of injury in the soldier caste of the subterranean termite species may be stringent enough that they override the impact that reproductive potential has on immune investment. Separating the independent effects reproductive potential, age and task risk remains elusive as these life history traits are all intertwined. It would be possible to examine individuals of different ages by analyzing the PO activity from different instars from each caste, this would provide information on how individual’s PO investment changes as adulthood is reached. However, distinguishing between caste and task may be impossible in these species as caste members all participate in similar work thus confounding the effects of task and caste on immunocompetence.

**Gender Bias**

Many species of insects exhibit sex-biased immunocompetence. Life history theory predicts differing investment in the immune system by males and females (Rolff, 2001a; Restif and Amos, 2010). The majority of studies on the social insects have, up until this point, found a female immune bias (see table 4a and references there in). Unfortunately the social Hymenopterans are not necessarily the best taxonomic group to study this question because several life history traits between the two sexes diverge in very important ways: while the diploid females live longer and are responsible for all of the colony labor, males are haploid, short lived, and do not contribute to colony work (Wilson, 1971). Here again, many of the supposed factors influencing immune function are intrinsically coupled and thus, their independent influence is impossible to address. Termites, however, are a better taxonomic group to address sex biases in
immunocompetence as both male and female reproductives are required for colony foundation, both sexes are diploid and both sexes have equivalent longevity. Within the sterile castes of many termite families, both the soldier and worker castes are also composed of males and females who participate equally in all tasks related to colony growth, maintenance, development and protection (Shellman-Reeve, 1997; Thorne, 1997). Therefore it is predicted that PO activity should be similar between the genders within a given species’ caste. The present results support this view: both male and female termites have been selected to invest similarly in immune function.

**Termite PO activity under fungal assault**

As stated earlier, PO is an integral part of the insect immune system. Under pathogenic assault, changes in PO activity should be expected and PO activity should vary throughout the course of infection. Several studies have shown that PO is critical to the successful survival of immune challenges (Shiao et al., 2001; Liu et al., 2007; Chouvenc et al., 2009a). It was hypothesized that non-lethal dosages of the entomopathogenic fungus *Metarhizium anisopliae* should trigger the PO cascade causing an increase in PO activity. Lethal dosages of this entomopathogenic fungus, however, likely disrupt immune function (Wang and St. Leger, 2006) causing a decrease in PO activity. These findings indicate that PO activity exhibits a complex temporal response through the first five days of infection, peaking in three of the four colonies treated with $2 \times 10^6$ and $2 \times 10^8$ conidia on day two post-exposure. This peak in PO activity corresponds with the time at which the fungal germ tube breaches the epidermis and gains access to the hemocoel (Chouvenc et al., 2009). Our findings are consistent with several other
experiments that found PO increased during the same period as a response to either an immune elicitor or infection (Gillespie et al., 2000b; Korner and Schmid-Hempel, 2004; Ryder, 2007; Chouvenc et al., 2009a)

PO plays a role in the early immune response of insects (Söderahall and Cerenius, 1998), thus measuring total PO activity is a reasonable way to measure immune response and immune investment. Of course, this is not a measure of the entire immune response, nor is it a measure of total immune investment. PO has been shown to change over the course of an insect’s life (Rolff, 2001b; Schmid et al., 2008; Armitage and Boomsma, 2010), in response to reproduction (Adamo et al., 2001; Gliksman and Yuval, 2010), and vary between the sexes (Adamo et al., 2001; Kurtz and Sauer, 2001; Rolff, 2001; Siva-Jothy et al., 2001; Fedorka et al., 2004). We conclude that feeding and foraging ecologies play the most critical role in shaping investment in the immune system. Just as interesting, we have found that the reproductive potential of a termite also influences investment in PO, although secondary to nesting ecology. New evidence has been provided that the immune system does not vary between most of the sexes in four termite species tested. Finally, PO activity was tracked through the course of fungal infection and one can distinguish a surge in PO activity that corresponds with a critical time at which fungal invasion into the hemocoel takes place.

