GENETIC, INDIVIDUAL, AND GROUP FACILITATION OF DISEASE RESISTANCE IN HONEY BEES (*Apis mellifera*) AND TWO SPECIES OF PAPER WASPS (*Polistes dominulus* and *P. fuscatus*)

A dissertation

submitted by

Noah Wilson-Rich

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in

Biology

TUFTS UNIVERSITY

May 2011

Adviser: Professor Philip T. Starks

ABSTRACT

Social insects are remarkable for their extreme form of group living. This includes a reproductive division of labor, overlap of generations, and cooperative care for the brood. The global success of social insects suggests that the benefits of eusociality outweigh its costs. In this dissertation, I investigated how three species of social insects resist disease at multiple levels of biological organization. My co-authors and I first present a comprehensive review of what is known about how social insects resist disease, scaling up from genes to proteins and cells, to individual behavior to groups and populations. Next, I provide the first critical test of the 'haploid susceptibility hypothesis' using naturally occurring genetic misfits in *Polistes dominulus* populations. This hypothesis assumes that males are likely to have fewer disease resistance alleles than females in haplodiploid species, and that this disparity has shaped the evolution of social behavior. My co-authors and I show that this hypothesis is not a significant contributor to the evolution of social behavior in *P*. dominulus. Instead, I note strong ecological influences on immune function, including temporal-, sex-, and caste-related factors. Time of emergence and behavioral role are the best predictors of immune function in *Polistes*. I then scale up from individual genetics in paper wasps to group genetics in honey bee hives. Interestingly, phenoloxidase activity and encapsulation response are not influenced by colony-level genetic diversity. Therefore, the mechanistic explanation linking genetic diversity to prior observations of increased disease resistance in genetically diverse colonies remains elusive. A main theme throughout this work is the use of empirical methods to quantify invertebrate immune function. I report results from a set of experiments, performed with my co-authors, demonstrating the applicability of a novel method for assaying the invertebrate encapsulation response.

This method uses a nylon monofilament coated with pathogen-associated membrane patterns (PAMPs). These implants, termed “PAMPlants”, now allow researchers to investigate questions relating to how invertebrates mount an immune response against different classes of microbes. Following this, I then report results from experiments documenting how cellular and humoral immune function develops with honey bee ontogeny. I draw conclusions within the context of behavioral differences between life stages, and also differences in pathogen pressure across these stages. The final data chapter investigates a possible immune mechanism for the noted success of the *P. dominulus* invasion into North America from Europe and Northern Africa. My co-author and I compared multiple measures of immune function between *P. dominulus* and its sympatric, native congener *P. fuscatus*. Surprisingly, *P. dominulus* had lower immune function than *P. fuscatus*. I interpret these results within the context of the enemy-release hypothesis as a putative explanation for the success of this non-native population. My results present a comprehensive report of immune function in three species of social insects, and provide important insights into past, present, and future methods for testing immune function in these truly remarkable beasts.

ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge the countless number of honey bees, paper wasps, and practice termites that I have worked with since I began exploring the wonderful world of social insects one decade ago. Respect for nature is of utmost importance, and I am very grateful for the opportunity to explore and study the natural world. Thank you to my undergraduate research mentor, Prof. Rebeca Rosengaus, for sparking my interest in social insects and how they resist disease. Thank you to my undergraduate academic advisor, Prof. Wendy Smith, for successfully steering me through my undergraduate biology curriculum and into a Ph.D. program rather than my original M.D. track. What sealed the deal for my pursuit of a Ph.D. was the entomology course co-instructed by Profs. Rosengaus and Smith. One day, I hope to pay back their favor to me by instructing an entomology course, too.

I would like to acknowledge my Ph.D. advisor, Prof. Philip Starks. Phil's guidance was invaluable, and I sincerely thank him for all the time, energy, and resources it took him to invest in me. Over the past six years, Phil has inspired me to think critically about ecology, evolution, and behavior, while he also pushed me to become a better scientist. In Prof. Starks's eight years of being at Tufts, it has been challenging and rewarding to conduct publishable research solely on funding from within Tufts and the NSF REU program, and so I am thrilled to have published eleven papers while a member of the Starks lab, nine of which were co-authored with Phil. It truly was the best of times and the worst of times. Ultimately, I am elated to have this final product to show for our work together.

Sincere thanks to my entire dissertation committee at Tufts: Drs. Juliet Fuhrman, Sara Lewis, and Colin Orians. Your patience, time, effort, and feedback have been invaluable, and I am so very grateful. Thank you so much to Dr. Heather Mattila at Wellesley College for joining my committee as the brave outside member! I have been a

fan of your work for years, and am honored to have you on board. I would also like to acknowledge members of my committees past, including Drs. Harry Bernheim and Jan Pechenik for their valuable participation in my qualifying exam process, as well as Dr. Rebeca Rosengaus for serving as an outside reader for the exam.

While external funding continues to remain elusive, I am grateful for the funds available to me while a member of the Starks Laboratory and of the Biology Department at Tufts University. The research in chapter 2 was supported by a grant to our collaborator, Dr. Marla Spivak from the NSF (Integrative Biology and Neuroscience 0319911). Chapters 3, 5, and 6 were funded through multiple NSF REU and RET monies (DBI-0649190), organized by Dr. Philip Starks and dispersed to undergraduate researchers in our lab, as well as the Tufts Summer Scholars Program. I am also grateful to have received four Graduate Student Research Awards from the Tufts Graduate School to help fund my research. Additional thanks to Joel Caren at North Carolina State University for genetic analyses in chapter 4, and to Rick Reault at New England Bees for assistance in specimen collection in chapter 6.

Special thanks to those not yet acknowledged who contributed so much through reviewing drafts, giving advice on methods, or sitting with me in writing groups, including: Shelley Adamo, Dan Ardia, Caroline Blackie, Sean Cahill, David DesRochers, James Giordani, Topher LaCross, Kerrin McCarthy, Marielle Postava-Davignon, Ryan Seaman, Jon Snow, Natasha Tigreros, Michael Simone-Finstrom, Bryan Wilson-Rich, Susan Wilson-Rich, for reviewing drafts of my writing and for inspiring me to continue writing. Last but most certainly not least, I am so grateful to my peers, friends, and family who have supported me and my research pursuits at Tufts University. Finally, to Misses Chrissy Bracken, Shoshoni Caine (my best friend at Tufts), Molly Dickens, Nealia House, Anne Madden, Tegan Morton, Randi Rotjan, Natasha Tigreros, and Misters Jason Heustis, Ansel Payne, Adam South, you are each so very important to me, and I

am honored to have had the opportunity to study beside you as graduate students at Tufts University.

For my mother, Lynne,

the hardest working single parent this world has ever known,

whose relentless dedication and encouragement

have produced a Jewish doctor of her very own.

TABLE OF CONTENTS

ABSTRACT…… 2

ACKNOWLEDGEMENTS…… 4

INDEX OF TABLES…….. 8

INDEX OF FIGURES…….. 9

CHAPTER:

1. Foreword: Socioecoimmunology…… 10

2. Introduction: Genetic, individual, and group facilitation of disease resistance in insect societies ……..... 18

Published in 2009: *Annual Review of Entomology* 54:405-423
Wilson-Rich N, Spivak M, Fefferman N, Starks P.

3. Genetic diversity at the individual level: A critical test of the 'haploid susceptibility' hypothesis with naturally occurring genetic misfits…….…… 48

For submission to: Proceedings of the Royal Society B: Biological Sciences
Wilson-Rich N, Foo B, Tien T, Hester F, Starks P.

4. Genetic diversity at the group level: Within- and across-colony effects of hyperpolyandry on immune function and body condition in honey bees (*Apis mellifera*)……..…… 69

For submission to: Behavioral Ecology and Sociobiology
Wilson-Rich N, Tarpy D, Starks P.

5. Recognition systems: In Metchnikoff's honor: An improved method for testing invertebrate immune function…….…….. 85

For submission to: Science
Wilson-Rich N, Taylor E, Lwanga L, Starks P.

6. Ontogeny: The ontogeny of immunity: Development of innate immune strength in the honey bee (*Apis mellifera*)…… 95

Published in 2008: *Journal of Insect Physiology* 54:1392-1399
Wilson-Rich N, Dres S, Starks P.

7. Invasion biology: The *Polistes* War: Weak immune function in the invasive *P. dominulus* relative to the native *P. fuscatus*.…… 117

Published in 2010: *Insectes Sociaux* 57:47-52
Wilson-Rich N and Starks P.

8. Conclusions and Future Directions…….. 131

BIBLIOGRAPHY…….. 140

INDEX OF TABLES

1. Mating number trends for honey bee (A. mellifera) queens (Chapter 4….. 77
2. Summary of results testing the ‘genetic diversity’ hypothesis (Chapter 4).. 79
3. Exhaustive list of all published studies using sterile, uncoated implants (either nylon monofilaments or sephadex beads) to assay the encapsulation response in invertebrates (Chapter 5)…….. SEE APPENDIX

INDEX OF FIGURES

1. Graphical representation of the approach to this review (Chapter 1)……22
2. Graphical representation of honey bee hygienic behavior (Chapter 2)…….29
3. Sex effects on immunocompetence in *P. dominulus* (Chapter 3)……...64
4. Ploidy effects on immunocompetence in *P. dominulus* (Chapter 3)…..…... 65
5. Different modes of immunity are affected differently by season of year in *P. dominulus* (Chapter 3)……..66
6. Combined sex and ploidy effects on immunocompetence in *P. dominulus* (Chapter 3)……..67
7. Hemolymph phenoloxidase (PO) activity is predicted by temporal, sex, and to a lesser degree, ploidy in *P. dominulus* (Chapter 3)…… 68
8. Encapsulation response does not correlate with increasing number of patrilines in honey bees (*A. mellifera*) (Chapter 4)……..…… 82
9. Phenoloxidase activity does not correlate with increasing number of patrilines in honey bees (*A. mellifera*) (Chapter 4)……..…… 83
10. Fat body mass does not correlate with increasing number of patrilines in honey bees (*A. mellifera*) (Chapter 4)…… 84
11. Single implants (Chapter 5)…… 91
12. Double implants (Chapter 5)…… 92
13. Hemocyte number varies with honey bee (*A. mellifera*) ontogeny (Chapter 6)…… 113
14. Encapsulation response does not vary with honey bee (*A. mellifera*) ontogeny (Chapter 6)……... 114
15. Hemolymph protein concentration (a) and phenoloxidase (PO) activity (b) vary with honey bee (*A. mellifera*) senescence (Chapter 6)…… 115
16. Fat body mass decreases as adult honey bees (*A. mellifera*) age (Chapter 6)…… 116
17. Native *P. fuscatus* have a stronger encapsulation response than invasive *P. dominulus* (Chapter 7)……..126
18. Native *P. fuscatus* have greater phenoloxidase (PO) activity than invasive *P. dominulus* (Chapter 7)…… 127
19. Honey bee (*A. mellifera*) hemocytes visualized through a light-contract microscope (Chapter 8)…… 136

CHAPTER 1

FOREWORD: SOCIOECOIMMUNOLOGY

CHAPTER 1.
Foreword: Socioecoimmunology

Eusocial insects are remarkable beasts. Their groups are intricately organized, with behavioral and reproductive divisions of labor, overlap of generations, and cooperative brood care. Since Darwin's age, researchers remain enticed by the apparent contradictions between natural selection and altruism apparent in eusocial systems. Humans have long been fascinated by social insects (see Wheeler 1928). Yet, in the later 21st century, E. O. Wilson changed the game. Wilson's (1975) seminal text defining the field of sociobiology widened the lens into their obscure world. More recently, Schmid-Hempel (1998) organized information relating to the noteworthy ability of eusocial insects to live in such tightly woven groups while resisting infection and disease.

Socioecoimmunology is the study of environmental variation in pathogens and parasites and its influence on individuals living in groups, their genetic structure, parasite/host dynamics, and social organization. This area of research offers a sociobiological perspective on the evolutionary ecology of disease and parasitism in social groups. Living in groups may increase the fitness of individuals by decreasing the costs associated with important life history activities. Fitness should increase if lower costs are associated with enemy defense, foraging, brood care, colonizing and competitive abilities, and the ability to adaptively modify the environment (Wilson 1975; Rosengaus et al. 1998). Group living also poses unique fitness constraints on individuals, including increased competition and increased risk of disease transmission due to close living quarters with closely related individuals (Schmid-Hempel 1998; Tarpy 2003). Biological evidence from the global success of social animals suggests that the benefits of social living can outweigh its costs.

In this dissertation, I employed immune measures to study the social and ecological factors influencing immune function in social insects. I studied three species of eusocial hymenopterans: *Apis mellifera*, *Polistes dominulus*, and *P. fuscatus* as primary study systems. Each chapter employed methods to quantify and compare measurements of immunocompetence (IC), defined as the ability of an organism to mount an immune response, with respect to each mode of immunity (i.e., behavioral, cellular, and humoral). As eusocial animals, honey bees and paper wasps live in complex societies with overlapping generations, a reproductive division of labor (e.g., one dominant laying foundress and subordinant workers in primitively eusocial paper wasps, and one queen with thousands of non-mating workers in highly eusocial honey bees), and cooperative care of the brood (Wilson 1975).

Animals living in groups have evolved adaptive mechanisms to decrease rates of disease transmission, including mutual grooming and removal of dead nestmates (Traniello et al. 2002). However, such hygienic behavior is expected to increase the rate of pathogen exposure between nestmates and actually may facilitate disease outbreak through increased frequency of physical contact between individuals (Fefferman et al. 2007). The high level of cohesion in eusocial animal colonies should increase the risk of disease outbreak as a result of close living quarters, high genetic relatedness between individuals, and continuous physical interactions between individuals within the same generation (horizontal transmission) and between generations (vertical transmission) (Schmid-Hempel 1998; Whiteman and Parker 2004; Godfrey et al. 2006). In response, eusocial insects in particular have evolved novel behavioral, physiological, and organizational adaptations to combat the increased risk of disease (Starks et al. 2000, Traniello et al. 2002, Hughes and Boomsma 2004, Wilson-Rich et al. 2007; Cremer et al. 2007).

Along with the assistance of a fabulous team of hard-working co-workers, I conducted experiments quantifying IC to investigate proximate and ultimate factors influencing disease resistance traits in eusocial insect colonies. I used a novel approach of combining multiple direct methods of quantifying immune function in honey bees and paper wasps. Results from this body of work link theoretical models (Fefferman et al. 2007), mechanistic studies (Schmid et al. 2008), and observational disease resistance studies (Tarpy 2003; Tarpy and Seeley 2006; Seeley and Tarpy 2007). Using a multiple methods approach, we investigated disease resistance traits of eusocial insects at complementary levels of biological organization. Here, we report results from tests of several socioecoimmunological hypotheses, as follows: 1) males in haplodiploid species have reduced disease resistance (the "haploid-susceptibility hypothesis") (chapter 3), 2) hyperpolyandry (when one queen mates with ≥ 10 males) increases colony-level disease resistance via a linear relationship between mating number and immune function (chapter 4), 3) individual honey bees induce an immune response after exposure to highly conserved molecules on microbial cell walls (chapter 5), 4) honey bee immune strength varies with development (chapter 6), and 5) in the absence of native pathogens, invasive species will have lower immune strength compared to native sympatric congeners (chapter 7).

In chapter 3, we focused our lens on the relationship between social insect immune function and its relationship to behavior by exploring the 'haploid susceptibility' hypothesis (HSH). The HSH was proposed as an explanation for how behavioral roles in haplo-diploid social systems evolved. It posits that haploid individuals are more susceptible to disease than polyploid individuals due to decreased genetic variability at key disease-resistance loci. The resulting decreased immunocompetence is hypothesized to have played a role in the evolution of social behavior by limiting the

behavioral repertoire haploids perform. Using multiple methods of testing *P. dominulus* immunity, we document immune response results in haploids and diploids, which did not support the HSH overall. Instead, our results showed that time of emergence and behavioral role may be the best predictors of immune function in *Polistes*. Our data also indicate strong ecological influences on immune function, including temporal-, sex-, and caste-related factors.

In chapter 4, we scale up the level of biological organization by examining the interaction between colony-level genetic diversity and immune function across multiple colonies in a population. We examined 22 honey bee colonies over two non-consecutive years (2006 and 2009) and performed within- and across-colony comparisons for multiple measures of immunity. Honey bee queens are known for their extreme number of males they mate with. We studied hives reared from naturally inseminated queens, and discovered the highest number of males a European honey bee queen has mated with, at 29! Behavioral ecologists have debated why queens mate with so many males, termed hyperpolyandry (≥10 males). We aimed to provide a mechanistic explanation for hyperpolyandry by showing a linear relationship of immune function with mating number. Surprisingly, genetic diversity has no effect on our measures of immune function, despite previous research results showing clear benefits of genetic diversity to colony health.

In chapter 5, we describe in detail a novel, biologically realistic technique for researchers to test *differential* immune function in honey bees. This method now allows for experiments that improve our understanding of how insect hosts respond to different types of pathogens (e.g., fungi, gram-positive bacteria, gram-negative bacteria) and the trade-offs associated with each type of infection. Further applications of results from this chapter will enable researchers to explore fundamental questions using new tools. How does the host's immune response act during co-infection? How does environmental

heterogeneity impact the ability of a host to fight infection? What impacts do different typ es of infections have on host behavior, sociality, and fitness? These are questions that only now we can begin to fully answer.

At this point, a more wide-ranging picture emerges about how social insects resist disease. Disease and immunity can both influence and be influenced by characteristics of the hosts and pathogens. While this finding is not anything new, *per se*, we have established confidence in our repertoire of methods and in multiple model systems – honey bees and paper wasps. In Chapter 5, we explored immune function in isolated, adult bees. How does the immune system develop to this point? We soon realized that even the most basic knowledge about the life history of the honey bee immune system was absent from the literature, including any results from encapsulation tests, phenoloxidase activity, and other direct tests of IC. In chapter 6, we rectified this. Again using a multiple measures approach, we document how the immune system develops in honey bees, from larvae to pupae to adult. Interestingly, our results provide valuable insights into applied biology as well as basic science, by presenting the following: 1) data that may be pertinent to explaining the current global phenomenon of colony collapse disorder, 2) suggestions for U.S. agricultural and apicultural practices, and 3) corrections to inaccurate prior assumptions about honey bee immune function.

Scaling out to a broader level of biological organization, in Chapter 7, we applied these multiple methods of testing immune function. What role does immune function play in the success of an invasive species? How much does the presence or absence of co-evolved pathogens and parasites impact invasion success? And furthermore, how much this 'enemy release' impact immune function? Here, we make use of a natural experiment of sorts – the successful establishment of *P. dominulus* across North America. Invasive species are of growing ecological concern, in part because of conflicts

arising with native congeners. The European paper wasp *P. dominulus* was first introduced to North America in the 1970s, and may be displacing at least one native species, *P. fuscatus*. Previous reports indicate that in native territories over half of *P. dominulus* colonies are infected by Strepsipteran parasites, which decrease host fitness. In North America, *P. fuscatus* are parasitized to a lesser degree (approximately one-third), but no infected colonies of invasive *P. dominulus* have been reported. Counter intuitively, our results indicate that *P. dominulus* has lower immune function for both immune measures assayed. Additionally, *P. dominulus* displayed less self-grooming activity than *P. fuscatus*. We briefly discuss possible immunological explanations for this invasion success, including the selective expression of low immunocompetence. Future study of native populations will provide a critical test of this hypothesis.

Ultimately, it was my goal, and that of my mentor (Phil Starks), committee members (Juliet Fuhrman, Colin Orians, and Sara Lewis), and many colleagues and coworkers, to produce a dissertation of high quality that will make significant advances to the fields of behavioral ecology, invertebrate immunology, sociobiology, and the budding new field of socioecoimmunology. In this work, we provide the first – and what may be the final – test of the haploid susceptibility hypothesis in *Polistes*, which has been debated in the behavioral ecology literature for years (chapter 3). We also report the first results from immunological tests aiming to identify the mechanism(s) responsible for how genetic diversity scales up to disease resistance at the colony level (chapter 4). We introduce methods of quantifying immune function that are new to the honey bee and paper wasp systems in every chapter, including one novel method (PAMPlants) that allows researchers to measure the differential immune response to different types of pathogens simultaneously (chapter 5). And we supply data sets previously lacking in the literature that facilitate our understating of how the immune system develops over the life

history of honey bees, and also perhaps relative to the natural history of the *P. fuscatus* invasion into North America (chapters 6 and 7, respectively). In the end, we conclude with exciting new frontiers in invertebrate immunological methods that we hope will become widely used over the next decade because of their relatively easy techniques and large data gathering potential. It is my goal that this cohesive work will advance science and inspire future students and researchers to continue in our shared pursuit of knowledge of the mysterious natural world we live in.

CHAPTER 2

INTRODUCTION: GENETIC, INDIVIDUAL, AND GROUP FACILITATION OF DISEASE RESISTANCE IN INSECT SOCIETIES

CHAPTER 2.
Introduction: Genetic, individual, and group facilitation of disease resistance in insect societies

Noah Wilson-Rich[1], Marla Spivak[2], Nina H. Fefferman[3], and Philip T. Starks[1]

[1]Department of Biology, Tufts University, Medford, Massachusetts 02155;

[2]Department of Entomology, University of Minnesota, St. Paul, Minnesota 55108

[3]Department of Public Health and Family Medicine, Tufts School of Medicine, Boston, Massachusetts 02111

Annu. Rev. Entomol. 2009. 54:405–23. doi: 10.1146/annurev.ento.53.103106.093301

ABSTRACT

In this review, we provide a current reference on disease resistance in insect societies. We start with the genetics of immunity in the context of behavioral and physiological processes and scale up levels of biological organization until we reach populations. A significant component of this review focuses on *Apis mellifera* and its role as a model system for studies on social immunity. We additionally review the models that have been applied to disease transmission in social insects and elucidate areas for future study in the field of social immunity.

INTRODUCTION

Living in groups may increase the fitness of individuals by decreasing the costs associated with important life-history activities, including foraging efficiency, cooperative brood care, colonizing and competitive abilities, defense from enemies, and the ability to adaptively modify the environment (Wilson 1975; Rosengaus et al. 1998). Living in groups also poses unique fitness constraints on individuals, including increased competition and increased risk of disease transmission due to close living quarters with closely related individuals (Schmid-Hempel 1998; Tarpy 2003). Evidence from the global success of social animals suggests that the benefits of social living may outweigh its costs (Wilson 1975; Moller 1996; Holway and Suarez 1999; Giraud et al. 2002; Starks 2003; Liebert et al. 2006).

Social immunity:
Collective defenses against parasites and pathogens

IC:
Immunocompetence

Immunity:
Traits that decrease susceptibility to parasites and pathogens

Antiseptic behavior:
Behavioral traits that decrease disease transmission and susceptibility

The success of social insects remains enigmatic in regard to their ability to resist disease. The high level of cohesion in eusocial animal colonies may increase the risk of disease outbreak as a result of close living quarters, high genetic relatedness among individuals, and continuous physical interactions between individuals both within and across generations (Schmid-Hempel 1998; Whiteman and Parker 2004; Godfrey et al. 2006; Fefferman et al. 2007). In response, eusocial insects in particular have evolved novel physiological, behavioral, and organizational adaptations to combat the increased risk of disease (Rosengaus et al. 1998; Starks et al. 2000; Traniello et al. 2002; Hughes and Boomsma 2004; Arathi et al. 2006; Cremer et al. 2007; Rosengaus et al. 2007; Wilson-Rich et al. 2007; Wilson-Rich et al. 2008). These collective defenses against

parasites and pathogens are examples of social immunity. As one would expect, disease resistance traits are under the influence of genetic and environmental constraints (Cotter et al. 2004; Bocher et al. 2007; Sadd and Schmid-Hempel 2007). The ability to mount an immune response can be quantified empirically by using measurements of immunocompetence (IC).We define IC as the ability of an organism to mount an immune response. Multiple forms of IC are seen throughout the biological world, as well as in behavioral, cellular, and humoral (noncellular) and behavioral processes (Ribeiro and Brehélin 2006). Eusocial insects are a unique system for studying the relationship between IC and behavioral ecology because of their behavioral processes, many of which mitigate susceptibility and thus bolster immunity. The term antiseptic behavior is introduced in this review to describe behaviors of individuals within a social insect colony that, analogous to the cellular and humoral processes within an individual, provide defenses against pathogens to decrease transmission and increase resistance to diseases. Some examples of antiseptic behavior include grooming, hygienic behavior, undertaking, avoidance, glandular secretions, and use of resins in the nest. Antiseptic behavior in social insects may provide another level of defense in addition to cellular and humoral IC. At the physiological level, eusocial insects are similar to nonsocial invertebrates in regard to their cellular and humoral immune physiology. Unfortunately, there have been remarkably few descriptive studies comparing the two, even though distinctions between social and nonsocial insects abound at the organismal and behavioral levels. In fact, examples of immune behavior exclusive to eusocial insects have been well described in the honey bee with regard to its mechanisms of antiseptic behaviors, which decrease disease transmission and susceptibility. Analogies between physiological and behavioral levels may also be drawn, for example, between cellular encapsulation of a foreign body and social aggregation of intruders by individuals within a social insect colony.

Here, we aim to synthesize some of the literature relating to disease resistance in eusocial animals, with special emphasis on the honey bee, *Apis mellifera*. The collective immune defense (or social immunity *sensu* Cotter et al. 2004) by social insects against parasites was recently reviewed by Cremer and colleagues (Cremer et al. 2007). As such, we do not focus solely on the group response to disease. Instead, we provide a multilevel approach, from gene to population, for a detailed understanding of the underlying mechanisms upon which natural selection may act (Figure 1). Although we organize this review from gene to population, the distinction across levels with respect to genetics is somewhat artificial. While acknowledging that phenotypes and extended phenotypes do not exist without genotypes, we believe the heuristic value of organizing the review from gene to population outweighs any disadvantages associated with our categorization.

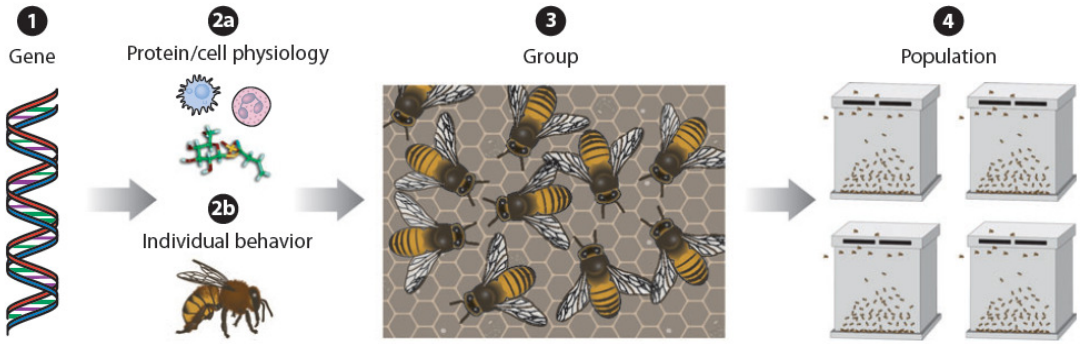

Figure 1. Graphical representation of the approach to this review. Social insects resist disease at multiple levels. Panel 1: Selection promotes genes that are most efficient in their ability to produce disease resistance phenotypes. Panel 2: Gene products include proteins and cells, which play vital roles in physiological immunity. Individual organisms engage in antiseptic behavior. Panel 3: Groups of individuals mount collective defenses, also known as social immunity. Panel 4: Populations provide important insight into large-scale disease dynamics, which can be studied using mathematical models.

WHAT ARE GENE-LEVEL DEFENSES AGAINST DISEASES?

Variation in pathogen intensity likely results in selection on disease resistance alleles. Solitary animals are limited in their ability to access social immunity and so should rely on physiological immune defenses more so than group living organisms. Indeed, recent evidence from the honey bee genome has shown that the genetic variation underlying the ability of honey bees to mount an immune response is likely to be lower compared to that of solitary insects (Evans et al. 2006), although additional research is necessary to fully support this. How do individuals within a densely populated society such as a honey bee colony compensate for this reduced immune ability? One way is through the evolution of defenses that emerge at the colony-level through the collective behaviors of individuals. One defense is hygienic behavior, in which individuals detect and remove diseased brood from the nest, resulting in colony-level resistance to pathogens and parasites. We review recent studies on honey bee genomics as they relate to disease resistance, the genetic basis of honey bee hygienic behavior, and allelic diversity (haploid susceptibility) to provide a model for similar research on the genetic basis of social immunity.

Honey bee hygienic behavior: The ability of honey bees to detect and remove diseased and parasitized brood in the nest

Genome-level studies: Honey bee genomics

Bioinformatics provide useful tools for answering questions related to allelic function and phylogenetic relatedness among genomes. The honey bee genome was recently sequenced by The Honeybee Genome Sequencing Consortium (Weinstock et al. 2006). This accomplishment provides the first genomic insight into the genetic makeup of a eusocial species.

Genetic diversity contributes to parasite resistance in ants (Schmid-Hempel and Crozier 1999), bumble bees (Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 1999; Baer and Schmid-Hempel 2001; Baer and Schmid-Hempel 2003), and honey bees (Tarpy 2003; Seeley and Tarpy 2007). Honey bee queens mate with an average of 7–17 drones (Winston 1987). Many hypotheses have been posited to explain this extreme polyandry in the mating system of the honey bee, including improved division of labor within a colony (Robinson 1992), heightened probability for sperm acquisition (Cole 1983), and decreased disease susceptibility via increased genetic diversity at disease resistance loci (Hamilton 1987; Sherman et al. 1988; Schmid-Hempel 1998; Tarpy 2006; Seeley and Tarpy 2007). The latter hypothesis, termed the 'polyandry versus parasitism hypothesis' (Sherman et al. 1988), posits that polyandry is a defense mechanism against pathogens and parasites. Tarpy (2003) and Seeley and Tarpy (2007) found that colonies headed by queens artificially inseminated with multiple males have decreased variation in their ability to resist infection to the diseases chalk brood and American foulbrood, respectively. This decrease in variation appears to guard against broad-scale disease susceptibility within a relatively genetically diverse colony.

In regard to disease resistance, honey bees possess fewer immune genes than fruit flies (*Drosophila melanogaster*) and mosquitoes (*Anopheles gambiae*), which affects every step of the immune response, from pathogen recognition to the production of immune proteins (Weinstock et al. 2006). This finding suggests a reduced flexibility in the abilities of honey bees to recognize and resist pathogens (Weinstock et al. 2006). Similarly, honey bees possess decreased variability in prophenoloxidase (proPO) genes compared with other insects, whereby honey bees have only one proPO

PO:
Phenoloxidase

Nest hygiene:
Any act that increases the cleanliness and sterility of a phenotype (e.g., cuticle) or extended phenotype (e.g., nest)

gene compared to three in *D. melanogaster* and nine in *A. gambiae* (Evans et al. 2006). proPO is the inactive zymogen precursor to phenoloxidase (PO), an important enzyme to innate immune function that is responsible for oxidation of tyrosine derivatives to toxic quinones and downstream polymerization into melanin. Although bees have intact pathways implicated in immunity (Toll, Imd, JAK/STAT, and JNK), these pathways seem to lack some of the flexibility seen in other insects for responding to and targeting pathogens. As one example, honey bees have half as many peptidoglycan recognition proteins as do *D. melanogaster*, *A. gambiae*, and *Tribolium castaneum* (flour beetles) (Weinstock et al. 2006) and fewer plausible exons for splice-site variation in peptidoglycan transcripts (Evans et al. 2006; Weinstock et al. 2006).

The honey bee genome also indicates differential expression of disease resistance peptides across castes and between developmental stages that are motile (adults) and those that are nonmotile (brood), suggesting another important link between physiological and behavioral IC. The proPO gene in honey bees is expressed more strongly in adults and older pupae than in younger pupae and larvae (Lourenço et al. 2005). An additional proteomic study of honey bee hemolymph showed the proPO zymogen was 50-fold more prevalent in the hemolymph of adult honey bee workers compared with larvae (Chan et al. 2006), and levels of the antibacterial peptide hymenoptaecin are also higher in adults than in larvae (Chan et al. 2006). Expression of peptidases, defensins, and transferrin homologues is preferentially upregulated in queens over workers (Grozinger et al. 2007), each of which is likely affected by differences in longevity and behavioral role. Together, these results suggest a relationship between behavioral capacity and the expression of disease resistance genes.

Honey bee sociality and nesting ecology likely play an important role in compensating for the limited number of immune genes and differential gene expression between castes. The high level of cooperative brood care and nest hygiene (defined as behaviors that increase sanitation of an individual or the nest) performed by adult honey bees likely augments hive disease resistance. Larvae and pupae are confined within the brood comb and adult bees perform nest hygiene (Winston 1987). Larvae are offered protection by the antibacterial properties of royal jelly (Bilikova et al. 2002; Klaudiny et al. 2005). Natural products stored in the colony (honey and pollen) are protected from bacterial decay by enzymatic secretion of glucose oxidase and the physical properties of honey (Visscher 1980). The inner hive is further protected from pathogens by deposition of propolis, which has antimicrobial properties (Bankova et al. 2000; Bastos et al. 2008).

Genetics of honey bee hygienic behavior

Hygienic behavior is defined as the ability to detect and remove diseased brood from the nest. Hygienic behavior is an antiseptic behavior and differs from undertaking (the removal of dead adult nestmates) and grooming [the removal of foreign objects and pathogens from oneself (autogrooming) or from another adult in the nest (allogrooming)]. Sumana and Starks (2004) show grooming occurs largely for cleaning purposes and not only to spread secretions such as cuticular hydrocarbons. Hygienic behavior in honey bees was first described in the 1930s when researchers sought to determine the mechanism by which some honey bee colonies were resistant to the highly infectious brood disease American foulbrood, caused by the bacterium *Paenibacillus larvae* (Figure 2) (Park 1937; Park et al. 1937). Park and colleagues (1939) observed that, ". . . the bees sometimes remove and dispose of [diseased] larvae very soon after they die,

thus eliminating the evidence." Their observations were confirmed by Woodrow and Holst (1942), who concluded, "The data show that resistance to American foulbrood in the honey bee colony consists in its ability to detect and remove diseased brood before the causative organism, *P. larvae*, reaches the infectious spore stage in the diseased larvae"; and that "The early removal of diseased larvae while they contain only the noninfectious rods of *P. larvae* prevents dissemination of disease in the colony, whereas removal of infected brood containing the highly infectious spores results in spread of disease to other larvae."

In 1964, Rothenbuhler and his students published a six-part series of articles on the behavioral genetics of hygienic behavior in honey bees (Jones and Rothenbuhler 1964; Rothenbuhler 1964a; Rothenbuhler 1964b; Thompson 1964; Trump et al. 1967; Momot and Rothenbuhler 1971). He developed a two-locus model of inheritance for hygienic behavior, which was recognized as a classic example of the effects of Mendelian inherited genes on behavior (Alcock 1993). The process of uncapping a cell containing dead brood and removing the contents was thought to be dependent on homozygosity for two recessive genes (*u* and *r*). Workers heterozygous at both loci should not be hygienic. Homozygosity at one of the two loci should result in workers that either uncap (*uu*) or remove (*rr*).

Other researchers have proposed that a three-locus model [*u*, *r1*, and *r2* (Moritz 1988) or *u1*, *u2*, and *r* (Gramacho 1999)] may better fit the original data set. More recently, using molecular techniques and quantitative trait loci (QTL) linkage mapping, Lapidge et al. (Lapidge et al. 2002) associated seven suggestive QTLs with hygienic behavior. Each putative QTL controlled only 9%–15% of the observed phenotypic variance in the character.

Honey bee hygienic behavior is also a mechanism of defense against the parasitic mite *Varroa destructor*. *A. cerana* (Peng et al. 1987) and some *A. mellifera* colonies are able to detect and remove pupae that are parasitized with these mites, particularly Africanized bees (reviewed in Boecking and Spivak 1999, but see Mondragon et al. 2005) as well as commercial lines available in the United States, for example, a line bred for Varroa Sensitive Hygiene (Harris 2007) and the MN Hygienic line (Spivak 1996). The removal process interrupts the mite reproductive cycle, thereby lowering the

Hygienic behavior

In 1956,W.C. Rothenbuhler first used the term hygienic behavior to describe a specific trait of honey bees: the ability to uncap and remove diseased brood from the nest. Currently, the term hygienic behavior is sometimes used to refer to general nest hygiene of social insects, such as trash removal and removal of dead adults (undertaking). Because of the extensive amount of research on the genetics, neuroethology, and applied ecology of honey bee hygienic behavior, we prefer to retain the usage of the term hygienic behavior to refer to the removal of diseased and parasitized brood from the nest by honey bees, dampwood termites, and other social insects that might perform this specific task. In our framework, hygienic behavior is an antiseptic behavior and a form of nest hygiene. The term nest hygiene refers to the broader collection of behaviors used by social insects to remove pathogens and parasites from the nest. In this way, hygienic behavior, undertaking, and trash removal are examples (subcategories) of nest hygiene.

mite population (Spivak and Reuter 1998; Spivak and Reuter 2001). It is unclear, however, if the detection of diseased brood by honey bees is influenced by the same loci as is the detection of mite-infested brood (Ibrahim and Spivak 2006).

One consideration for these and future studies on the genetics of hygienic behavior is that all worker honey bees are able to, and do, perform the motor tasks of uncapping and removing diseased brood at some point in their adult life. The genetic difference among colonies lies in how quickly individual bees within a colony detect the presence of diseased brood within the nest. Individual bees with low-threshold responses to the cues from the diseased brood rapidly initiate the removal process. Future research would benefit from studies on the genetic differences in the detection of diseased brood, and more studies are needed on the neuromodulation of olfactory sensitivity and responsiveness among bees within lines bred for hygienic behavior.

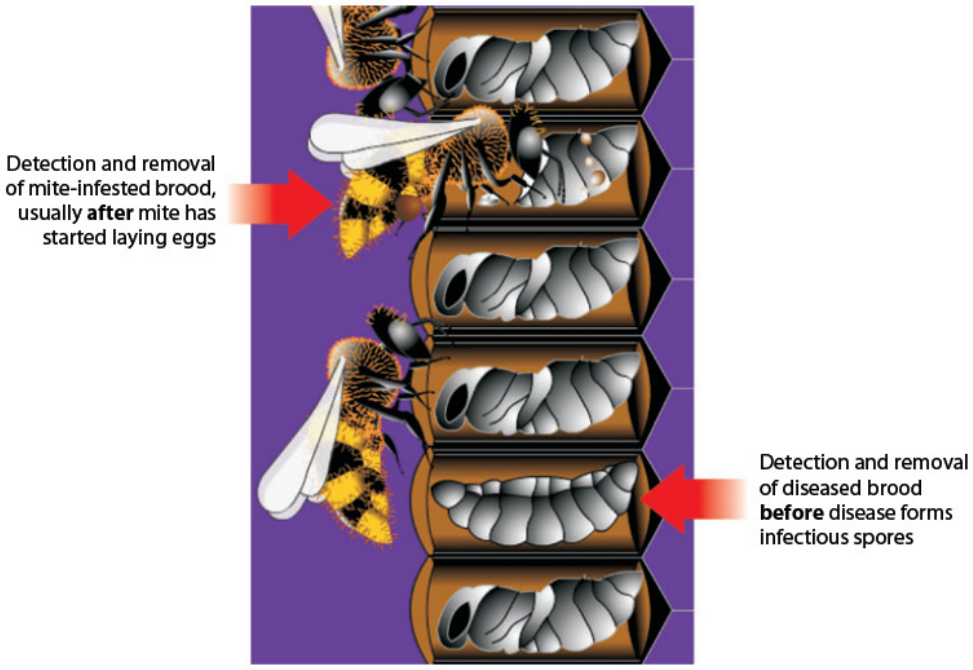

Figure 2. Graphical representation of honey bee hygienic behavior.

Allelic diversity: The haploid susceptibility hypothesis

Haplo-diploid systems are ideal for scaling up from single gene effects to collective impacts because these systems have adults that are either haploid or diploid. Comparing across haploid and diploid conspecifics may thus shed light on the collective impacts of some genetic traits. And indeed, data do suggest that males of some eusocial hymenopterans are more susceptible to certain pathogens, for example *Varroa* mites in honey bees (Santillán-Galicia et al. 2002), although the multiple hypotheses explaining increased male susceptibility remain to be tested, e.g., developmental time, size, or location in the brood comb.

This susceptibility may drive other observable characteristics. For example, isolation of hymenopteran males has been observed in honey bees and paper wasps. Honey bee drones accompany swarms at a lower rate than expected given the number of males in the colony (Ratnieks and Miller 1993), and paper wasp males in *Polistes dominulus* are isolated from returning foragers (Starks and Poe 1997). The haploid susceptibility hypothesis suggests that haploid males exhibit increased disease susceptibility, and that this vulnerability may have been a factor in the evolution of behavioral interactions in social animals (O'Donnell and Beshers 2004). This hypothesis assumes that decreased diversity at disease resistance loci negatively influences the survival of haploid organisms (as haploids and homozygous diploids have one type of defense loci, whereas heterozygotes have two and thus possess a heterozygote advantage).

The theoretical implications behind the haploid susceptibility hypothesis were elaborated upon by O'Donnell and Beshers (2004) in the context of male behavioral roles in eusocial Hymenoptera. Empirical support for this hypothesis includes lower IC in haploid male eusocial insects (in the wood ant *Formica exsecta*, (Vainio et al. 2004), in

the leaf-cutting ant *Acromyrmex echinatior* (Baer and Schmid-Hempel 2003), in the bumble bee *Bombus terrestris* (Gerloff et al. 2003)]. However, other studies have shown that haploid males are not more susceptible to disease than diploid females (for *B. terrestris*, see Ruiz-González and Brown 2006). No alternative explanations elucidate why these males have lower IC than diploid females, including differences in life history (Baer et al. 2005), behavioral role (Vainio et al. 2004), and coevolution between parasites and the predominantly female physiology of eusocial colonies (Ruiz-González and Brown 2006). One method of addressing embedded confounding factors when comparing haploid males with diploid females would be to include diploid males—a common genetic misfit in *Polistes* wasps (Liebert et al. 2004; Liebert et al. 2005)—thereby controlling for morphological differences between males and females.

WHAT ARE INDIVIDUAL SYSTEM-LEVEL DEFENSES AGAINST DISEASE?

Cellular and humoral immune cascades prevent infection within the hemocoel of insects. Scaling up another level of organization, individuals within some social insect colonies are able to detect diseased nestmates, which stimulates the expression of antiseptic behavior. Antiseptic behavior includes grooming, hygienic behavior of honey bees, undertaking, avoidance behavior, and metapleural gland secretion spreading.