The collective results in this comparative study contribute to the understanding that termite PO activity is influenced by life history traits including nesting ecology and caste, but not gender. By measuring investment in PO activity we have begun to piece together the evolutionary puzzle of termite ecological immunity thus helping to uncover why termites are such an evolutionarily successful taxonomic group. Comparative studies of insect immune investment will guide our understanding of the role that life history traits play in shaping the
immune system and help better elucidate the mechanisms behind insect ecological immunity in general and in social insects, in particular.
Appendix A

Bradford Assay Results

Linear regression (SPSS v13) revealed that protein concentration varied with mass in a species-dependent manner (P<0.001). Therefore, Pearson correlations for mass vs. protein concentration were calculated for each species separately. *Zootermopsis angusticollis* protein concentration was not statistically significantly correlated with mass (n=32, P=0.292, figure A1). Protein concentration had a strong, positive correlation with mass in *R. flavipes* before controlling for caste variation R=0.780, n=14, P=0.001, figure A1). However, further grouping by caste revealed no statistically significant protein concentration correlations with mass (R=0.811, n=14, P=0.01). Termite caste not only have distinct functions in the colony they also have unique morphologies. Members of the same termite species, but different castes can vary greatly in mass thus it was important to run regression models in which caste was a variable examining protein content. Protein concentration in *N. acajutlae* had a positive and very strong correlation with mass (R=0.954, n=25, P<0.001, figure A1), further analysis also revealed a statistically significant correlation between protein concentration and mass even when grouping by caste for soldiers (R=0.981, P<0.001). *Nasutitermes corniger* exhibits a positive and very strong correlation between protein concentration and mass (R=0.942, n=19, P<0.001, figure A1) as well as a statistically significant correlation between mass and protein concentration for the worker caste (R=0.959, P<0.001).

*Effect of species, caste and sex on protein concentration*

*Zootermopsis angusticollis*
Linear regression of *Z. angusticollis* including caste (pseudergate, nymph and soldiers), colony of origin, mass, and gender as variables revealed only colony of origin (P=0.001) was statistically significantly correlated with protein concentration ($R^2=0.520$; see table A1 for the values of n).

*Reticulitermes flavipes*

Linear regression of *R. flavipes* including caste (pseudergate, nymph, and soldiers) and mass as variables, produced an $R^2$ value of 0.658. This model did not include gender as a variable because it is not possible to morphologically determine gender in the castes used. Only one colony of *R. flavipes* was used for this model. None of the variables were a statistically significant predictor of protein concentration.

*Nasutitermes acajutlae*

Linear regression of *N. acajutlae*, including caste (worker and soldier) and mass as variables, produced an $R^2$ value of 0.963. Caste (P=0.001) and mass (P=0.020) were both significant predictors of protein content, as protein concentration increases so does mass. In this case caste and size are also very difficult variables to distinguish between because the worker’s average size (2.71mg) is 287% larger than the soldier’s average size (0.94mg). This model did not include gender because gender varies by caste in this species, thus it is impossible to distinguish between these two variables. There was only one colony available to sample.
*Nasutitermes corniger*

Linear regression of *N. corniger*, including caste (worker and soldier) and mass as variables, yielded an $R^2$ value of 0.919. Caste ($P=0.023$) and mass ($P=0.05$) were both significant predictors of protein content. Again, in this case, caste and size are very difficult to distinguish because the average size of workers (3.06mg) is 306% greater than the average size of the soldiers (1.0mg).

Table A1: Number of individuals used during the Bradford assay. Those castes that do not exist for a given species are marked with n/a.

<table>
<thead>
<tr>
<th></th>
<th><em>Zootermopsis angusticollis</em></th>
<th><em>Reticulitermes flavipes</em></th>
<th><em>Nasutitermes acajutlae</em></th>
<th><em>Nasutitermes corniger</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Soldier</td>
<td>n=7</td>
<td>n=3</td>
<td>n=15</td>
<td>n=9</td>
</tr>
<tr>
<td>Worker</td>
<td>n/a</td>
<td>n/a</td>
<td>n=10</td>
<td>n=10</td>
</tr>
<tr>
<td>Pseudergate</td>
<td>n=8</td>
<td>n=3</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Nymph</td>
<td>n=17</td>
<td>n=7</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>
Figure A1: Scatter plot of protein content (measured by Bradford assay) and individual mass of Zootermopsis angusticollis (no correlation between mass and protein content), Reticulitermes flavipes (no correlation after grouping by caste), Nasutitermes acajutlae termites (strong positive correlation), and Nasutitermes corniger (strong positive correlation).


—. 2009b. Susceptibility of seven termite species (Isoptera) to the entomopathogenic fungus *Metarhizium anisopliae*. *Sociobiology*, 54:723-748.


Thorne, B. L., J. F. A. Traniello, E. S. Adams, and M. Bulmer. 1999. Reproductive dynamics and colony structure of subterranean termites of the genus *Reticulitermes* (Isoptera