Antiseptic behavior displayed by eusocial insects is likely to influence the selective pressure on other modes of IC (i.e., cellular and humoral), assuming each mode of IC is costly. As such, physiological immune strength in eusocial insects likely differs from that of nonsocial insects, whereas the qualitative aspects appear similar. One method of investigating differences in cellular and humoral IC between insects of varying degrees of sociality is to assay IC within a phylogenetic lineage, thereby controlling for differences relating to evolutionary history. A similar approach by Stow

and colleagues (Stow et al. 2007) provided support for this hypothesis by documenting more antimicrobial compounds on the cuticle of eusocial bees compared with subsocial bees. More antimicrobial cuticular compounds might alleviate the heightened risk of disease transmission facilitated by colonies of closely related individuals.

Cellular immunity

Cellular immunity is common to all animals possessing mobile blood cells, including social insects. The cellular immune processes of social insects are similar to those of nonsocial invertebrates. Invertebrates possess many types of blood cells, termed hemocytes. Three types of hemocytes are common to all insects: prohemocytes, granulocytes (also called granular hemocytes), and plasmatocytes (Chapman 1971). Recently, Manfredini and colleagues (Manfredini et al. 2008) confirmed that these three types of hemocytes constitute the cellular component of hemolymph in the eusocial paper wasp, *P. dominulus* (Hymenoptera), although descriptive hemocyte analysis in other eusocial species remains to be reported. Prohemocytes are the stem cells of the circulatory system and may differentiate into other types of hemocytes (Lavine and Strand 2002). Granulocytes release chemotactic factors into the hemolymph to attract plasmatocytes and play an important role in clotting, healing wounds, and immune processes such as nodulation and encapsulation (Ribeiro and Brehélin 2006). These cells are likely the first hemocytes to recognize a foreign body (FB) (Lavine and Strand 2002). Plasmatocytes are homologous in function to vertebrate macrophages in that they may phagocytose small FBs or mark larger ones

Cellular immunity:
The cellular antipathogenic component of the hemolymph

FB:
Foreign body

Humoral immunity:
The non-cellular antipathogenic component of the hemolymph

as nonself for subsequent isolation. Nodulation occurs when plasmatocytes aggregate onto one another to form a nodule on the FB, thereby marking it for isolation and/or excretion (Ribeiro and Brehélin 2006). A nodule typically occurs when the FB is too large to be encapsulated. Encapsulation occurs when plasmatocytes recognize a FB and differentiate into flattened cells called lamellocytes. These cells attach to the FB, deactivate it with toxic quinones, and encapsulate it by producing a hard layer of melanin around it.

Humoral Immunity

As with cellular immunity, humoral immune processes in eusocial insects are similar to those in nonsocial invertebrates. Humoral immunity is defined as the noncellular antimicrobial component of the hemolymph. PO is an important enzyme in the invertebrate immune response, which catalyzes the oxidation of dopamine precursors to toxic quinones. Reactive quinines are toxic to microbes and directly contribute to pathogen neutralization (Lu and Jiang 2007). PO also catalyzes the polymerization of quinones to melanin, a protein that hardens and darkens around a FB to further isolate it within host hemolymph. The precursor to PO (proPO) is constitutively expressed in the hemolymph (Cerenius and Söderhall 2004).

Social insects may possess a unique humoral immunity advantage against pathogens. Dampwood termites (*Zootermopsis angusticollis*) constitutively express proteins in the hemolymph with some degree of antimicrobial activity; some of these proteins are inducible and may be transferred between individuals within a colony (Rothenbuhler 1964). Antimicrobial proteins are produced by hemocytes and fat bodies in response to recognition of broad classes of microbes (e.g., gram-negative and gram-positive bacteria and fungi) (Hoffman 2003). Over 170 insect immune proteins have

been identified (Saito et al. 2004). Some antimicrobial proteins are less selective in their activity and are effective against all bacteria (e.g., cecropins) (Otvos 2000). These proteins are produced when pathogen-recognition receptors on host hemocytes bind to pathogen-associated membrane patterns. Transcriptional activation of peptides effective against gram-positive bacteria (e.g., defensins) and fungi (e.g., drosomycins and metchnikowins) occurs through the Toll signal transduction pathway, whereas the IMD pathway produced peptides effective against gram-negative bacteria (e.g., drosocin, diptericins, attacins, and cecropins) (Otvos 2000; Tzou et al. 2002; Hoffman 2003). The *de novo* synthesis of inducible immune proteins may be assayed both qualitatively and quantitatively through combined SDSPAGE and isoelectric focusing gel methods (Rosengaus et al. 2007).

Limitations to Individual Defense

The foraging and nesting ecology of eusocial insects exposes them to a diversity of pathogens, which are defended against through multiple modes of immunity. Immunity does not come without costs (Dudley and Milton 1990; Giorgi et al. 2001), and these costs should confer a selective advantage to those with the most efficient defenses. Each mode of immunity (e.g., behavioral, cellular, and humoral) may be activated exclusively or concomitantly. Energy should be preferentially invested into the most effective type(s) of immunity, especially when resources are limited. The favored mode should be the one most efficient at reducing pathogen virulence while augmenting host survival and reproduction.

The costs of mounting an immune response have seldom been assessed, but when investigated they are shown to be high (Hughes and Cremer 2007). For example, bats spend considerable time grooming accompanied by a significant increase in oxygen

consumption (Giorgi et al. 2001). Fly-swatting behavior in howler monkeys consumes 24% of their total metabolic budget (Dudley and Milton 1990). Our understanding of the energetic cost of immunity in eusocial insects remains remarkably limited. Immune solicitation in bumble bees has been associated with increased food consumption (Tyler et al. 2006; but see Schmid-Hempel and Schmid-Hempel 1998) and memory loss (Mallon et al. 2003; Riddell and Mallon 2005). Foraging activity is associated with decreased encapsulation response (König and Schmid-Hempel 1995). Because immunity is multimodal, mixed results relating to the costs of immunity do not necessarily refute one another if different immune pathways were measured; modes of immunity likely differ in energetic requirements. Furthermore, because immune pathways do not necessarily correlate with one another, accurate conclusions of IC parameters are likely to result from studies that investigate multiple immune processes (Wilson-Rich et al. 2008).

WHAT ARE COLLECTIVE DEFENSES AGAINST DISEASES?

Naturally, the costs of immunity may be alleviated through group facilitation (e.g., nest hygiene and antiseptic behavior). Social immunity refers to the collective disease defense mechanisms of a collaborative group (Cremer et al. 2007). Many different physiological and behavioral mechanisms can contribute to these social immune defenses. Some behaviors effect protection against pathogens only at the group level, whereas others may be individually protective as well (hence antiseptic). Behavioral structuring (age and caste) and spatial nest compartmentalization are excellent examples of collective pathogen defenses (Schmid-Hempel 1998). Behavior related to social immunity is commonly observed in animal societies and is most easily explored in those societies that rely on both social interactions and societal organization to survive,

i.e., those societies that lend themselves well to the term superorganism (Wilson and Sober 1989).

Antiseptic behavior is a vital component of behavioral defenses enabled by sociality. Some additional examples include removal of infected larvae from among the healthy brood in honey bees, construction of nests from antimicrobial materials in wood ants (Christe et al. 2003; Castella et al. 2008), social transfer of antipathogenic proteins in dampwood termites (Traniello et al. 2002), and social fever in response to disease in honey bees (Starks et al. 2000). Behavioral fever is a mechanism of behavioral IC that occurs when poikilothermic individuals manually increase their body temperature in response to pathogen exposure. In this process, body temperature is increased beyond the optimal range of pathogen development and is noted to occur both by individual movement to warmer areas and through group facilitation (i.e., huddling). Behavioral fever has been observed in solitary, gregarious, and eusocial organisms [e.g., cockroaches (Bronstein and Connor 1984), lizards (Vaughn et al. 1974), locusts (Wilson et al. 2002), and honey bees (Starks et al. 2000)]. Fever is considered an adaptive trait, as it increases host survival and fitness (Kluger 1979; Nesse and Williams 1995).

In honey bees, behavioral fever is induced by adults positioned over brood comb in response to infestation by *Ascosphaera apis* (Starks et al. 2000). Fungal spores are introduced into the colony by foraging adults, who vector the spores to larvae via feeding regurgitated nutrients. *A. apis* germinates in the larval gut when colony temperature falls below 32◦C for more than two hours (Bailey and Ball 1991). Although the process of temperature upregulation in this system is known, the mechanism by which *A. apis* infests colonies is just beginning to be understood. Indeed, given the energetic costs associated with the production of fever, one might hypothesize a benefit in localizing that response.

Thermoregulation in general is used by the honey bee to defend against disease. Developing brood are highly vulnerable to changes in temperature (Winston 1987). Honey bee workers participate in complex behaviors that limit both the magnitude and frequency of temperature fluctuations away from the ideal conditions (Heinrich 1980; Heinrich 1985; Starks and Gilley 1999; Starks et al. 2000, 2005; Siegel et al. 2005). The optimal temperature for honey bee brood development is 32◦C–36◦C (Heinrich 1980; Heinrich 1985; Seeley 1985; Winston 1987; Bujok et al. 2002), and prolonged exposure to temperatures outside this range can cause developmental abnormalities, disease, and even death (Fukuda and Sakagami 1968; Winston 1987; Bailey and Ball 1991; Bujok et al. 2002; Jones et al. 2005).

Maintaining optimal hive temperatures requires significant energy and coordination of adult workers. Cooling the hive is achieved by wing fanning, which may be performed in conjunction with spreading water to induce evaporation, and heating the hive is done by isometrically contracting thoracic muscles (Heinrich 1980, 1985). Fine-tuned local heating is achieved by individual bees heating their thoraces and placing them close to cells to increase temperature of specific brood cells (Bujok et al. 2002; Kleinhenz et al. 2003). Honey bees can thwart temperature fluctuations by congregating in response to localized temperature stress (hot or cold), a behavior termed shielding (Starks and Gilley 1999; Siegel et al. 2005; Starks et al. 2005). In this stereotyped behavior, bees perch and are stationary on the hive wall with their ventral side facing the heat stress (Starks and Gilley 1999; Starks et al. 2005).

Case study: Honey bee hygienic behavior

Hygienic behavior is defined specifically as the removal of diseased and parasitized brood from the nest and is one model system of social immunity. Studies of

this antiseptic behavior in honey bees scale levels of biological organization ranging from its genetic basis, to its neuromodulation, which facilitates the detection of pathogens by individual bees, to the assembly of individual-level responses, to colony-level social immunity.

Based on the premise that the genetic basis of honey bee hygienic behavior lies in how quickly individual bees within the colony detect the presence of diseased brood within the nest, Spivak and students conducted a series of experiments to test the hypothesis that some individual honey bees are particularly responsive to olfactory-based stimuli associated with diseased brood. Bees with the greatest olfactory sensitivity to diseased brood odors might first detect the problem and initiate the removal response. To test this hypothesis, Spivak and Gilliam (1998a, 1998b) bred a line of bees derived from Italian strains of *A. mellifera* L. for rapid-hygienic behavior, and a complementary line for nonhygienic or slow-hygienic behavior. Although Spivak used the terms hygienic behavior and nonhygienic behavior for the bred lines in all her publications, the terms rapid-hygienic behavior and slow-hygienic behavior are better descriptors of the differences between the lines and we recommend they be used in all future research. The rapid-hygienic line, called the MN Hygienic line, is currently sold commercially throughout the United States.

Individual bees from the rapid-hygienic line exhibited significantly increased sensitivity to the odor of chalkbrood disease at lower concentrations compared with bees from the slow-hygienic line, based on electrophysiological recordings of nerve impulses from the antennae [electroantennogram recordings (Masterman et al. 2001)]. Proboscis-extension response conditioning showed that bees from the rapid-hygienic line discriminated between the odors associated with healthy brood and brood infected with chalkbrood at a significantly lower stimulus level compared with bees from the slowhygienic line (Masterman et al. 2000, 2001). The combined results provide

supportive evidence for differential detection and behavioral response thresholds between the two lines of bees. Even within the line bred for rapid-hygienic behavior, there was significant variation in olfactory sensitivity and responsiveness among bees that tend to uncap dead brood and bees that tend to remove dead brood, which may lead to partitioning of the uncapping and removal tasks (Gramacho and Spivak 2003).

Spivak, Mesce, and colleagues (2003) further hypothesized that heightened olfactory sensitivity of these hygienic bees may be mediated by the sensitizing effects of particular biogenic amines in the bee brain. Because the neuromodulator octopamine (OA) enhances the response of bees to olfactory stimuli (Mercer and Menzel 1982; Hildebrandt and Muller 1995) and plays a pivotal role in olfactory-based behavior (Hammer 1993; Hammer and Menzel 1995), they examined whether bees from the rapid-hygienic and slow-hygienic lines differed with regard to their OA expression. The staining intensity of octopamine-immunoreactive (OA-ir) neurons in the deutocerebral region of the brain, medial to the antennal lobes, was compared in the brains of rapid-hygienic and slow-hygienic bees collected while performing hygienic behavior or in the brains of same-age bees not performing the behavior at the time of collection. The probability of having highly expressed OA-ir neurons was significantly greater in bees collected while performing hygienic behavior than in same-age bees not performing the behavior, independent of genotype, indicating that OA may play a part in modulating the behavior (Spivak et al. 2003).

Oral administration of OA increased olfactory sensitivity in individual bees selected for slow-hygienic behavior based on electroantennogram recordings but had no effect on bees selected for rapid-hygienic behavior (Spivak et al. 2003). In turn, oral administration of epinastine, a highly specific OA antagonist, reduced the sensitivity of bees selected for rapid-hygienic behavior but had no effect on bees selected for slow-hygienic behavior. Combined, these results suggest that the two lines may differ in the

distribution and responsiveness of their OA receptors, which is most consistent with the previous electrophysiological and anatomical studies (Spivak et al. 2003).

The chemosensory and neuroethological data provided mechanistic underpinnings for behavioral studies of individual bees within colonies in the field. Bees performing hygienic behavior are middle-aged, on average 15.7 ± 6.9 days (Arathi et al. 2000); they are significantly younger than foragers from the same colony. If bees that detect and remove diseased brood are older than the majority of bees that feed larvae, it would suggest that the age-based division of labor among hygienic and nurse bees within a colony may help reduce disease transmission.

The performance of hygienic behavior depended on the proportion of bees in the colony from the rapid-hygienic line. When colonies were composed solely of bees from the rapid hygienic line, some bees performed the task of uncapping cells at higher frequencies than the task of removing cell contents, and another group performed both tasks to the same extent (Arathi et al. 2000; Arathi and Spivak 2001). An individual bee's persistence (defined as the number of times an individual was observed performing uncapping or removal tasks) was significantly lower in colonies composed of bees from the rapid-hygienic line than in a colony with a minority of bees from that line. Only 18% of the bees, on average, in the rapid hygienic colonies were observed performing any component of hygienic behavior at one time. Despite the lack of persistence and the low number of bees engaged in the behavior, the rapid-hygienic colonies were significantly more efficient in achieving the task (removing 100% of the dead brood within a specified time) compared with colonies with fewer bees from this line. When the bees from the rapid hygienic line were in the minority, they were observed uncapping and removing the freeze killed brood well beyond middle age and tended not to partition the hygienic behavior components into subtasks (Arathi and Spivak 2001).

Bees from the slow-hygienic line were significantly less likely to perform hygienic behavior in the presence of rapid-hygienic bees. They also tended to recap cells that had been uncapped by bees from the rapid-hygienic line, contributing to colony-level inefficiency of the mixed genotype colonies (Arathi et al. 2006). An explanation backed by the chemosensory data is that slow hygienic bees recap cells containing diseased brood because they have reduced sensitivity to olfactory cues associated with diseased brood. Thus, slow-hygienic bees may perceive a hole in the pupal cap but may not necessarily detect that the brood within the cell is dead or diseased, and respond by resealing the hole with wax instead of continuing the process of uncapping. The delay in removing diseased brood allows the pathogen to reach the infectious stage (Woodrow and Holst 1942), facilitating disease transmission. This hypothesis remains to be tested experimentally.

Studies of the chemosensory, neural, and behavioral profiles of bees from the rapid- and slow-hygienic lines provided the framework for explaining hygienic behavior on the basis of a response-threshold model. This model, used to explain aspects of the division of labor within a social insect colony, suggests that individuals encounter different stimuli, and those with lower response thresholds perform tasks specifically associated with those stimuli (Bonabeau and Theraulaz 1999; Beshers and Fewell 2001). All bees can perform uncapping and removal behaviors, but bees that detect abnormal brood odors at a low stimulus level may rapidly initiate uncapping behavior, resulting in the removal of diseased brood before it becomes infectious. Slow-hygienic bees, with less olfactory sensitivity, detect and discriminate abnormal from normal brood only when the stimulus level is higher and thus tend to recap brood that has been uncapped, and proceed with the full process of uncapping and removal only after the brood is dead or infectious, leading to disease transmission. Colonies composed of a majority of rapid-hygienic bees have a larger proportion of bees with relatively high

olfactory sensitivity for diseased and dead brood (Gramacho and Spivak 2003) and tend to partition tasks between uncapping and removal (Arathi et al. 2000; Arathi and Spivak 2001; Gramacho and Spivak 2003). Task partitioning, and the tendency not to recap brood that has previously been uncapped (Arathi et al. 2006), leads to greater efficiency at the colony level. In contrast, a colony with a high proportion of slow-hygienic bees tends to take longer to detect infected brood and may then proceed to uncap, recap, and uncap these cells multiple times, and remove the diseased brood much later, if at all. This repetitive performance of the initial subtask of uncapping cells increases the probability that these bees will make repeated contact with the pathogen, resulting in an increased probability that the pathogen is transmitted throughout the colony.

POPULATION-LEVEL ANALYSIS: MODELING ON ANTISEPTIC BEHAVIOR IN SOCIAL INSECTS

Mathematical and computational modeling provides a method for exploration of aspects of disease-defensive behavior inaccessible to direct empirical manipulation. These models are used to examine multiple scales of effect, providing a quantitative, controlled, manipulable framework. These types of investigations into the efficacy of a broadly defined set of individual- and colony-level behaviors have already provided great theoretical insight into the social processes of disease defense, although only a few such examinations currently exist. Exploiting the known structure of social insect behaviors, disease-specific etiological rates, and nesting ecologies, the techniques employed have used empirically determined measurements to create individual-, cellular automata-, and differential equation-based models. Each of these methods makes different assumptions and therefore provides a different mathematical perspective of the problem. Together,

and in conjunction with empirical studies, these techniques can provide a quantitative understanding of the effects of social immunity on social insect disease dynamics.

Individual-based models focus on mobile individual actors, who interact with each other according to a set of predetermined rules. These rules can apply to interactions with other individuals and can also extend to the consideration of location within an explicit spatial structure. By definition, these models apply best to the examination of how individual behaviors lead to organized, colony-wide differences in disease load and mortality costs. Cellular automata–based models examine spatially explicit processes, in which the state of each location (or cell) is determined by a function of the current state of all neighboring locations. Naturally, these models are well suited to study the spatiotemporal propagation of disease throughout social insect colonies (Naug and Camazine 2002; Pie et al. 2004), considering the state of a cell to be defined by the state of individuals occupying the physical space represented. In the examination of the impact of social immunity, cellular automata models focus at the colony level by incorporating effects among local interactions (e.g., transmission of disease between individuals in adjacent cells or among individuals within the same cell) and then by measuring the colony-wide differences in either the number of infected individuals or the disease-related mortality over time achieved by incorporation of those local effects.

Fundamentally, both cellular automata and individual-based models can be considered mathematically based frameworks for empirical experimentation. The formulation of neither provides any theoretically meaningful result. Just as with laboratory-based experimentation, the results must be analyzed (frequently with the use of statistics) and interpreted within the context of the manipulations and alterations included in the individual behaviors examined and their assumed effects.

In contrast, models of disease spread composed of systems of differential equations provide theoretically meaningful results. By assuming average rates of

transmission and contact, and removing the individual levels of effect from consideration, these models yield important insights into threshold values for colony level assumed behavioral effects. They assume the average colony-level effect of incorporating the behaviors and then examine the resulting disease outcomes: colony death; epidemic outbreaks; low, constant endemic disease presence; or clearing pathogen presence from the population entirely. Models using these more analytical epidemiological formulations can be used in conjunction with the cellular automata– and individual-based models to provide a complete understanding of the local (among small groups of individuals) and global (colony-wide) effects of individual behaviors.

Fefferman and colleagues (2007) used a set of individual based models to perform behavioral knockout experiments of behaviors hypothesized to enhance social immunity and found that the early removal of infected brood in a colony of dampwood termites (hygienic behavior) and social-contact-generated immune protection offered protective benefits against disease risks, although each was found to confer maximal protection under different timescales of effect. Allogrooming also acts as an effective antiseptic behavior, despite the potential to cause increased disease transmission. Behavioral structuring was also examined to determine whether it effectively contributed to social immunity: Colony demography and the spatial segregation of etiologically distinct subgroups did not confer substantial protective benefits.

Naug and Camazine (2002) employed a set of cellular automata models to examine the social immune contribution of division of labor, colony demography, and nest architecture. By experimentally altering the density of infective individuals and varying the behavioral rates of interactions among different subgroups, they determined that these behavioral partial segregations were effective social immune defenses only when combined with assumed differential replacement rates of individuals within each subgroup.

Pie and colleagues (2004) constructed both a set of cellular automata models and a differential equation model to examine behavioral structuring aspects of social immunity. The theoretical model demonstrated that the likelihood of epidemics was significantly increased by increasing the density of individuals in the colony, and the experimental models demonstrated that nest architecture was increasingly protective against the spread of disease as the spatial segregation among nest chambers increased. These results showed that increasing homogeneous contact rates among nestmates increased the disease risks for the colony, implying that any behaviorally segregating structuring (e.g., division of labor, caste, or developmentally dependent segregation) could be considered an element of social immunity. Although some of these results seem to contradict those of the empirical models in Naug and Camazine (2002), it is important to recall the important influences of the different model assumptions employed: Pie and colleagues (2004) did not assume that individuals dying from the disease were automatically replaced by new, susceptible individuals. This difference can be interpreted as comparing a timescale for the duration of infection with that for the egg laying brood rearing rate of the colony, and the differences in the results, rather than contradicting each other, therefore only provide a more complete understanding of the sensitivity of the host-pathogen dynamic to the behavioral and ecological conditions of the entire system.

Sumpter and Martin (2004) used purely theoretical modeling to examine the dynamics of viral epidemics in mite-infested honey bee colonies. (For a review of coinfection by mites and viruses in honey bee colonies, see Ball and Allen 1988.) By incorporating specific variables to represent bee behaviors, they isolated the theoretical thresholds of impact caused by certain behaviors (e.g., honey bee hygienic behavior) to the epidemic spread of both mite and viral presence. They found that, taken in isolation, no collective behavioral responses provided any stable protective effects against either

macro- or micro-parasites. However, they did determine that honey bee hygienic behaviors that succeeded in reducing the mite infestation to less than 15% of the initial number of mites per bee would be effective at reducing the viral transmission below a critical threshold level, thereby preventing viral epidemics.

Each of these models examined aspects of the effect of individual- and colony-level behaviors on the spread of infectious disease within a social insect colony. The results from these studies of a broadly defined set of behaviors contributing to collectively protective social immunity provide, however, only limited understanding of the possible effects of these responses. Further work, involving both theoretical and experimental models, will continue to provide insight into the protective effects of social immunity (composed collectively from cellular, humoral, and behavioral immunity, and from antiseptic and nest hygienic behavior) in ways that empirical investigations could not achieve directly.

Behavioral immunity: Any behavioral act that decreases susceptibility to infection

CONCLUSIONS

Organisms are in a continuous coevolutionary arms race with some pathogens and parasites. Because disease is both a variable and a constant selective force, animals have evolved a myriad of methods of combating infection. Examining disease from an evolutionary perspective is becoming increasingly common. In no animal models can we more precisely examine disease-prevention techniques, and their colony- and population-level implications, than in social insects. The ability to breed for increased nest hygiene and antiseptic behavior attests to the genetic diversity underlying disease resistance behaviors, and the ability to examine colonies in the wild allows for within-colony, within-population, and across population studies. A multilevel review of disease

resistance behavior and physiology in insect societies provides a theoretical, evolutionarily sound, and biologically relevant foundation for examining disease resistance in other systems—systems unlikely to be as tractable

CHAPTER 3

A CRITICAL TEST OF THE 'HAPLOID SUSCEPTIBILITY' HYPOTHESIS WITH NATURALLY OCCURRING GENETIC MISFITS

CHAPTER 3.
A critical test of the 'haploid susceptibility' hypothesis with naturally occurring genetic misfits

Noah Wilson-Rich*, Bill Foo, Tien Tien, Faith Hester, and Philip T. Starks

Department of Biology, Dana Laboratories, Tufts University, Medford, MA 02155, USA

ABSTRACT

The haploid susceptibility hypothesis (HSH) was proposed as an explanation for how behavioral roles in haplo-diploid social systems evolved. It posits that haploid individuals are more susceptible to disease than polyploid individuals due to decreased genetic variability at key disease-resistance loci. The resulting decreased immunocompetence is hypothesized to have played a role in the evolution of social behavior by limiting the behavioral repertoire haploids perform. Here, we report results from a critical test of this hypothesis by conducting three immune function assays: 1) total hemolymph protein concentration, 2) hemolymph phenoloxidase activity, and 3) encapsulation response. Our data show that the HSH is *not* a significant contributor to the evolution of social behavior in *Polistes dominulus*. Instead, we note strong ecological influences on immune function, including temporal-, sex-, and caste-related factors. Time of emergence and behavioral role are the best predictors of immune function in *Polistes*.

INTRODUCTION

The fields of Evolutionary Biology and Behavioral Ecology often use rare cases to advance general theory. For example, W.D. Hamilton (1964) famously used the relatedness asymmetry that results from the relatively rare haplo-diploid genetic system of hymenopterans to help explain the evolution of eusociality, which in turn supported his more general kin selection theory. Eusocial systems, a relatively common occurrence within the order Hymenoptera, are characterized by an overlap of generations, cooperative care of the young, and a reproductive division of labor (Wilson 1971). This reproductive division of labor, which in its extreme form results in sterile workers, was a major concern of Darwin's given its obvious implications regarding the theory of natural selection (Darwin 1859).

Hamilton's insights resolved Darwin's concerns and led to a series of attempts to describe the evolution of eusocial behavior. One such explanation was through the creation of reproductive skew models, which combine genetic, behavioral, and ecological factors to predict when groups should form and how reproduction should be partitioned between cooperating members (e.g., see Keller and Reeve 1994; Reeve et al. 2000). With respect to *Polistes* wasps, at least, reproductive skew models fail to predict the degree of the reproductive division of labor (Liebert and Starks 2006; Nonacs et al. 2006).

The influence of relatedness on who helps – males or females – has only recently been challenged by suggesting that male-female behavioral differences evolved due to inherent differences in susceptibility to disease, and not necessarily due to Hymenopteran relatedness asymmetries (O'Donnell and Beshers 2004). As males are typically haploid, and females typically diploid, males are likely to have fewer disease

resistance alleles than females. Prior investigations of immunocompetence between haploids and diploids suggest haploid are more susceptible to disease (van Zon et al. 1964; Moret and Schmid-Hempel 2001; Santillán-Galicia et al. 2002; Gerloff et al. 2003; Vainio et al. 2004). However, tests of this 'haploid susceptibility' hypothesis have resulted in mixed results, most likely due to difficulties with the confounding factors relating to sex and caste (hypothesis supported: Baer et al. 2005; Baer and Schmid-Hempel 2006; hypothesis not supported: Ruiz-González and Brown 2006; Rutrecht and Brown 2008).

These confounding factors are not insurmountable. *P. dominulus* is an invasive wasp that produces both haploid and diploid males (Liebert et al. 2004, 2005), thus allowing us to disentangle sex from the analysis. Ploidy and sex are determined in *P. dominulus* via single-locus complementary sex determination (CSD), whereby hemizygous individuals are male and heterozygous individuals are female. Information on genetic diversity is particularly relevant to introduced Hymenoptera because of this sex-determining mechanism.

Occasionally, homozygous individuals are produced that result in the male phenotype (see Liebert et al. 2004, 2005). Diploid males are often sterile, and therefore are generally considered part of a population's segregational genetic load, which is the reduction in a population's average fitness due to the segregation of alleles into homozygotes that have low fitness. Diploid males are mostly produced early in the colony cycle during the worker phase, which helps mitigate the caste issue in a system where season of eclosion influences role ('worker' versus 'reproductive') (Mead et al. 1990). On rarest occasions, triploid individuals are produced from the unreduced male parental gamete, resulting in a female if the maternal allele is different from that of the paternal alleles or male if the maternal allele is the same.

Without the use of polyploid males, the confounding relationship between ploidy and sex cannot be disentangled, and thus the haploid susceptibility hypothesis (HSH) cannot be critically tested. However, comparing immunocompetence across haploid, diploid, and triploid conspecifics may shed light on collective influence of some genetic traits. The frequency of naturally occurring diploid males, and the apparently normal behavior of these males, allows for a unique opportunity to test the HSH using *Polistes* wasps.

METHODS

Immunocompetence is the ability of an organism to mount an immune response (Wilson-Rich et al. 2009). Measures of immunocompetence are a strong indicator of the degree and effectiveness of the host's ability to thwart pathogens and parasites (Pye 1974; Carton and David 1983; Leonard et al. 1985; Ochiai and Ashida 1988; Paskewitz and Riehle 1994; Gorman et al. 1996; Washburn et al. 1996; Ashida and Brey 1997; Doums and Schmid-Hempel 2000; Siva-Jothy 2000; Kraaijeveld et al. 2001a, 2001b; Trudeau et al. 2001; Wilson et al. 2001; Beck and Strand 2007). The immunocompetence of individual wasps was measured using three complementary tests of cellular and humoral immune function: total hemolymph protein concentration, hemolymph phenoloxidase activity, and encapsulation response to a novel foreign body.

Specimen collection:

We collected 106 *P. dominulus* nests and 411 individuals over three years (2007-2009) from sites across Massachusetts, southern New Hampshire, and southeastern

New York. Nests were identified as *P. dominulus* visually and then collected with resident wasps. Each nest was immediately transported to the International Social Insect Research Facility (ISIRF) at Tufts University, Medford, MA. Foundresses were removed from the nest because of their unknown pathogen exposure history to control for unknown effects on immune function. Pupae were allowed to eclose as adults for subsequent immunocompetence experiments.

A range of 1-30 individual *P. dominulus* adults eclosed per colony (mean ± SD = 3.9 ± 4.6 wasps). Each new adult was fed a diet of 50% sucrose solution and waxworm larvae *ad libitum*. These wasps were allowed a period of 48 hours for hardening and darkening of the cuticle. This step enabled us to control for the form of the phenoloxidase enzyme (PO) we assayed during tests of immune function (hemolymph PO and not wound PO or granular PO, which are both used in cuticular development; see Ashida and Brey 1997). No parasitism or outward sign of disease was noted on any wasp or nest included in this study. Of note, not all wasps collected were included in the genetic and immune studies due to a variety of factors, including survival, hemolymph quantity and quality, and grooming out implants.

Genetics:

The ploidy status of each individual was determined using microsatellite genetic analysis following the methods described by Johnson and Starks (2004), and Liebert and colleagues (2004, 2005). All analyses were conducted in the Starks Laboratory in the Dana Laboratories at Tufts University, Medford, MA. DNA was extracted from 291 frozen adult wasps. Female individuals were genotyped twice at three loci (Pdom1, Pdom25, and Pdom117) (primers developed by Henshaw 2000). All males were

genotyped across 8 loci (Pdom1, Pdom2, Pdom25, Pdom93, Pdom117, Pdom121, Pdom122, and Pdom127b) to reduce the probability of a polyploid male being homozygous at a particular microsatellite site. An individual was determined to be diploid or triploid if it displayed multiple alleles at any particular microsatellite loci. Males that were polyploid at only one primer were re-analyzed at this locus to prevent misidentification.

Immunology:

Encapsulation response (ER): We first performed a standard encapsulation response assay as a direct measurement of each wasp's ability to neutralize a foreign body using methods developed by König and Schmid-Hempel (1995) and Wilson-Rich and coworkers (2008, 2010). We elicited an ER by inserting a nylon 'pseudoparasite' (Cox-Foster and Stehr 1994) between each wasp's abdominal sternites to mimic the behavior of Strepsipteran endoparasites within the genus *Xenos*. *Xenos* are parasites of *Polistes* that protrude through the intersegmental membrane and reduce host fitness (Beani 2006).

Hemolymph collection: Hemolymph was then collected by first ice anesthetizing individual wasps and then piercing the abdomen using sterile technique. The resulting drop of liquid was collected, and any fluid which appeared yellow or brown was discarded to avoid gastric or other non-hemolymph fluid. One microliter of hemolymph was collected and transferred to a microcentrifuge tube (0.5ml, BD Falcon) containing nine microliters of sterile phosphate buffered saline and frozen until later protein and PO analyses.

Hemolymph protein concentration: Proteins in the hemolymph play in important role in an insect's immune response, including the anti-fungal drosomycins and metchnikoffins, and antibacterial cecropins, defensins, attacins, diptericins, and others (Tzou et al. 2002). The protein concentration of each hemolymph sample was determined using the Bradford method (Bradford 1976; Wilson et al. 2001; Lee et al. 2006; Wilson-Rich et al. 2008; Wilson-Rich and Starks 2010).

Phenoloxidase activity: We assayed PO activity using slightly modified methods originally developed by Wilson-Rich and coworkers (2010). To control for variation in hydration state of individuals, hemolymph protein concentration was controlled at 0.02mg/ml and added in varying volumes to the reaction mixture (Parkinson and Weaver 1999). The enzymatic substrate L-dopa, a tyrosine derivative, was then added to each solution to reach a final concentration of 0.03M. Next, melanin production was recorded using a Bio-Rad Benchmark Plus microplate spectrophotometer and Microplate Manager software (Bio-Rad, version 5.2.1). PO activity was quantified by recording the change in sample absorbance at 492nm every 30 seconds for 9 minutes (the linear phase of reaction; personal observation). Lastly, PO activity was quantified as the slope of the linear phase of reaction (Rolff and Siva-Jothy 2002).

Statistics:

Each of the three IC measures (total hemolymph protein concentration, phenoloxidase activity, and ER) was compared across sex, ploidy status (haploid, diploid, or triploid), season of year (spring/early summer or late summer/early fall), and nest of origin. We investigated the effects of the following independent variables on each immune measure: 1) sex, 2) ploidy, 3) ploidy + sex, 4) ploidy + season + sex. Immune

function was analyzed using a univariate general linear model (GLM) ANOVA. Colony of origin was incorporated as a covariate in order to control for pseudoreplication. Post-hoc pairwise comparisons were computed where appropriate using Tamhane's T2 tests, whereby equal variance between data sets was not assumed. Tests of equal variation between data sets were run using Levene's tests. All statistical tests were run with the computer program SPSS for Windows (v. 11).

RESULTS

Genetics:

Genetic analysis of up to eight microsatellite loci revealed the presence of 240 diploid females, 4 triploid females, 60 haploid males, 37 diploid males, and 3 triploid males. There was a nest effect across for all immunocompetence measures (N=106 nests total, total hemolymph protein concentration: $H = 128.1$, df = 75, $p < 0.001$, hemolymph PO: $H = 128.8$, df = 43, $p < 0.001$, ER: $H = 107.8$, df = 52, $p < 0.001$). To control for this nest effect, colony of origin was incorporated into the GLM as each individual measure.

Immunology:

Total hemolymph protein concentration (HPC): We analyzed data from 254 wasps taken from 76 colonies (mean ± SD = 3.34 ± 4.27 wasps per nest). Sex did significantly predict HPC ($F = 223.268$; df = 1, 75; $P < 0.05$) (Figure 3, top), as did ploidy ($F = 18.154$; df = 2, 75; $P < 0.001$) (Figure 4, top), both of which were interestingly independent of colony of origin. Diploids had the lowest activity (mg protein / ml

hemolymph = 2.06 ± 1.72), haploids had moderate activity (mg protein / ml hemolymph = 5.41 ± 5.68), and triploids had the highest activity (Vmax = 8.37 ± 11.28). Pairwise comparisons revealed diploids had significantly lower HPC than haploids (mean difference = -2.97, SE = 0.48, Tamhane's T2 $P < 0.001$). There was also a trend for HPC to change with season of year ($F = 54.037$; df = 1, 75; $P < 0.01$) (Figure 5, top). Early season wasps had less concentrated protein levels than late season wasps.

When sex was factored with ploidy, the GLM showed a highly significant effect on HPC ($F = 16.740$; df = 3, 75; $P < 0.001$), which was not affected by colony of origin (Figure 6, top). Diploid females had significantly less concentrated protein than haploid males (mean difference = -3.848, SE = 0.451, Tamhane's T2 $P < 0.001$) and diploid males (mean difference = -2.445, SE = 0.716, Tamhane's T2 $P < 0.05$). Diploid females had less HPC than triploid males, although not significantly so. When all three independent variables were combined (ploidy + sex + season), the GLM significantly predicted HPC ($F = 11.340$; df = 7, 74; $P < 0.001$) (Figure 7, top). Again, this relationship was not affected by colony of origin. Post-hoc analyses revealed only one significant difference between cohorts: early season diploid females (i.e., workers) had less HPC than early season haploid males (mean difference = -3.68, SE = 0.835, Tamhane's T2 $P < 0.001$).

Phenoloxidase (PO) activity: We analyzed data from 120 wasps taken from 42 colonies (mean ± SD = 2.86 ± 2.05 wasps per nest). Overall, there was not a significant difference between males and females with regard to PO activity (Figure 3, middle). Ploidy was a significant factor contributing to PO ($F = 6.176$; df = 2, 41; $P < 0.05$) (Figure 4, middle). Colony of origin also contributed to this effect ($F = 5.308$; df = 2, 41; $P < 0.05$). Diploids had the lowest activity (Vmax = 15.63 ± 36.10), haploids had moderate activity (Vmax = 30.33 ± 42.95), and triploids had the highest activity (Vmax = 85.00 ±

24.02). Post-hoc comparisons showed PO activity for diploids was significantly lower than haploids (mean difference = -3.34, SE = 0.48, Tamhane's T2 $P < 0.001$), while other pairs were not statistically different. Season of year was also a significant factor contributing to PO ($F = 330.761$; df = 1, 43; $P < 0.05$) (Figure 5, middle). Early season wasps (Vmax = 1.58 ± 2.54) had less PO activity than late season wasps (Vmax = 60.26 ± 47.66).

When sex was factored with ploidy, the GLM showed a significant effect on PO activity ($F = 2.834$; df = 3, 41; $P < 0.05$) (Figure 6, middle); yet, none of the pairwise comparisons was significantly different. There was also a colony effect on the sex + ploidy interaction ($F = 2.317$; df = 3, 41; $P < 0.05$). When all three independent variables were combined (ploidy + sex + season), the GLM significantly predicted PO activity ($F = 17.761$; df = 6, 41; $P < 0.001$) (Figure 7, middle). Interestingly, colony of origin was no longer important. Post-hoc analyses revealed just one cohort that repeatedly stood apart. Late season diploid females (i.e., reproductive gynes) had higher PO activity than all early season cohorts measured (versus early haploid males: mean difference = 71.85, SE = 14.11, Tamhane's T2 $P < 0.005$; versus early diploid males: mean difference = 69.35, SE = 14.21, Tamhane's T2 $P < 0.005$; versus early diploid females: mean difference = 71.59, SE = 14.11, Tamhane's T2 $P < 0.005$).

Encapsulation response: We analyzed data from 105 wasps taken from 45 colonies (mean ± SD = 2.33 ± 1.46 wasps per nest). Sex was a significant factor contributing to ER ($F = 9.345$; df = 1, 52; $P < 0.005$), with females higher than males ($F = 4.523$; $df = 1, 52$; $P < 0.05$; Figure 3, bottom), as was ploidy ($F = 6.862$; $df = 2, 44$; $P < 0.01$) (Figure 4, bottom). Colony of origin significantly affected both of these relationships (for sex: $F = 2.110$; df = 1, 52; $P = 0.050$; for ploidy: $F = 15.898$; $df = 2, 44$; $P < 0.05$). Triploids had the lowest value (OD = 28.80 ± 0.52), haploids had the middle value (OD =

54.29 ± 30.25), and diploids had the highest value (OD = 66.35 ± 28.37); however, post-hoc comparisons showed no significant differences between any pairs. Season of year was also a significant factor contributing to ER ($F = 9.984$; df = 1, 52; $P < 0.005$) (Figure 5, bottom). Late season wasps had lower ERs than early season wasps.

When sex was factored with ploidy, the GLM showed a trend on ER ($F = 2.209$; df = 3, 44; $P < 0.1$) (Figure 6, bottom); likewise, none of the pairwise comparisons were significantly different. There was also a colony effect on the sex and ploidy interaction ($F = 3.981$; df = 3, 44; $P < 0.05$). When all three independent variables were combined (ploidy and sex and season), the GLM significantly predicted ER ($F = 5.148$; df = 6, 44; $P < 0.001$) (Figure 7, bottom). Colony of origin was no longer a significant factor. Post-hoc analyses revealed two cohorts that stood apart. Late season diploid females had lower PO activity than early season diploid females (mean difference = - 33.75, SE = 7.53, Tamhane's T2 $P < 0.01$). Likewise, late season diploid males had lower ER all early season cohorts measured (versus early haploid males: mean difference = -40.56, SE = 6.84, Tamhane's T2 $P < 0.1$; versus early diploid males: mean difference = -53.29, SE = 11.54, Tamhane's T2 $P < 0.05$; versus early diploid females: mean difference = -56.47, SE = 6.28, Tamhane's T2 $P < 0.05$).

DISCUSSION

Overall, our results do not support the haploid susceptibility hypothesis. In fact, direct ploidy comparisons show the opposite trend – diploid susceptibility – which is clearly not the full story (see Figure 4). Instead, other ecological factors have strong influences on the immunocompetence of *Polistes* wasps. First, a robust temporal effect was noted in both direct immune measures, albeit in different directions (Figure 5).

Humoral (cell-free) assays – total hemolymph protein concentration and hemolymph PO activity – both increased from early season to late season. Encapsulation showed the opposite movement of a decrease in this immune process from early to late season. Together, these findings suggest a trade-off in innate immune defense from largely cellular to solely humoral mechanisms. Pathogen pressure for these invasive populations of *P. dominulus* in the northeast United States are poorly documented; however, we speculate that this shift likely reflects the type of parasites that infect these hosts during different seasons of the year. The enemies of *P. dominulus* may have a stronger influence on immunocompetence than haploid susceptibility, as pathogen landscapes clearly pose selection pressure on host immune defenses (Manfredini et al. 2010).

A second notable pattern emerged across all three immunocompetence tests when sex, ploidy, and season were analyzed together (see Figure 7). Both sexes and all ploidy states showed the same seasonal trend described above. Late season diploid females had significantly greater PO activity than all early season cohorts (early haploid males, early diploid males, and early diploid females). A similar seasonal effect was found across cohorts in the ER results. Late season diploid males had significantly lower ERs than all early season cohorts. Additionally, late season diploid females had significantly lower ERs than early season diploid males. These analyses further support our conclusion that timing of eclosion, and subsequent predicted behavioral role, are more important predictors of *Polistes* immune function than ploidy alone.

The haploid susceptibility hypothesis suggests that haploid males exhibit increased disease susceptibility, and that this vulnerability may have been a factor in the evolution of behavioral interactions in social Hymenopterans (O'Donnell and Beshers 2004). This hypothesis assumes decreased diversity at disease resistance loci

negatively impacts the survival of haploid organisms (as haploids and homozygous diploids have one type of defense loci while heterozygotes have two). Empirical support for this hypothesis includes lower immunocompetence in haploid male eusocial insects [e.g., the wood ant *Formica exsecta* (Vainio et al. 2004); the leaf-cutting ant *Acromyrmex echinatior* (Baer et al. 2005); and the bumble bee *Bombus terrestris* (Gerloff et al. 2003)]. However, some studies have shown haploid males are not more susceptible to disease than diploid females [e.g., the bumble bee *Bombus terrestris* (Ruiz-González and Brown 2006)]. Here, we critically tested the HSH by controlling for confounding factors (e.g., behavioral role, sex, and season) using multiple measures of immunocompetence. An alternative hypothesis is that decreased ER in late season wasps compared to early season wasps could reflect the natural aging process of *Polistes*; this topic remains to be explored.

This study advances our understanding of haploid susceptibility because we used multiple measures of immunocompetence. We also included three types of naturally occurring genetic misfits into our sample: diploid males, triploid males, and triploid females. Notably, no account of triploid males has been reported until now. Combined, our approach enabled a robust test of the HSH. If we compared males and females only and not included genetic misfits, then we would have supported the HSH with ER results (Figure 3). Even if we included genetic misfits but relied on just one immune tests, then the HSH would have been supported based on PO results alone (Figure 7). Clearly, neither of these approaches provides the full story. A single measure of immune strength may provide misleading, and almost certainly incomplete, information. Even the one prior report where the immunocompetence of genetic misfits – diploid males – were compared to haploid males and diploid females, Gerloff and coworkers (2003) relied on just one measure, ER. Relying on one method constricts the

lens into actual disease defense capability (see Adamo 2004a), and likely contributed to prior studies supporting this hypothesis.

Across our three measures, we found that sex and time of year play the most important roles in predicting immunocompetence in *P. dominulus*. These results are not surprising given the primitively eusocial nature of *P. dominulus*, where sex and time of year are major predictors of behavioral role (Mead et al. 1990). The life history of *P. dominulus* involves one or more reproductively dominant female foundresses that start a new nest in the springtime. These females mated the previous fall, from which they lay fertilized eggs to produce worker females. During this early season, workers typically do not mate but instead assist the foundress(es) in various tasks such as nest building, brood care, and foraging. Later in the season, reproductives (both female and male) emerge, while the workers continue to focus on their colony rearing tasks. The reproductives mate, and only late season females overwinter while all other castes die. The individuals with the longest lifespan have the strongest humoral immune activity. Interestingly, a parallel can be drawn from honey bee workers, where the oldest individuals have the highest PO activity, putatively because they are more likely to be in contact with pathogens and parasites (Wilson-Rich et al. 2008).

By combining our results with our knowledge about *P. dominulus* life history, a broader picture of what influences immunocompetence and behavior becomes more apparent. Clearly, behavioral role in *P. dominulus* is affected by season and sex. Our results show that genetic misfits are present throughout the year, and by incorporating these polyploidy cohorts into our analysis we were able to show that ploidy plays a less important role in selecting for disease defense.

Future studies should 1) determine the behavioral role of genetic misfits (and early haploid males) within the parameters of the colony cycle, and 2) continue to incorporate multiple approaches when investigating immunocompetence, including complementary analysis of innate and acquired tests. Results reported here indicate that temporal and sexual factors play an important role in the degree to which individuals can thwart pathogens. This susceptibility, in turn, selects for host defense more strongly than ploidy. Our data show that ploidy plays a role in immunocompetence, but that role is secondary to the environment. In particular, time of emergence and thus whether an individual is an individual or a reproductive or a worker, is the single best predictor of immune function.

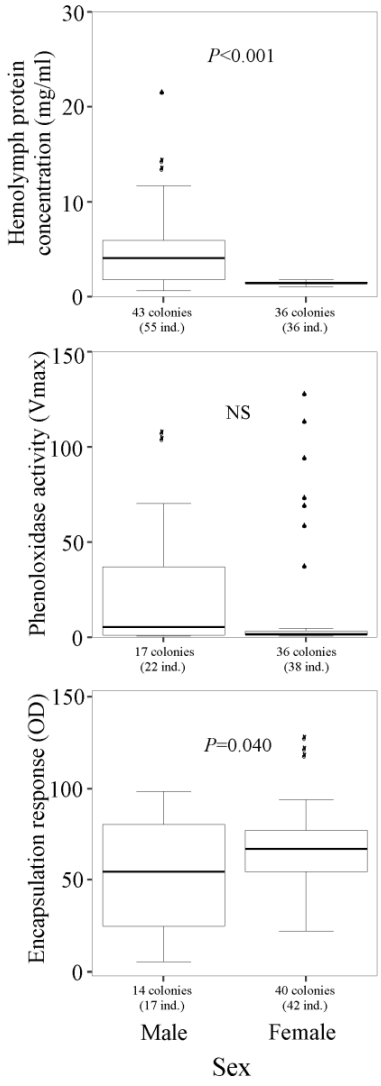

Figure 3. Sex effects on immunocompetence in *P. dominulus*. High values for all three measures indicate a strong ability to mount an immune response. Bars represent median values, boxes enclose the middle 50% of the data, and the whiskers enclose 95% of the data extend to the points beyond which outliers (shown as symbols above bars) reside. Asterisks denote outliers. Letters indicate *P*-values differences below 0.05 as calculated using univariate generalized linear models.

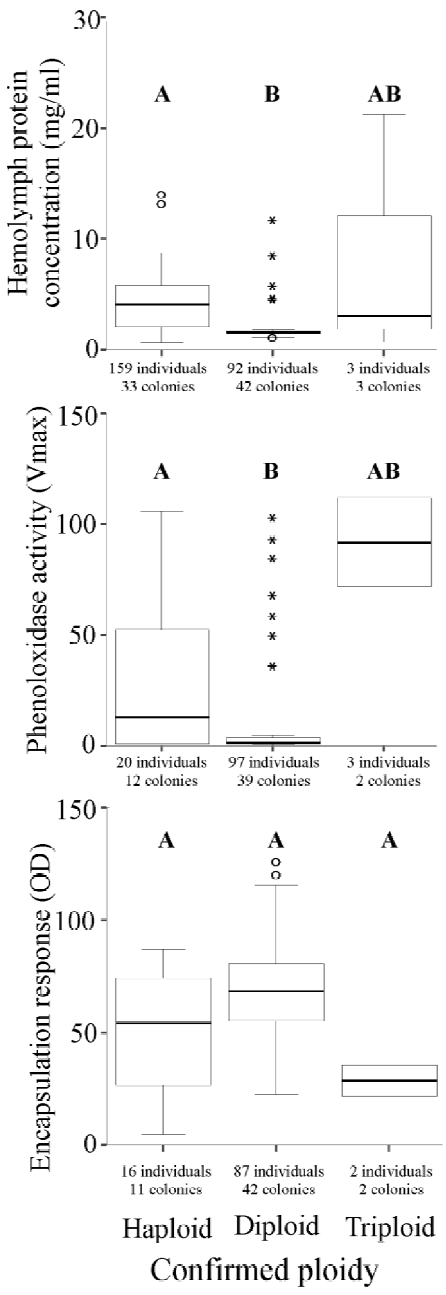

Figure 4. Ploidy effects on immunocompetence in *P. dominulus* (Chapter 3). The sexes are pooled. High values for all three measures indicate a strong ability to mount an immune response. Bars represent median values, boxes enclose the middle 50% of the data, and the whiskers enclose 95% of the data extend to the points beyond which outliers (shown as symbols above bars) reside. Circles denote outliers. Asterisks denote extremes. Letters indicate *P*-values differences below 0.05 as calculated using univariate generalized linear models followed by post-hoc Tamhane's T2 tests.

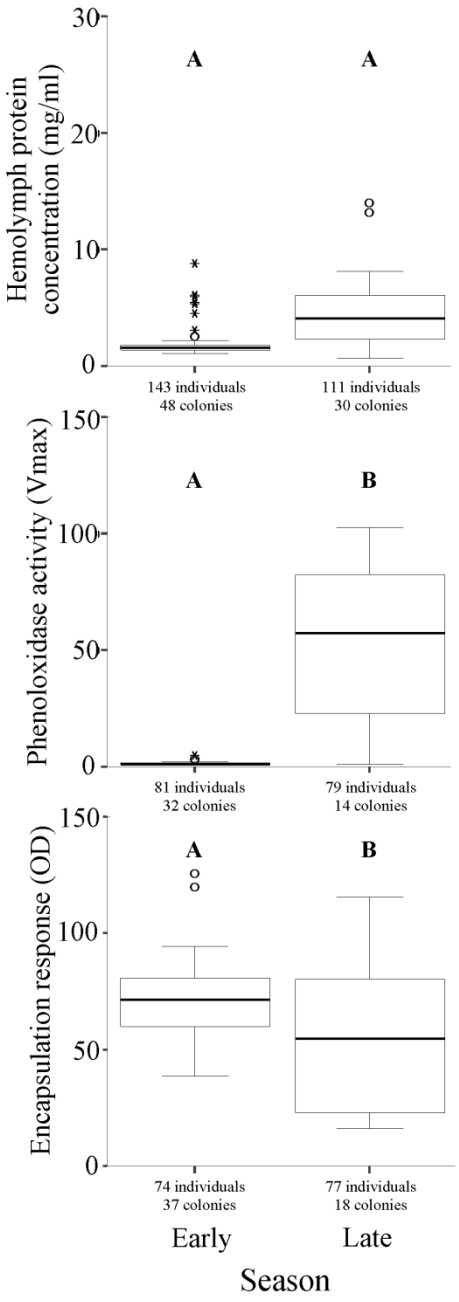

Figure 5. Different modes of immunity are affected differently by season of year in *P. dominulus*. High values for all three measures indicate a strong ability to mount an immune response. Bars represent median values, boxes enclose the middle 50% of the data, and the whiskers enclose 95% of the data extend to the points beyond which outliers (shown as symbols above bars) reside. Circles denote outliers. Asterisks denote extremes. Letters indicate *P*-values differences below 0.05 as calculated using univariate generalized linear models.

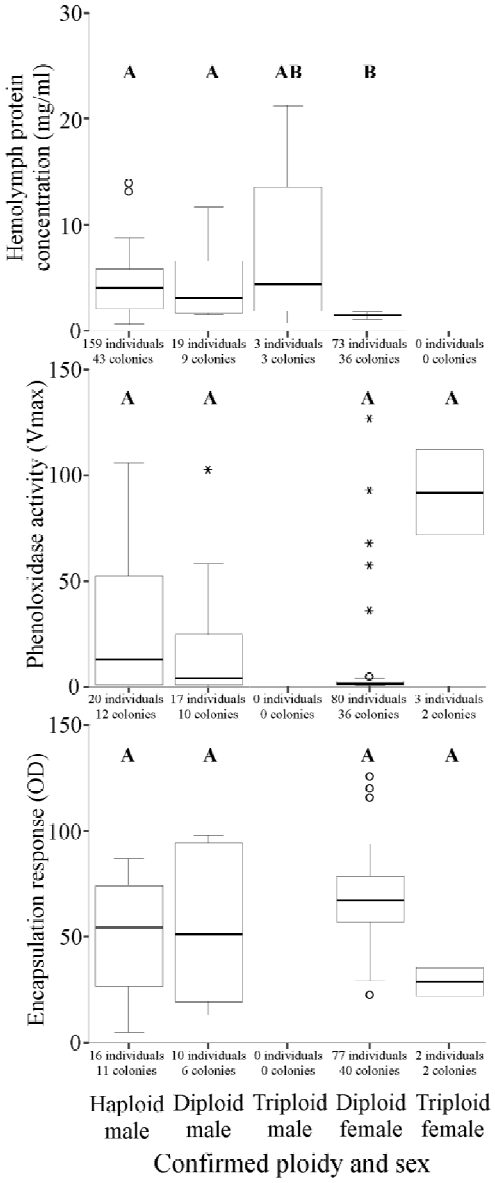

Figure 6. Combined sex and ploidy effects on immunocompetence in *P. dominulus*. High values for all three measures indicate a strong ability to mount an immune response. Bars represent median values, boxes enclose the middle 50% of the data, and the whiskers enclose 95% of the data extend to the points beyond which outliers (shown as symbols above bars) reside. Circles denote outliers. Asterisks denote extremes. Letters indicate *P*-values differences below 0.05 as calculated using univariate generalized linear models followed by post-hoc Tamhane's T2 tests.

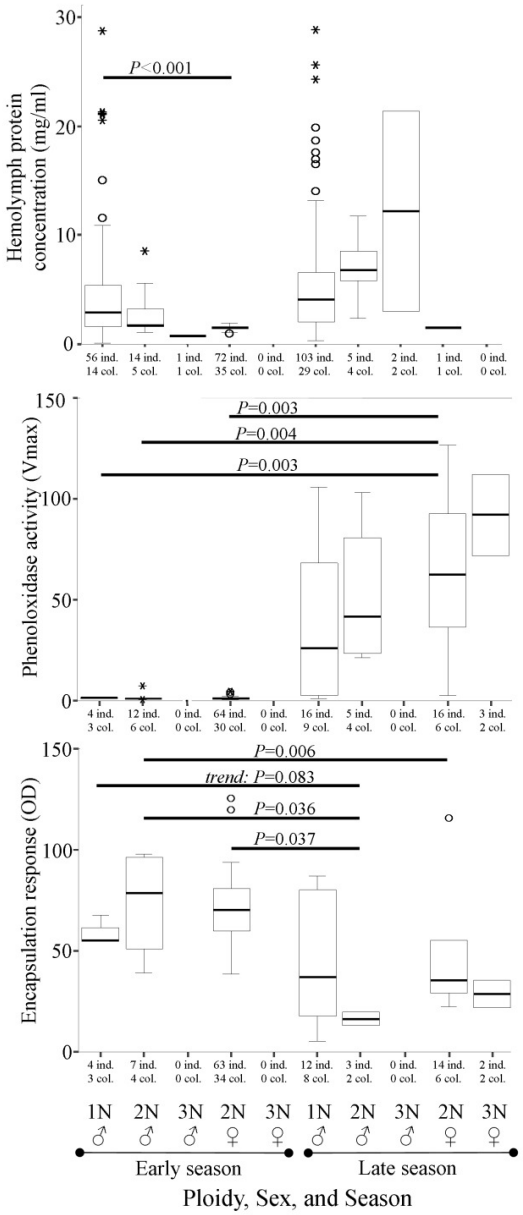

Figure 7. Hemolymph phenoloxidase (PO) activity is predicted by temporal, sex, and to a lesser degree, ploidy in *P. dominulus*. High values for all three measures indicate a strong ability to mount an immune response. Bars represent median values, boxes enclose the middle 50% of the data, and the whiskers enclose 95% of the data extend to the points beyond which outliers (shown as symbols above bars) reside. Circles denote outliers. Asterisks denote extremes. *P*-values were calculated using univariate generalized linear models followed by post-hoc Tamhane's T2 tests.

CHAPTER 4

WITHIN- AND ACROSS-COLONY EFFECTS OF HYPERPOLYANDRY ON IMMUNE FUNCTION AND BODY CONDITION IN HONEY BEES (*Apis mellifera*)

CHAPTER 4.
Within- and across-colony effects of hyperpolyandry on immune function and body condition in honey bees (*Apis mellifera*)

Noah Wilson-Rich[1], David R. Tarpy[2], and Philip T. Starks[1]

[1]Department of Biology, Tufts University, Medford, MA 02155

[2]Department of Entomology, North Carolina State University, Raleigh, NC 27695

ABSTRACT

Honey bees (*Apis mellifera*) have become a model system for studies on the influence of genetic diversity on disease. Honey bee queens mate with a remarkably high number of males – up to 29 in this study – from which they rear a colony of highly integrated workers. Polyandry with up to 10 males was originally explained as a mechanism to reduce the production of diploid males, which are functionally sterile. Recent evidence has shown a significant benefit of genetic diversity on colonies with respect to disease resistance at this mate number and above. Here, we explored the relationship between the level of polyandry (effective genetic diversity) and early mechanisms of cellular and humoral immune defense (encapsulation response and phenoloxidase activity). We also investigated an effect of patriline on fat body mass, a measure of body condition. We hypothesized that phenoloxidase activity, encapsulation response, and fat body mass would show a linear relationship with mating number. Surprisingly, genetic diversity, although clearly a benefit to colony health, has no effect on these immune measures, and no consistent effect on body condition.

INTRODUCTION

Insect societies have long been used for evolutionary study of cooperation (e.g., Hamilton 1964a, 1964b) and conflict (e.g., Trivers and Hare 1976). Hamilton's inequality made sense of Darwin's concern of sterile workers within the eusocial Hymenoptera, and explains how self sacrificing could be beneficial among related individuals (Hamilton 1964a, 1964b; see Payne et al. 2010). Kin selection theory (Hamilton 1964a, 1964b), as applied to eusocial insects, explains that for workers to provide assistance, the benefits of performing a behavior should outweigh the costs after being devalued by the value of relatedness between the actor and the recipient ($rB > C$). While high levels of relatedness seem necessary for this to occur, this is not always the case.

Haplodiploidy is a genetic phenomenon in many social insect systems, including the insect order Hymenoptera, whereby males are typically haploid and females are typically diploid. Within Hymenoptera, sexual phenotypes arise through the complementary sex determination mechanism, whereby males are hemizygous at the sex determination locus, and females are heterozygous. On rare occasion, diploid males are produced when a queen mates with a drone who shares the same sex determination allele as her, resulting in 50% of her offspring that are homozygous at this locus. Typical (haploid) males are produced from unfertilized eggs, and as such are related to their mother by 100%, but have no fathers. Females are produced from fertilized eggs, and are related to their mother and their father equally, by 50%. In colonies reared from singly mated queens, workers (females) are related to each other by 75%, but related to their brothers by only 25%.

Trivers and Hare (1976) showed conflict between the queen and her daughters with respect to investment into sons or daughters, based on this relatedness asymmetry.

The overall investment in workers over males was 3:1, whereas the queen's relatedness should prefer a 1:1 investment. In a special case of his kin selection hypothesis, Hamilton (1964a, 1964b) pointed out that there is a clear benefit for daughters to help their mothers raise sisters in colonies reared from a singly mated queen. This is because of the relatedness asymmetry within haplodiploid systems, such as in the Hymenoptera. Sister workers are related to each other by 3/4, but that relationship diminishes when queens mate multiply. The resulting decreased in mean colony relatedness (r) is, therefore, decreased when multiple patrilines are present.

Interestingly, polyandry does occur in some social Hymenoptera, and sometimes at surprisingly high levels. Polyandry occurs when the queen mates with more than one male, thus reducing the mean r among workers (see Schmid-Hempel 1998; Palmer and Oldroyd 2000; Strassman 2001). In honey bees (*Apis mellifera*), queens are known to mate with many males (1 – 28, Tarpy and Nielson 2002; 8 – 27, Palmer and Oldroyd 2000), with colonies consisting of an average of 12 subfamilies (Tarpy et al. 2004). Given what we know about kin selection theory and queen-worker conflict, the phenomenon of polyandry in honey bee colonies is therefore puzzling. Mean colony r does not effectively decrease after approximately 10 successful matings; there is an asymptote in this relationship. Therefore, polyandry up to this threshold likely originated as a mechanism to avoid the production of sterile, diploid male honey bees (Page 1980; Tarpy and Page 2001).

Diploid males are considered evolutionary dead ends that have no function, as they reduce colony fitness by consuming resources and contributing nothing (Woyke 1963; Page 1980; also see Tarpy and Page 2002). In honeybees, the diploid drone condition is invariably lethal, since diploid male larvae are eaten by workers within 72h of eclosion, possibly because of a substance secreted by diploid drone larvae which

causes cannibalism (Woyke 1963; Winston 1987) or, perhaps, by their larger size. Artificially reared diploid drones display underdeveloped testes which produce little semen (Woyke 1969, 1973). Cannibalism of diploid male honey bees may be an adaptive trait to prevent wasting resources on individuals not providing fitness benefits. Diploid male avoidance is effectively achieved after mating with up to 10 drones (Tarpy and Page 2001).

Recent data show hyperpolyandry in honey bees provides the additional benefit of disease resistance to the colony (Tarpy 2003; Tarpy and Seeley 2006; Seeley and Tarpy 2007). Tarpy (2003) showed that colonies reared from queens inseminated from 24 drones had lower variance in their ability to resist *Ascosphaera apis*, the fungal pathogen that causes chalk brood disease in honey bee brood, as compared to queens inseminated by one drone. This observation indicates that multiple matings increase the odds that a colony will survive chalk brood disease, whereas a colony reared from a monogamous queen was more of a gamble for how it would resist this disease. Tarpy and Seeley (2006) followed up this study by setting up hives reared from queens inseminated with either one or 10 drones. They found that colonies with higher genetic diversity had lower intensity of all measured brood diseases (chalk brood, sacbrood, American foulbrood, and European foulbrood). This observation was confirmed yet again the following year after artificial infection with the bacterium *Paenibacillus larvae*, the causative agent of the highly virulent American foulbrood disease (Seeley and Tarpy 2007). Colonies reared from queens inseminated with sperm from 10 drones had lower disease intensity and higher colony strength (i.e., more brood, heavier, and more populated).

In this study, we aimed to determine the influence of colony-level genetic diversity on two immune function mechanisms that could be responsible for the recent

observations linking genetic diversity to disease resistance. We hypothesized that immune function serves to explain the results we see in genetically diverse versus genetically monomorphic hives. With recent advances in our understanding of honey bee immunity (Wilson-Rich et al. 2008; reviewed in Wilson-Rich et al. 2009), we can test broad defenses and first explore the earliest cellular and humoral immune mechanisms that pathogens and parasites would encounter once in the honey bee hemocoel. We predict the results will show correlation between level of genetic diversity (number of patrilines), and both immune function (phenoloxidase activity and encapsulation response) and body condition (fat body mass) with regard to both average values as well as the variation around the average values. We examined these factors across colonies (by number of effective patrilines) and also across patrilines (within colonies). Specifically, across colonies, we predicted that as genetic diversity increased, the mean immune function would also increase, while the variation around the mean would decrease. We predicted that body condition would positively correlate with genetic diversity across colonies. Within colonies, we predicted to see a patriline effect on immune function and body condition, whereby different sibling groups would vary from each other.

METHODS

Specimen collection: 1,124 honey bees were collected from 22 colonies at the Cummings School of Veterinary Medicine at Tufts University in North Grafton, MA, USA during two non-consecutive years. In 2006, guarding and foraging adults were collected from 12 colonies (N = 266 individuals, mean ± SD = 22.17 ± 5.18 individuals per colony, range 12 - 28). In 2009, guarding and foraging adults, as well as brood, were collected from 10 colonies (N = 858 individuals total, mean ± SD = 85.80 ± 8.09 individuals per

colony, range 66 – 95 per colony). Brood collection involved mixed samples of larvae and pupae, as the immune function measures used (phenoloxidase activity and encapsulation response) are the same across both developmental stages (Wilson-Rich et al. 2008).

Immunology and body condition: Phenoloxidase activity (PO), encapsulation response (ER), and fat body mass (FB) were performed following the methods described by Wilson-Rich and colleagues (2008). In 2006, 133 adults were assayed for ER and 310 adults for FB. In 2009, 256 individual brood were assayed for PO and 299 adults for FB. Not all individuals collected produced usable data based on limitations of each respective assay, such as inability to collect hemolymph for PO or failure to retrieve the implant for ER. A highly conservative threshold was implemented for PO analyses, whereby the Vmax of enzyme linear phases were used only if $r^2 \geq 0.9$. Multiple measures were conducted in an effort to gain more information about the effects of genetic diversity across the dynamic immune system (see Adamo 2004a). We assessed two measures (ER, PO) of immune function and one measure of body condition (FB) for a holistic view of immunocompetence (see Wilson-Rich et al. 2009).

Genetics: Colony-level genetic diversity and within-colony patrilines were quantified blindly after samples were collected for immune tests. The subfamily of each individual was determined using polymorphic microsatellite genetic analysis (Tarpy et al. 2010). Because of their generally high mutation rate, microsatellite markers may have a large number of alleles, which make them particularly suited for paternity analysis (Estoup et al. 1995). All analyses were conducted in the Lake Wheeler Honey Bee Research Facility in Raleigh, NC, USA. DNA was extracted from 1,124 honey bees (N = 266 adults from the 2006 collection and N = 858 brood from the 2009 collection).

Statistics: Immune function was compared both across and within colonies based on the number of patrilines, and patriline of origin, respectively. For across colony comparisons, we created a univariate generalized linear model (GLM) to control for any effect of subfamily differences within colonies. The GLM ANOVA incorporated each respective immune measure as discrete dependent variables, the number of subfamilies in each respective colony as the independent variable, and patriline as a covariate nested in each colony. We did not analyze genetics from adults in 2009 (only brood genetics were conducted for the 2009 samples), so these individuals were compared separately from the GLM, and Kruskal-Wallis H tests were performed instead. For within colony comparisons, we first determined whether each data set was normally distributed using Shapiro-Wilk tests. We then performed either ANOVAs or Kruskal-Wallis H tests across patrilines followed by post-hoc pairwise comparisons, depending upon the normality of the distribution of each respective data set from Levene's tests. Comparisons of variation were conducted also using Levene's tests for unequal variance. Furthermore, we calculated partial eta-squared values (as in Wilson-Rich et al. 2008) to determine the proportion of variance in each immune measure that may be explained by colony-level genetic diversity. All statistics were calculated using SPSS for Windows (v. 11).

RESULTS

Genetics: Across the 22 colonies, we found a range of 8—29 patrilines per colony (mean ± SD: 16.5 ± 6.3) (table 1). In 2006, the number of patrilines ranged from 9 – 15 (mean ± SD: 11.0 ± 2.3), while in 2009, the number of patrilines ranged from 11 – 29 (mean ± SD: 20.8 ± 5.2).

Table 1. Mating number trends for honey bee (*A. mellifera*) queens. These data are an overview of genetic results from 22 honey bee colonies at the Tufts University apiary in North Grafton, MA, USA. The number of patrilines for each colony was determined using the methods of Tarpy and coworkers (2010). Overall, hives averaged 16.5 ± 6.3 patrilines, which is greater than previously reported. Further, it is likely that we underestimated the number of patrilines based on our limited sample size.

Year	Number of bees collected per colony	Number of subfamilies	Number of bees per patriline (mean ± SD)
2006	18	9	2.00 ± 1.32
2006	28	15	1.87 ± 0.64
2006	22	12	1.83 ± 1.19
2006	20	8	2.50 ± 2.00
2006	25	11	2.27 ± 1.56
2006	27	12	2.25 ± 2.30
2006	27	15	1.80 ± 1.08
2006	18	11	1.64 ± 0.81
2006	17	12	1.42 ± 0.67
2006	28	9	3.11 ± 2.26
2006	24	9	2.67 ± 3.04
2006	12	9	1.33 ± 0.71
2009	85	11	7.73 ± 7.38
2009	85	29	2.93 ± 1.96
2009	84	25	3.36 ± 2.46
2009	84	17	4.94 ± 3.11
2009	91	23	3.96 ± 2.75
2009	95	17	5.59 ± 5.47
2009	94	25	3.76 ± 4.47
2009	89	23	3.87 ± 3.05
2009	66	20	3.30 ± 4.01
2009	85	18	4.72 ± 3.48

Across colony comparisons: A total of 998 honey bees were used for immune function tests. We tested the encapsulation response of 133 adult bees taken from six colonies in 2006 (mean ± SD = 21.16 ± 4.40 bees per colony). Overall, the number of patrilines was not a significant factor contributing to encapsulation response ($F = 0.350$; df = 5, 14; $P > 0.5$) (Figure 8). Variation around the average encapsulation response differed significantly with number of subfamilies ($F = 2.011$; df = 62, 64; $P < 0.01$). The

number of patrilines contributed remarkably little to predicting encapsulation response (eta-squared = 0.092).

We assayed phenoloxidase activity on brood from seven colonies in 2009 (N = 229 individuals, mean ± SD = 32.71 ± 9.40 bees per colony). Overall, the number of patrilines was a significant factor contributing to phenoloxidase activity ($F = 7.908$; df = 6, 25; $P < 0.001$) (Figure 9). Variation around the average phenoloxidase activity also differed significantly with number of patrilines ($F = 1.680$; df = 96, 132; $P < 0.01$). Post-hoc pairwise comparisons of colony median and variance revealed haphazard relationships, with no clear directional trend for how patriline number influences phenoloxidase activity activity (Figure 9). The number of patrilines in a honey bee hive contributed to 1/2 of the predictive value determining phenoloxidase activity (eta-squared = 0.498).

We quantified fat body mass on 264 adults from 12 colonies in 2006 (mean ± SD = 22.00 ± 5.34 bees per colony). Overall, the number of patrilines was a significant factor contributing to FB ($F = 9.701$, df = 11, $P < 0.001$) (Figure 10). Variation around the average fat body mass also differed significantly with number of patrilines ($F = 1.925$, df = 130, 33; $P < 0.001$). In 2009, 266 adults from 10 colonies were assayed for FB (mean ± SD = 26.60 ± 7.73 bees per colony). Again, the number of patrilines was a significant factor ($H = 17.969$, df = 9, $P = 0.05$). The number of patrilines in a honey bee hive contributed to just over 2/3 of the predictive value determining fat body mass mass (eta-squared = 0.680).

Within colony comparisons: Looking within each colony, we compared each measure of immune function across patrilines. Surprisingly, there was no significant patriline effect on either encapsulation response or phenoloxidase activity, in any of the

colonies (ANOVA $P > 0.05$ or KW $P > 0.05$). We did note a patriline effect in the fat body mass analysis in about half of the colonies (5/12 colonies: colony F, $F = 2.52$, df = 26, $P = 0.05$; colony K, $F = 3.33$; df = 8, 15; $P < 0.05$).

Table 2. Summary of results testing the 'genetic diversity' hypothesis (Chapter 4). We hypothesized that immune function explains the results seen in genetically diverse versus genetically monomorphic hives (Tarpy 2003; Tarpy and Seeley 2006; Seeley and Tarpy 2007). We predicted that phenoloxidase activity, encapsulation response, and fat body mass would show a linear relationship with mating number. Surprisingly, genetic diversity, although clearly a benefit to colony health, has no effect on these immune measures, and no consistent effect on body condition, as noted here.

Immune function:	Hypothesis supported?	Significance
1) Encapsulation response		
-Across colonies	No	No effect of patriline number
-Within colonies	No	No patriline effect
2) Phenoloxidase activity		
-Across colonies	Unclear	Colony effect significant, although not correlated with patriline number (Figure 9)
-Within colonies	No	No patriline effect
3) Fat body mass		
-Across colonies	No	No effect of patriline number
-Within colonies	Yes, for 2/12 colonies	Weak patriline effect

DISCUSSION

We found no support for the polyandry vs. parasitism hypotheses in either of the immune function parameters measured (table 2). Remarkably, patriline of origin was not a predictor of either phenoloxidase activity or encapsulation response in any of the 22 colonies analyzed. There was an effect of subfamily number on body condition within

only 20% of the colonies. There was a significant patriline effect on fat body mass in two colonies. Fat bodies are vitally important for honey bees for energy and production of antimicrobial proteins (Zachary and Hoffman 1984; Hultmark 1993; Hetru et al. 1998). We did note an increase in mean number of patrilines, from 12.0 ± 6.41 (Tarpy and Nielson 2002) to 15.5 ± 6.28. We also observed an upward shift in the range of number of patrilines, from 1 – 28, (Tarpy and Nielson 2002) and 8 – 27 (Palmer and Oldroyd 2000) to 8 – 29, presented here.

Genetic diversity increases disease resistance, but not through these mechanisms. The immune systems we studied, phenoloxidase activity and encapsulation response, are important lines of defense to host insects after a pathogen enters the host hemocoel (reviewed in Wilson-Rich et al. 2009). We know that honey bee colonies reared from hyperpolyandrous queens have greater relative fitness against infectious disease compared to monandrous queens (Tarpy 2003; Tarpy and Seeley 2006; Seeley and Tarpy 2007). The mechanism of disease resistance, therefore, remains elusive. Multiple additional mechanisms of immune function should be investigated to gain an advanced understanding of how colony-level genetic diversity increases fitness by reducing disease.

Genetic diversity possibly provides more lines of defense against pathogens and parasites, assuming that more, different alleles coding for disease resistance phenotypes are entering the population. These additional and different defenses likely keep disease intensity in check (see Hughes and Boomsma 2006). It is surprising that encapsulation response and phenoloxidase activity are not affected by colony-level genetic diversity. Yet, these innate mechanisms may be *too* important for fighting pathogens and parasites. Perhaps their vital importance makes them indispensible to all

individuals, and do not show variation. The one mode of immune defense with the most flexibility in phenotype is, of course, behavior.

Behavior is the most obvious mechanism for how genetic diversity may scale up to disease resistance, and as such, requires future study. Age polyethism is also influenced by patriline. Honey bee colonies benefit from multiple subfamilies because the genotypes of workers affect the probabilities of initiating and ending behavior associated with colony division of labor (Calderone and Page 1988; Robinson and Page 1988; Frumhoff and Baker 1988; Page et al. 1989; Strassman 2001; Mattila et al. 2008; Mattila and Seeley 2010). This relationship between patriline number and colony fitness should then scale up to show hives with increased genetic diversity better withstand disease, assuming patriline also affects immune function.

Ultimately, the high variation during a mating flight combined with queen's inability to assess the number of times they mate may result in extreme final mating numbers (Tarpy and Page 2001). Extreme polyandry may not have a direct benefit, but instead very low costs to mating multiply (Tarpy and Page 2001). However, this non-adaptive hypothesis is unlikely given lower disease intensity and greater fitness in colonies with high genetic diversity (Tarpy 2003; Tarpy and Seeley 2006; Seeley and Tarpy 2007). Alternate hypotheses for hyperpolyandry in honey bee queens include sperm limitation (Cole 1983), sperm competition (Parker 1984), and increased caste differentiation (Robinson 1992; Fuchs and Moritz 1998; Oldroyd and Fewell 2007, 2008; Mattila and Seeley 2010). Ultimately, these hypotheses each explore mechanistic explanations for the benefits of a known adaptive behavior, and as such are not necessarily mutually exclusive.

Future tests may include examining induced immune effector molecules, either through studying their post-transcriptional (Manfredini et al. 2010a) or post-translational (Rosengaus et al. 2007) gene products. Constitutive and induced mechanisms of cellular immune function can be studied using advanced methods of differential hemocyte counts, types, and functional tests (Manfredini et al. 2008, 2010b). Additionally, actual disease resistance tests will provide valuable information about the actual biological relevance of each immune measure (Manfredini et al. 2010a).

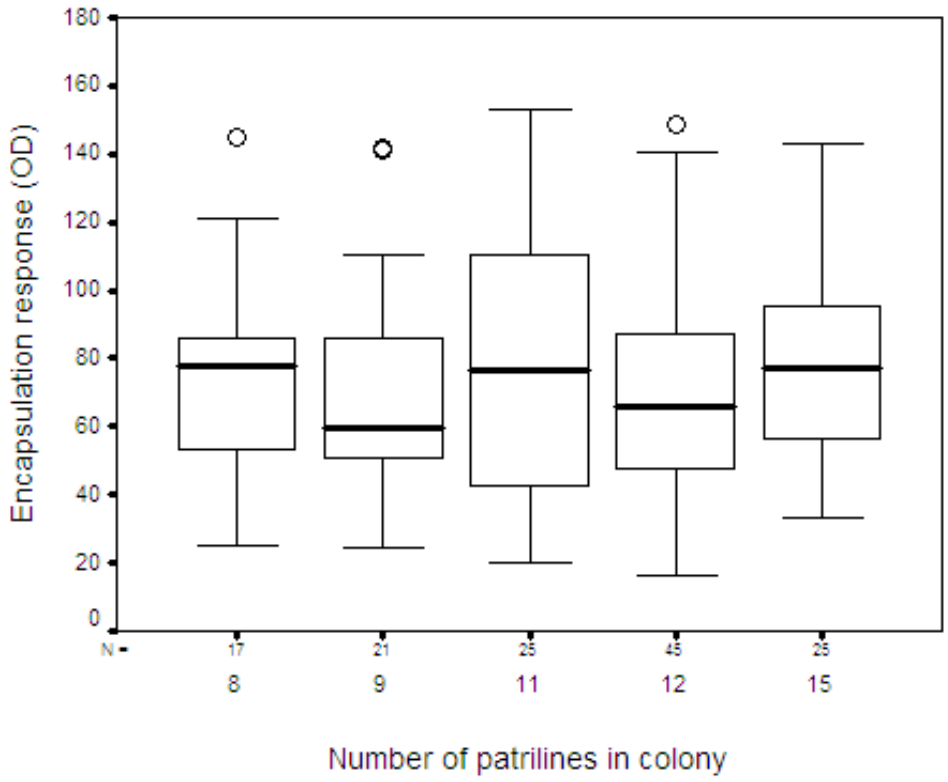

Figure 8. Encapsulation response does not correlate with increasing number of patrilines in honey bees (*A. mellifera*). Boxes represent middle 50% of data, lines in boxes show median values, and whiskers represent 95% of data, beyond which outliers reside. Circles show outliers and asterisks show extremes. Sample sizes are noted along the x-axis as the number of individuals in each colony with representative number of patrilines stated beneath it.

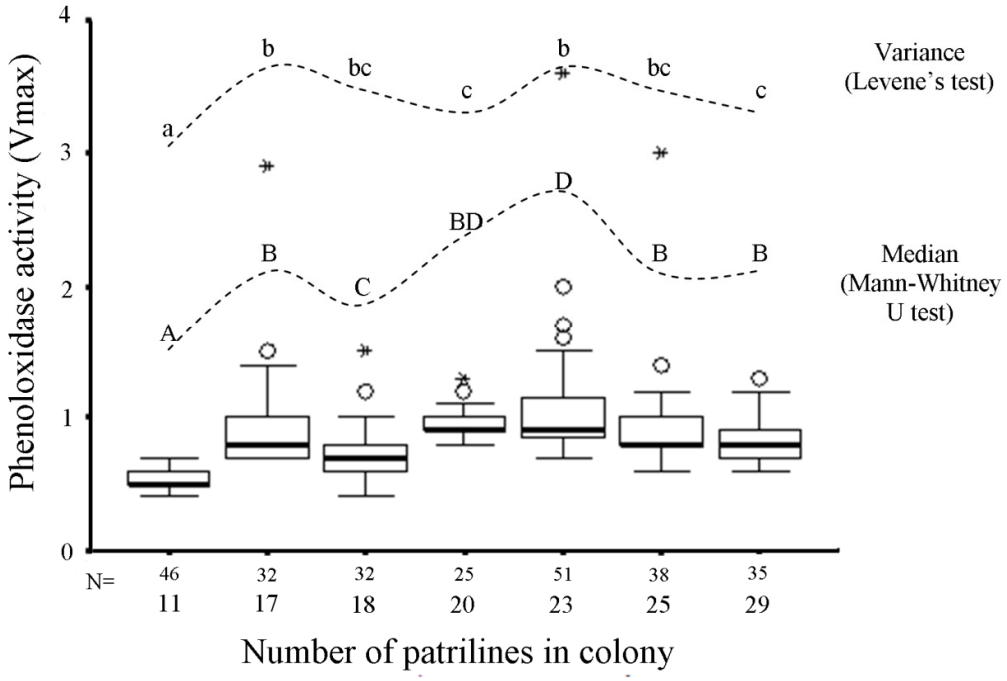

Figure 9. Phenoloxidase activity does not correlate with increasing number of patrilines in honey bees (*A. mellifera*). Different letters indicate different medians and variances identified through pair-wise comparisons. Lines hovering above boxes suggest trends across data sets for medians and variations around the median. Boxes represent middle 50% of data, lines in boxes show median values, and whiskers represent 95% of data, beyond which outliers reside. Circles show outliers and asterisks show extremes. Sample sizes are noted along the x-axis as the number of individuals in each colony with number of patrilines stated beneath it.

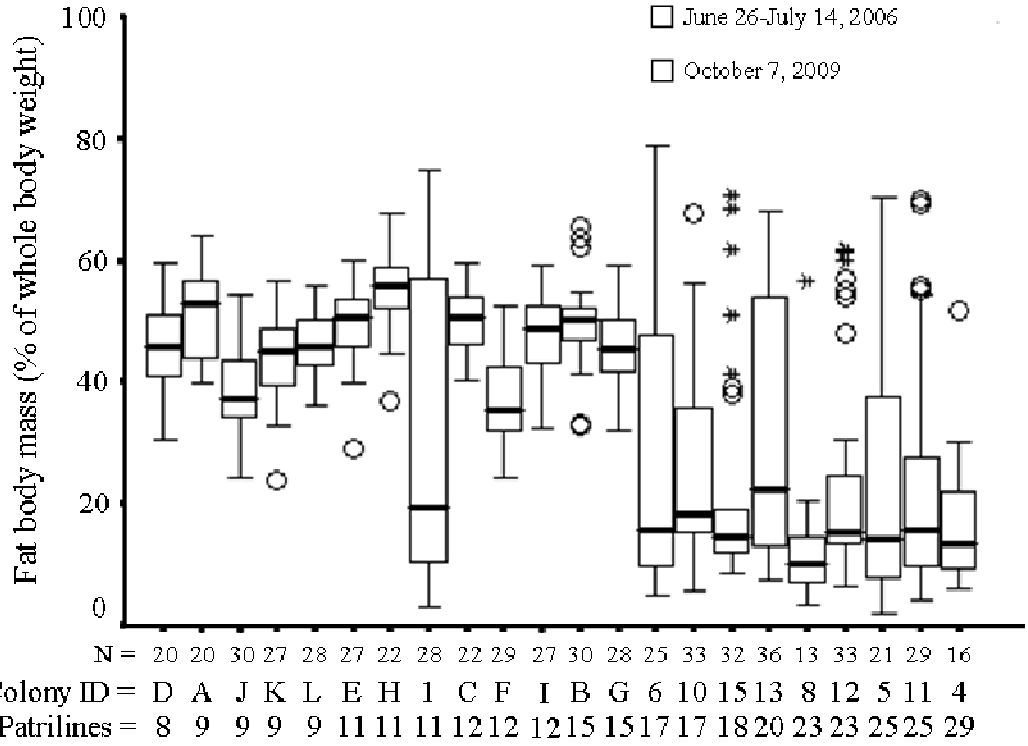

Figure 10. Fat body mass does not correlate with increasing number of patrilines in honey bees (*A. mellifera*). Boxes represent middle 50% of data, lines in boxes show median values, and whiskers represent 95% of data, beyond which outliers reside. Circles show outliers and asterisks show extremes.

CHAPTER 5

IN METCHNIKOFF'S HONOR: AN IMPROVED METHOD FOR TESTING INVERTEBRATE IMMUNE FUNCTION

CHAPTER 5.
In Metchnikoff's honor:
An improved method for testing invertebrate immune function

Noah Wilson-Rich, Erica Taylor, Lillian Lwanga, and Philip T. Starks

Department of Biology, Dana Laboratories, Tufts University, Medford, MA 02155, USA

ABSTRACT

Since Metchnikoff's first observations of the invertebrate encapsulation immune response (1882 in the starfish *Astropecten*, 1884 in *Daphnia*), researchers have developed empirical tools to quantify immune function in animals with primitive immune systems. Interestingly, all previous tests were done in a sterile vacuum, as if pathogen specificity did not matter. Here we present data on a biologically realistic technique to test how a host will respond to specific classes of pathogens and parasites. To mimic natural hemocoelic invaders in honey bees, we coated nylon monofilaments with pathogen-associated membrane pattern (PAMP) molecules. These implants ("PAMPlants") induced stronger responses than the sterile, non-coated implants previously used. The strength of our results indicates that PAMPlants are a significant improvement on the previous naïve methods. In addition, given the honey bees' variable responses to PAMPlants, our data raise the possibility that they react differently to different classes of pathogens.

INTRODUCTION

In 1908, Élie Metchnikoff won the Nobel Prize in medicine for his discoveries about immune function in invertebrates (Metchnikoff 1884; as translated and reprinted in Magasanik et al. 1984; Beck and Habicht 1996; Da Silva 2002 and references therein). While vacationing with his family on the Sicilian coast in 1882, Metchnikoff impregnated a starfish larva with the thorn of a rose, thus inventing the first method of studying the invertebrate encapsulation response (ER) (Beck and Habicht 1996). Upon recognition of a foreign body, the host immune system responds first with granular hemocytes, which release chemotactic factors into the hemolymph near the wound. Plasmatocytes migrate toward the foreign body, and either phagocytose it or aggregate around it. The latter process involves a morphological alteration by flattening into lamellocytes – scale-like cells that surround, isolate, and neutralize foreign bodies too large to be engulfed by a single cell.

Nearly a century later, Metchnikoff's methods for measuring invertebrate immune function were resurrected and used to answer questions relating to ecological immunity – the study of abiotic and biotic factors influencing disease resistance traits. This renaissance was facilitated because the immune system of invertebrates is simpler than that of vertebrates (see Beck and Habicht 1996). Recent empirical ER studies (summarized in Table 3) using the Metchnikoffian methods of König and Schmid-Hempel (1995) have elucidated relationships between immune function and many ecological and behavioral factors, including, but not limited to, sex, behavioral role, dominance, female mate choice, foraging ability, energetic cost, diet, habitat type, infection risk, co-infection, individual and colony condition, ontogeny, and invasion biology (König and Schmid-Hempel 1995; Siva-Jothy 2000; Wilson-Rich et al. 2008; see Table 3 in appendix).

The immune response is multi-faceted and insects have many weapons other than ER for thwarting disease. The ER mode of immunity, however, is particularly important and is associated with disease resistance to virally infected cells (Washburn et al. 1996; Trudeau et al. 2001), parasitoids (Carton and David 1983; Kraaijeveld et al. 2001a) and parasites (Doums and Schmid-Hempel 2000). Indeed, the phenoloxidase cascade is similarly important, as it plays a complementary role in the ER process by contributing to resistance to viruses (Wilson et al. 2001; Beck and Strand 2007), bacteria (Pye 1974; Ashida and Brey 1997), fungi (Ochiai and Ashida 1988), parasites (Leonard et al. 1985; Paskewitz and Riehle 1994; Gorman et al. 1996; Siva-Jothy 2000), and parasitoids (Wilson et al. 2001).

In this paper, we report results from a novel method for testing immune function using foreign bodies coated with pathogen-associated molecular patterns (PAMPs). PAMPs are highly conserved structural components of the microbial cell wall, including β-glucans (B13G) on fungi, lipopolysaccharides (LPS) on gram-negative bacteria, and peptidoglycans (PGN) on gram-positive bacteria (reviewed in Nürnberger et al. 2004; Zipfel et al. 2005). Selection should favor host immune systems that use PAMPs as recognition molecules (see Postel and Kemmerling 2009). Yet, all prior ER studies used implants (monofilaments or beads) that were sterile and uncoated. Our method allows for finer investigation of the ability of a host to identify and respond to a specific class of pathogen. We used honey bees (*Apis mellifera*) because of their well-documented immune mechanisms, including ER (Wilson-Rich et al. 2008; reviewed in Wilson-Rich et al. 2009). Previous reports may hold little specific biological relevance because of the absence of recognition molecules, whereas PAMPlants mimic natural biological systems. Indeed, our results indicate that previous data significantly underestimated the honey bee's immune strength.

METHODS

To quantify the ER of honey bees to PAMPs, we modified a standard encapsulation response implant technique (König and Schmid-Hempel 1995). Sterile cuts of nylon monofilament (2mm long, 0.004mm diameter, Scientific Anglers Tippet, 3M) were dipped through one of five different solutions with varying concentrations of PAMP solution or control solution. PAMPs were selected based on their being the predominant molecules found on the cell wall of their representative microbes. Phosphate-buffered saline (control, PBS), lipopolysaccharide in PBS (predominant on gram-negative bacterial cell wall; LPS; 0.1mg/ml, 1mg/ml, 10mg/ml), peptidoglycan in PBS (predominant on gram-positive bacterial cell wall; PGN; 1mg/ml, 10mg/ml, 100mg/ml), and β-1,3-glucans in PBS (predominant on fungal cell wall; B13G; 1mg/ml, 10mg/ml, 100mg/ml). As such, there was a proportional fold increased in PAMP concentrations across treatments. Bovine serum albumin in PBS (control, BSA, 1mg/ml) was added in 2009 for the double implant trials as a second negative control, as honey bees likely did not evolve with exposure to this cow protein. All implants were allowed to dry overnight in sterile Petri dishes.

Coated implants served as effective pseudo-parasites – non-self haemoceolic invaders with molecular cues on their outermost layer. The *Varroa* mite is a common parasite of honey bees that implants itself through the host cuticle (see Spivak 1996). Our implants mimic this natural process, while focusing on the immune response by the honey bee host to the bacterial and fungal pathogens commonly transmitted by *Varroa* (reviewed in Sammataro et al. 2000). As such, each PAMP coat was predicted to elicit a higher ER relative to sterile controls due to the evolved relationship between pathogens and host immune system. PBS and BSA monofilaments were negative controls, and were expected to induce the lowest relative ER.

We performed a series of standard encapsulation response assays (König and Schmid-Hempel 1995; Wilson-Rich et al. 2008). First, bees were ice-anesthetized. Next, nylon monofilaments were implanted between the third and fourth ventral intersegmental membrane (N=53 singly implanted, and N=71 doubly-implanted, individuals), each of which remained in situ for four hours. Honey bees in 2008 received a single implant, while those in 2009 received double implants. These duly-implanted wasps each received one PAMPlant, and one control implant dipped in saline only. This design provided a self-controlled comparison. BSA treatments were included in the double-implant experiment in 2009 only.

Implants were removed with fine forceps after re-anesthetizing honey bees on ice, and then analyzed for darkness, measured by the mean gray value of each image. Images were taken using a fluorescence-detecting Olympus VX40 and image capturing and analysis software (Optronics Magna Fire-SP, v1.0_5, and ImageJ [NIH], respectively). The fluorescence allowed us to detect the melanin due to its auto-fluorescent properties, and omit all non-melaized matter that was superfluous to the encapsulation process. Darker implants indicated a stronger ER, and thus greater immune function according to this measure.

Mean ER values from singly implanted samples were compared using ANOVA followed by post-hoc Tukey HSD pairwise comparisons when appropriate. We also employed an ANOVA followed by post-hoc Bonferroni tests to test for a colony effect, as bees were collected from three different colonies. Doubly implanted mean values within PAMP treatments but between coated and uncoated were compared using paired t-tests within individual honey bee. All statistics were calculated using SPSS for Windows v. 11.0.

RESULTS

Overall, the PAMPlants induced higher ERs than the control implants. There were no significant effects of colony of origin or PAMP concentration on ER. As such, data from all samples were combined within the singly- and paired-implant groups. In 2008, for honey bees with a single implant, ER was significantly higher than control in response to each of the three PAMPs ($F = 8.707$, df = 3, 47, $P < 0.001$). There were no significant differences in encapsulation response across the three PAMP treatments (Figure 11).

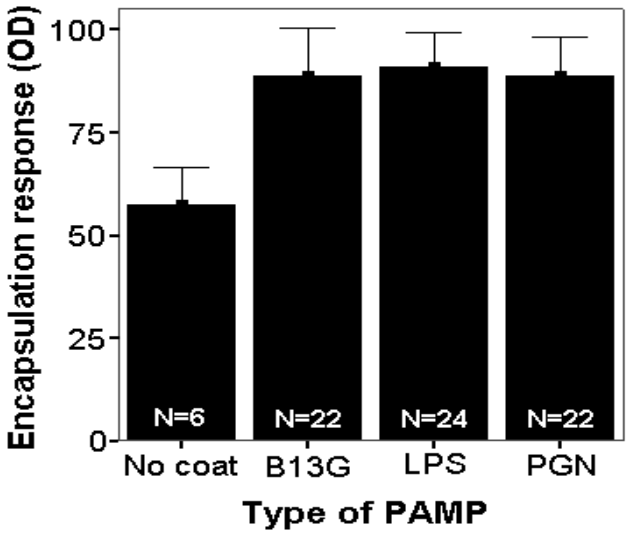

Figure 11. Single implants. Worker honey bees were implanted with one coated or uncoated monofilament. The optical density (OD) of each implant was quantified using fluorescence microscopy and imageJ software to measure the encapsulation response around the implants. Asterisk indicate $p<0.05$ using Tukey HSD pairwise comparisons, showing all coated implants ("PAMPlants") nearly doubled the immune response compared to uncoated implant. B13G = β-1, 3-glucan (fungal PAMP), LPS = lipopolysaccharide (gram-negative bacteria PAMP), PGN = peptidoglycan (gram-positive bacteria PAMP).

In 2009, bees receive two implants, and the BSA treatment was added. The same pattern emerged seen in the singly-implanted bees in 2008, showing upregulation

to PAMP-coated implants across coat types (Figure 12; $F = 11.582$; df = 4, 52; $P < 0.001$). Two of the three PAMPlants (B13G and LPS) showed significantly higher ERs compared to paired controls ($p<0.05$ for both), while PGN showed no significant differences. Likewise, BSA, the secondary control, showed no overall difference from the paired uncoated implant. There was no effect of side (left versus right) on ER.

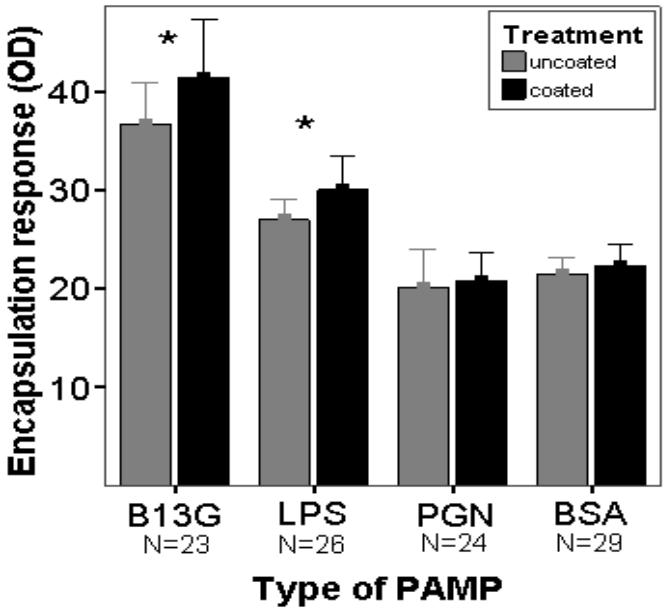

Figure 12. Double implants. Worker honey bees were implanted with two nylon monofilaments. One monofilament was uncoated while the other was coated in one of four types of molecules: B-1, 3-glucan (B13G), lipopolysaccharide (LPS), peptidoglycan (PGN), or bovine serum albumin (BSA). The optical density (OD) of implants was quantified using fluorescence microscopy and imageJ software to measure the encapsulation response around the implants. Asterisks indicate $p<0.05$ using paired t-tests. Sample sizes (N) indicate the number of bees doubly implanted.

DISCUSSION

In using a simple technique, Metchnikoff discovered the process of phagocytosis in invertebrates. His discovery laid the foundation for modern advancements in assay methods. Here we report results from a novel, direct method of testing immune function

in invertebrates. Honey bees encapsulated coated foreign bodies more strongly than those not coated with molecules found on pathogens (i.e., PAMPs). These PAMPs are conserved molecules found on and in the cell wall of bacteria and fungi (see Postel and Kemmerling 2009). This finding supports the idea that selection should favor hosts that first recognize PAMPs as foreign cues, and then induce an appropriate immune response to clear them.

The singly implanted honey bees showed a greater immune response to PAMPlants than control treatment (uncoated). The doubly implanted honey bees showed a stronger immune response to B-1, 3-glucans and lipopolysaccarides PAMPs compared to paired, doubly implanted, uncoated controls. Interestingly, the immune response of honey bees to single implants was nearly twice as much of double implanted honey bees. One possible explanation for this finding is that the immune response was divided between the two foreign bodies, albeit unevenly. Implants coated with the non-naturally associated PGN and BSA were slightly more encapsulated than their uncoated pairs, although not significantly so.

As opposed to the single implant trials, the data in our paired implant trials were not uniform. Honey bees seem to show a stronger response to fungi over gram-negative bacteria and even more so over gram-positive bacteria. Data presented here indicate that all previous results on honey bee ER were muted relative to the natural response (e.g., see Wilson-Rich et al. 2008), and are an important step in the exploration of the real-world effects of different classes of microbes on host immunity. The improved PAMPlant technique we report enables researchers to explore the mechanism associated with defense against specific classes of microbes on host disease resistance by more realisticly designing the implants.

Understanding host-pathogen co-evolutionary dynamics is a grand challenge in modern science. PAMPlants provide researchers with a tool to test hypotheses relating to disease resistance. Areas of exploration include dynamics during co-infection by different types of pathogens, energetics of immunity, fitness costs of different pathogens, sexual selection handicaps, pathogen evasion of host immunity, and exciting trade-offs between defense, growth, and reproduction. Coated implants are a novel and biologically-relevant approach to investigating all of these topics and more. This improved method allows us to open up new doors for future research, one century after Metchnikoff's first discovery.

It is quite possible that our view of invertebrate immunity is oversimplified (see Wilson-Rich et al. 2010's comments on Rolff and Reynolds 1999). Progress in this field will be limited unless biologically relevant techniques are adopted. Ours is one such technique. Each study in Table 3 documents a missed opportunity to understand the degree to which immune function interacts with sex, behavior, dominance, mate choice, foraging ability, energetics, diet, habitat type, infection risk, co-infection, individual and colony condition, ontogeny, and invasion biology.

CHAPTER 6

THE ONTOGENY OF IMMUNITY:

DEVELOPMENT OF INNATE IMMUNE STRENGTH

IN THE HONEY BEE (*Apis mellifera*)

CHAPTER 6.
The ontogeny of immunity:
Development of innate immune strength in the honey bee (*Apis mellifera*)

Noah Wilson-Rich, Stephanie T. Dres, and Philip T. Starks

Department of Biology, Dana Laboratories, Tufts University, Medford, MA 02155, USA

Journal of Insect Physiology 54:1392-1399. doi:10.1016/j.jinsphys.2008.07.016

ABSTRACT

Honey bees (*Apis mellifera*) are of vital economic and ecological importance. These eusocial animals display temporal polyethism, which is an age-driven division of labor. Younger adult bees remain in the hive and tend to developing brood, while older adult bees forage for pollen and nectar to feed the colony. As honey bees mature, the types of pathogens they experience also change. As such, pathogen pressure may affect bees differently throughout their lifespan. We provide the first direct tests of honey bee innate immune strength across developmental stages. We investigated immune strength across four developmental stages: larvae, pupae, nurses (1-day old adults), and foragers (22-30 day old adults). The immune strength of honey bees was quantified using standard immunocompetence assays: total hemocyte count, encapsulation response, fat body quantification, and phenoloxidase activity. Larvae and pupae had the highest total hemocyte counts, while there was no difference in encapsulation response between developmental stages. Nurses had more fat body mass than foragers, while phenoloxidase activity increased directly with honey bee development. Immune strength was most vigorous in older, foraging bees and weakest in young bees. Importantly, we found that adult honey bees do not abandon cellular IC as has recently been proposed. Induced shifts in behavioral roles may increase a colony's susceptibility to disease if nurses begin foraging activity prematurely.

INTRODUCTION

Social insects have evolved adaptive mechanisms to decrease rates of disease transmission, including mutual grooming and removal of dead nest mates (Traniello et al. 2002). However, hygienic behaviors may also increase the rate of pathogen exposure between nestmates and actually facilitate disease outbreak (Fefferman et al. 2007). Eusocial animals have a high potential risk of spreading infection among individuals from the same colony because they live in highly integrated groups with an overlap of generations. Additionally, the high level of cohesion in eusocial animals may increase the risk of disease outbreak as a result of close living quarters, high genetic relatedness between individuals, and continuous physical interactions between individuals within and across generations (Schmid-Hempel 1998; Whiteman and Parker 2004; Godfrey et al. 2006). In response, eusocial insects have evolved novel behavioral, physiological, and organizational adaptations to combat the increased risk of disease (Wilson 1971; Wilson 1975; Rosengaus et al. 1999; Starks et al. 2000; Traniello et al. 2002; Hughes & Boomsma 2004; Wilson-Rich et al. 2007; Cremer et al. 2007; Fefferman et al. 2007; Aubert and Richard 2008; Wilson-Rich et al. 2009).

Honey bees (*Apis mellifera*) are highly successful eusocial insects with nearly cosmopolitan distribution (Sheppard and Meixner 2003). Honey bees defend themselves from an especially diverse range of pathogens, including bacteria, fungi, viruses, protozoa, mites, flies, beetles, and nematodes (Bailey and Ball 1991; Schmid-Hempel 1998; Evans and Pettis 2005). Behavioral differences between honey bee brood and adults are likely to play an important role in disease susceptibility to different pathogens. Disease resistance capacity can be empirically tested and quantified using measures of immunocompetence (IC). We define IC as the ability of an organism to mount an immune response, either in cellular, humoral, or behavioral form (see König and Schmid-

Hempel 1995; Siva-Jothy 1995; Schmid-Hempel and Ebert 2003; Adamo 2004a; Rantala and Roff 2005; Wilson-Rich et al. 2009).

Variation in pathogen-specific selective pressure may result in IC dissimilarities across developmental stages. We hypothesize that selection has maximized disease resistance abilities at each developmental stage. Given that larvae and pupae are confined within a comb cell, brood are limited in their ability to move away from approaching parasites or to otherwise avoid pathogens through behavioral mechanisms. As such, brood likely rely on cellular and humoral mechanisms of defense (Evans et al. 2006). Alternatively, adult honey bees display a range of hygienic and antipathogenic behaviors including grooming and removal of infected nestmates (Spivak 1996). Adult behavior is influenced by age, through an ontogenetic process termed temporal polyethism (Winston 1987; Starks et al. 2005). During this progression, young adults ("nurse bees") feed larvae until they develop into pupae, which are capped with wax and isolated until eclosion as nurse bees. Nurses typically remain in the hive and perform hygienic activities and tend to the brood, while older adult bees (i.e., foragers) leave the hive to collect pollen and nectar (reviewed by Winston 1987).

The majority of disease phenotypes are expressed either in brood or in adults, but seldom in both (Bailey 1968a), although many pathological microorganisms may commonly be present at tolerable levels in the hive (Bailey 1968b). Honey bee brood (larvae and pupae) are infected by different pathogens than are adults. Brood are susceptible to bacterial disease (e.g., American and European foulbrood, caused by *Paenibacillus larvae* and *Streptococcus pluton*, respectively; Govan et al. 1998, 1999), fungal disease (e.g., chalk brood, caused by *Ascophaera apis*; Gilliam et al. 1983; Johnson et al. 2005), and viral disease (e.g., sacbrood, caused by the SBV virus; Ghosh et al. 1999). Adults are affected by protozoan disease (e.g., nosema, caused by *Nosema apis*; Gatehouse & Malone 1998), hemophilic mite parasitism (e.g., the tracheal mite,

Acarapis woodi and the Varroa mite, *Varroa destructor (formerly jacobsoni)*; Sammataro et al. 2000; Evans et al. 2007), and viral disease (e.g., acute and chronic bee-paralysis viruses and Israeli acute paralysis virus; Bakonyi et al. 2002, Ribière et al. 2002; Cox-Foster et al. 2007). Of note, at least one adult parasite, the Varroa mite, also affects brood. Pathogens are likely present at multiple developmental stages, yet the disease phenotype is most clearly observed at only one.

Here, we detail our findings from a study of honey bee cellular and humoral IC over four developmental stages: larvae, pupae, nurses (1 day old bees), and foragers (22-30 day old adults). To examine if honey bee IC varies with developmental stage, we assayed physiological IC across honey bee developmental stages: total hemocyte concentration, encapsulation response, fat body mass, and phenoloxidase activity. Due to differences in pathogen pressure and behavioral capacity, we predicted that larvae and pupae would have higher physiological IC than nurses and foragers. Because disease resistance is difficult to measure (Luster et al. 1993; Keil et al. 2001; Adamo 2004a; Rantala and Roff 2005), we took multiple measures of immune strength to achieve a broad spectrum analysis of honey bee IC.

METHODS

Specimen collection: One frame of brood and forty foraging adult honey bees (*A. mellifera*) were collected from 10 colonies among three field sites in Massachusetts (N=1200 bees total collected, though not all were used due to challenges specific to each particular assay; see below for details). All source colonies were briefly inspected for symptoms of bacterial, fungal, or viral disease; only healthy bees were collected. Specimens were collected from four colonies of Italian bees at the International Social Insect Research Facility (ISIRF) at the Tufts University Cummings School of Veterinary Medicine in North Grafton, Massachusetts, four colonies of Carnolian bees at the Bee-

Cause Apiary in Dunsboro, Massachusetts, and two colonies of Italian-Carnolian mixed breed at the Bee-Cause Apiary in Tyngsboro, Massachusetts. All specimens were transported to Tufts University in Medford, Massachusetts. At the laboratory, larvae and pupae were excised from the brood frame. Each brood frame was then immediately isolated and incubated for 24 hours at 32-33˚C. One-day old nurse bees were collected after the incubation period (for similar methods see Starks et al. 2005). Because stress may influence some measures of immunocompetence (see Braude et al. 1999; Adamo and Parsons 2006), animals were exposed to similar conditions within each developmental stage so as to minimize differences in stressful handling within groups.

Hemolymph collection: Hemolymph was collected immediately from larvae and pupae by puncturing the soft cuticle with fine forceps sterilized in 95% ethanol. Nurses and foragers were ice anesthetized before hemolymph was collected by severing the abdomen and collecting samples from the proximal abdominal opening. Five microliters of hemolymph was collected from the resulting bubble of hemolymph, transferred to a 96-well plate with individual cells containing 95 microliters PBS (Sigma, pH 7.4), and frozen at -20˚C to disrupt hemocytes for later analyses of humoral immunity (Gilliam and Shimanuki 1970; Wilson et al. 2001; Chan et al. 2006). One additional microliter of hemolymph was collected with a micropipette and transferred to a microcentrifuge tube (0.5ml, BD Falcon) containing nine microliters of sterile deionized water and used for total hemocyte count. Any fluid which appeared yellow or brown was avoided as this was likely not hemolymph but gastric fluid (Chan et al. 2006).

Total hemocyte count: A total hemocyte count was performed as an indirect measurement of baseline cellular immunocompetence (Wilson et al. 2001, 2002; Lee et al. 2006). Hemocyte counts have been shown to correlate positively with encapsulation response (Rantala et al. 2000; but see Doums et al. 2002), phenoloxidase activity

(Cotter et al. 2004) and parasitoid resistance (Eslin and Prévost 1998; Kraaijeveld et al. 2001a), and also correlate negatively with aging (Amdam et al. 2004, 2005; Schmid et al. 2008). To perform this test, we added the diluted hemolymph solution to an improved Neubauer hemocytometer (Fisher Scientific), where all hemocytes were counted under a light microscope. Hemocytes counts in sterile, deionized water were reliable and repeatable (personal observation).

Encapsulation response: A standard encapsulation response assay (Konig and Schmid-Hempel 1995; Schmid-Hempel and Schmid-Hempel 1998; Rantala et al. 2000; Vainio et al. 2004; Rantala and Roff 2005; Lee et al. 2006; Kapari et al. 2006; Haviola et al. 2007) was used as a direct measurement of an insect's ability to neutralize a foreign body that cannot be ingested by phagocytosis. On the one hand, the ability to encapsulate a novel foreign body correlates positively with resistance to virally-infected cells (Washburn et al. 1996; Trudeau et al. 2001), parasitoids (Carton and David 1983; Kraaijeveld et al. 2001b) and parasites (Doums and Schmid-Hempel 2000), as well as with male dominance (Rantala and Kortet 2004) and female mate choice (Rantala et al. 2002; Rantala and Kortet 2003; but see Rantala et al. 2003). On the other hand, encapsulation correlates negatively with the total number of hemocytes (Doums et al. 2002), suggesting the encapsulation process requires hemocytes be removed from circulation. Cellular encapsulation may occur when the cuticle is punctured by a foreign body, as first noted in Metchnikoff's Nobel Prize winning (1908) experiments, and may be induced when a parasite invades the host hemocoel (Wilson et al. 2002). We induced an encapsulation response in honey bees by mimicking the behavior of the common *Varroa* mite (Sammataro et al. 2000) with a nylon 'pseudoparasite' (Cox-Foster & Stehr 1994). Nylon line (0.004mm diameter, Scientific Anglers Tippet, 3M) was cut by hand with a razor blade into approximately 2mm long segments and sterilized in 95% ethanol.

Cuts were implanted individually inside larvae and pupae medially on the ventral side so that approximately 1mm of the nylon cut remained outside the body wall. Nurses and foragers were first ice anesthetized and then immobilized using a 'threading technique', whereby curved forceps press down on all six legs to expose the ventral side. Each specimen was implanted with a nylon cut through the medial ventral intersegmental membrane between the 3rd and 4th sternites (Allander & Schmid-Hempel 2000).

After implantation, adult bees (i.e., nurses and foragers) were moved to a 1.5 milliliter microcentrifuge tube with holes poked through cap to isolate bees from grooming activity so that the implant remains in place, while maintaining access to air (Wilson-Rich and Starks 2010). Specimens were left at room temperature for four hours, after which a glass slide was created of thread in glycerol medium (see Kapari et al. 2006; Calleri et al. 2006, 2007). Explanted threads were photographed at 400x magnification using an Olympus VX40 fluorescence detecting microscope and image capturing software (Optronics Magna Fire-SP, v1.0 x5). Three pictures were taken of each explant to accurately quantify a 3-dimensional process using 2-dimensional tools (Rantala et al. 2000; Rantala and Kortet 2003; Contreras-Garduño et al. 2006; Kapari et al. 2006; Haviola et al. 2007). Each image was captured through a multi-wavelength filter (emittance range approximately 400-600nm) to detect melanin, an autofluorescent protein produced within encapsulating cells (Calleri et al. 2007). The excitation wavelength of melanin has been previously reported at 488nm (Kozikowski et al. 1984; Meredith and Sarna 2006). This allowed for the control of any non-melanized debris which may have accumulated on the monofilament. The mean gray value was calculated for the inserted portion of the thread and all capsules on it using image analysis software (ImageJ 1.34s, National Institutes of Health, USA; Rasband 2007), and compared to an

unimplanted control thread (Allander and Schmid-Hempel 2000; Rantala et al. 2000; Haviola et al. 2007).

Phenoloxidase activity: The phenoloxidase (PO) pathway is a central component to invertebrate immune reactions occurring in the hemolymph (Söderhäll and Cerenius 1998; Lourenço et al. 2005). PO activity assays are a commonly used to quantify immune enzyme activity via melanin production in the absence of cells (Wilson et al. 2001, 2002; Rolff and Siva-Jothy 2002; Cotter et al. 2004; Lee et al. 2006; Mullen and Goldsworthy 2006). Although PO plays an important role in cellular IC (among other roles in cuticular sclerotization and quinone production; Lavine and Strand 2002), PO may work solely as an independent humoral immune protein during melanotic encapsulation (Gillespie et al. 1997). Moreover, PO activity and the ability to encapsulate a novel foreign body are heritable (Cotter and Wilson 2002), and so natural selection should favor those with the most effective immune response.

PO is produced when its zymogen prophenoloxidase (proPO) is activated in response to any of many triggers, including wounding, mechanical agitation, and various chemicals (Ashida and Brey 1997). PO acts by oxidizing tyrosine derivatives to form toxic quinones, which are then polymerized into melanin. Measurements of immunity using PO have provided valuable insights into invertebrate immunology (Nigam et al. 1997). There is a well-documented relationship between PO activity and resistance to viruses (Wilson et al. 2001; Beck and Strand 2007), bacteria (Pye 1974; Ashida and Brey 1997), fungi (Ochiai and Ashida 1988), parasites (Leonard et al. 1985; Paskewitz and Riehle 1994; Gorman et al. 1996; Siva-Jothy 2000), and parasitoids (Wilson et al. 2001). It is important to note that recent evidence from mutant, PO-deficient *Drosophila* have raised important questions about the exact role of PO in defense, as these flies

were able to survive a microbial infection as well as wild-type flies (see Leclerc et al. 2006).

The protein concentration of each diluted hemolymph sample was determined by a standard protein quantification assay (Bradford 1976; Wilson et al. 2001; Lee et al. 2006). To control for variation in hydration state of individuals, hemolymph protein concentration was controlled at 0.02mg/ml and added in varying volumes to the reaction mixture (Parkinson & Weaver 1999). The enzymatic substrate L-dopa, a tyrosine derivative, was added to each solution to reach a final concentration of 0.03M. Absorbance measurements were recorded at 492nm before and immediately after L-dopa was added. Data were recorded using a Bio-Rad Benchmark Plus microplate spectrophotometer and Microplate Manager software (Bio-Rad, version 5.2.1). L-dopa is a chromogenic substrate and appears colorless when dissolved in water; however, in the presence of PO melanin is produced and the solution turns brown.

PO analysis for each bee was repeated three times and a mean Vmax was calculated. PO activity was quantified by recording the change in sample absorbance at 492nm every 30 seconds for 9 minutes. Phenolthiocarbamide was then added to each well to inhibit PO activity and ensure melanin production was a result of PO activity alone (Eshete and LoVerde 1993; Parkinson and Weaver 1999; Wilson et al. 2001; Adamo 2004b; Zettervall et al. 2004; Richards et al. 2005). Absorbance readings then continued for 9 more minutes. Within the spectrophotometer, all temperatures were maintained at a set range between 32-34˚C to mimic brood comb conditions (Winston 1987). PO activity was quantified as the slope of the linear phase of reaction (Rolff and Siva-Jothy 2002).

Fat body quantification: Fat body quantification is an indirect measurement of induced humoral immunocompetence (Ellers 1996; Doums et al. 2002). The fat body is functionally analogous to the vertebrate liver and produces antipathogenic proteins (Faye and Wyatt 1980; de Verno et al. 1984; Ellers 1996; Gillespie et al. 1997; Lavine and Strand 2002; Brown et al. 2003). As such, a relative comparison of fat body size serves as an indirect assessment of induced humoral immune strength and of overall condition. Adult abdomens were severed from thoraces and dried for three days at room temperature. Abdomens were weighed and washed in ethyl ether for 24 hours to dissolve fat. Larvae and pupae were not included in this assay because of their lack of hardened cuticle. Abdomens were then dried for three days and weighed again. The fat body was calculated as the percent change in abdominal weight after the ethyl ether wash (Ellers 1996; Doums et al. 2002).

Morphometric analysis: Measurements were taken of head width (McMullan and Brown 2006), forewing length (Lobo et al. 1989; Diniz-Filho and Malaspina 1995; Mostajeran et al. 2006), femur length and tibia length (Mostajeran et al. 2006) for both the nurses and foragers in order to detect any size differences between colonies or developmental stage. Digital images of body parts were captured using a Nikon SMZ 1500 dissection microscope and the image capturing software Spot (Diagnostic Instruments, v. 4.5.9). Morphometric data were collected using imageJ (Rasband 2007).

Statistical methods: IC measures (total hemocyte count, encapsulation response, total hemolymph protein, phenoloxidase activity, and fat body mass) were not normally distributed and non-parametric statistics were used. Each IC measure was compared between developmental stage (larvae, pupae, nurses, foragers) and races (Italian, Carnolian, Italian/Carnolian mix) using Kruskal-Wallis tests followed by pairwise comparisons with Mann-Whitney U tests. Eta-squared values were also calculated to

determine the proportion of variance in each IC measure that may be explained by developmental stage or race. All statistical tests were run with the computer program SPSS for Windows (v. 11).

RESULTS

Total hemocyte count: The median total hemocyte count for each honey bee developmental stage was significantly different from the other three life stages (Figure 13). In general brood displayed more hemocytes than adults. Pupae had significantly greater cell density than all other developmental cohorts (Mann-Whitney U, $p<0.001$). Larvae had the second highest density of hemocytes (Mann-Whitney U, $p<0.001$). Foragers had the second lowest density of hemocytes (Mann-Whitney U, foragers versus nurses: $p<0.05$). Hemocyte counts were not normally distributed (Shapiro-Wilk test: $p<0.001$). Of note, there was a large amount of intercolonial variation with regards to total hemocyte counts (data not shown). No colony or race effect was found.

Encapsulation response: Each developmental stage displayed a very similar ability to encapsulate a foreign body. There were no obvious trends in any of the colonies sampled (N=10 colonies, 324 individuals). When the data were combined across colonies, there was strikingly little difference in encapsulation ability between developmental stages (Figure 14; Kruskal-Wallis, df=3, $p>0.05$). The encapsulation response of individuals within each developmental stage was not normally distributed (Shapiro-Wilk test, $p<0.05$). Although there was a large amount of intercolonial variation with regards to the ability to encapsulate a foreign body, variation in encapsulation response was not explained by developmental stage (eta-squared = 0.013) or by race (eta-squared = 0.003).

Phenoloxidase activity: Hemolymph protein concentration was quantified as a preliminary step of the PO assay to control for differences in hydration state between specimens. A general decline in protein concentration was noted from juvenile to adult (Figure 15a; Kruskal-Wallis, df=3, $p<0.001$; Mann-Whitney U, $p<0.001$ between all developmental stages). Nurses and foragers were the only cohort pair that showed no significant difference in hemolymph protein concentration. The concentration of total protein in the hemolymph of individuals within each developmental stage was not normally distributed (Shapiro-Wilk test, $p<0.001$).

While juvenile bees had greater protein density, foragers has the highest PO activity. PO activity followed the opposite trend from the protein concentration, whereas PO activity increased with ontogeny (Figure 15b; Kruskal-Wallis, df=3, $p<0.001$). A common trend was seen across 90% of colonies sampled (N= 9 out of 10 colonies, 214 individuals total). One colony displayed slightly decreased PO activity in foragers compared to nurses, though both cohorts remained higher than juvenile bees within that colony. The median maximum reaction velocity (Vmax) of PO was greatest in foragers (compared to larvae and pupae, 9.7x greater activity, Mann-Whitney U, $p<0.001$; compared to nurses, 3.2x greater activity, $p<0.001$). Nurses had the second highest PO activity (compared to larvae and pupae, 3.0x greater activity, $p<0.001$). There was no significant difference between larvae and pupae with regards to PO activity. The PO activity of individuals within each developmental stage was not normally distributed (Shapiro-Wilk test, $p<0.01$). About a third of the variation seen in PO activity could be explained by developmental stage (eta-squared = 0.297), though race made a minimal contribution to variation (eta-squared = 0.016).

Fat body quantification: The difference between nurses and foragers differed across colonies, with all but one colony showing nurses with more fat mass than

foragers (Figure 16; Mann-Whitney U: $p<0.001$). The fat body mass of individuals within each developmental stage were not normally distributed (Shapiro-Wilk test, $p<0.001$).

Morphometric analysis: Morphometric measurements showed no significant differences between colonies within races with regards to any quantified body parts (head width, femur length, tibia length, and forewing length). As such, the data between colonies of the same race were combined. All morphometric data were normally distributed, so parametric statistics (1-way ANOVAs followed by Tukey's HSD post-hoc tests) were used for analysis. Racial differences were noted with regards to size (head width: $F(2, 266)=3.73$, $p=0.025$; femur length: $F(2, 266)=11.81$, $p<0.001$; tibia length: $F(2, 266)=20.87$, $p<0.001$; forewing length: $F(2, 266)=5.09$, $p=0.007$). Head width of Italians were larger than Carnolians (mean difference=0.029mm, SE=0.012, Tukey's HSD $p=0.038$). Femur length of Italians were larger than both Carnolians (mean difference=0.0745mm, SE=0.0175, Tukey's HSD $p<0.001$) and mixed breeds (mean difference=0.0885mm, SE=0.0224, Tukey's HSD $p<0.001$). Tibia length of Italians were larger than both Carnolians (mean difference=0.0588mm, SE=0.0153, Tukey's HSD $p<0.001$) and mixed breeds (mean difference=0.1243mm, SE=0.0224, Tukey's HSD $p<0.001$). Tibia length also differed between Carnolians and mixed breeds, with Carnolians being larger (mean difference=0.0655mm, SE=0.0190, $p=0.002$). Forewing length of Italians were larger than Carnolians (mean difference=0.1225mm, SE=0.0405, $p=0.008$). However, there was no correlation between race or size and encapsulation response or PO activity (Pearson Correlation: race and encapsulation response, $r=0.018$, $df=324$, $p=0.74$; race and PO activity, $r=-0.093$, $df=212$, $p=0.175$; head width and encapsulation response, $r=0.071$, $df=176$, $p=0.348$; head width and PO activity, $r=0.055$, $df=89$, $p=0.611$). Any difference was controlled for (e.g., concentration of

protein used for PO assay) or irrelevant (e.g., size differences did not correlate with IC measures).

DISCUSSION

In this paper we report how the immune system of honey bees varies between four distinct life stages: larvae, pupae, nurse, and forager. We hypothesized that there would be a difference in IC with developmental stage, based on differences in host behavior and pathogen pressure between developmental stages (Winston 1987; Bailey and Ball 1991; Schmid-Hempel 1998). Results from total hemocyte count, fat body quantification, and PO activity (Figures 15a, b) support this hypothesis. However, these data contrast with our expectation that larvae and pupae would have higher cellular (vis-à-vis total hemocyte count and encapsulation response) and humoral IC (vis-à-vis fat body mass and PO activity) than nurses and foragers. Instead, larvae and pupae had low cell counts and PO activity (though hemolymph protein concentration decreased). Encapsulation response did not change with development. Nurse bees had greater fat body mass than foragers, indicating greater ability of antipathogenic peptide production in young adult bees compared to older bees (Brown et al. 2003). Our results are in line with previous studies investigating honey bee immunity. For example, Schmid et al. (2008) reported an increase in PO activity and a decrease in hemocyte count as adult bees develop from nurses to foragers.

The decline in hemocytes has been shown to be age-dependent and not task-dependent (Schmid et al. 2008), so the immunodeficiency of foraging nurses remains independent of hormonal control. However, our results contrast with Schmid and coworkers (2008) as we show the average encapsulation response of honey bees remains stable across developmental stages. The similarity between the ability of each developmental stage to encapsulate a novel foreign object was not significantly

influenced by size, race, or nest of origin. Adult bees were immobilized and isolated from other bees during this procedure, and it may be argued that this procedure stressed the bees and therefore indirectly influenced their immune response; however, stress is expected to weaken immunocompetence (Adamo and Parsons 2006). If stress does influence encapsulation ability, then our results are a conservative estimate of adult bee cellular immunocompetence.

As such, it appears that adult honey bees do not abandon cellular IC as previously suggested (Bedick et al. 2001; Amdam et al. 2004, 2005; Schmid et al. 2008). Although our study and Schmid and coworkers' (2008) study found a significant decrease in total hemocytes with development, neither study limited cell counts to those with immune function. If non-immune hemocytes (prohemocytes) are reduced while hemocytes with immune activity (granulocytes and plasmatocytes) are conserved, then this would support our interpretation that cellular IC remains as a viable mode of immunity. This hypothesis may be tested by counting each type of hemocyte (see Manfredini et al. 2008).

The non-cellular component of the honey bee immune system also changes with development. However, a fairly uniform humoral response might be predicted given evidence from the honey bee genome. Honey bees possess fewer immune sequences than found in other insect genomes, including the fruit fly, *Drosophila melanogaster* and the mosquito, *Anopheles gambii* (Evans et al. 2006). The noted small number of immune alleles impacts every step of the immune response, from pathogen recognition to the production of immune proteins (The Honeybee Genome Sequencing Consortium 2006). This finding implies a reduced flexibility in the abilities of honey bees to recognize and resist pathogens (The Honeybee Genome Sequencing Consortium 2006), yet does not explain the high intercolonial variability seen in our encapsulation response data. Likewise, honey bees possess only one proPO (PO precursor) gene compared to three

Drosophila melanogaster proPO genes and nine *Anopheles gambiae* proPO genes, which may contribute to the consistent direct relationship between ontogeny and PO activity between colonies (Evans et al. 2006). The proPO gene is expressed more strongly in adults and older pupae than in younger pupae and larvae (Lourenço et al. 2005). This is also in line with our findings of PO activity, which were taken from diluted hemolymph samples and so actual PO activity may be higher than noted in our results. Moreover, Chan and coworkers (2006) found that the actual proPO zymogen was 50-fold more prevalent in the hemolymph of adult honey bee workers compared to larvae, which also agrees with our results. Additional proteomic study of honey bee hemolymph showed the antibacterial peptide hymenoptaecin is expressed in lower baseline amounts in larvae versus adults (Chan et al. 2006), a trend reflected in the PO data reported here.

Although the encapsulation response is maintained across developmental stages, there was a noticeable amount of intercolonial variation. Larvae and pupae had high encapsulation in some colonies, but very low in others. Explanations for this variation could not be found in morphometric or race analyses. This assay was the clearly the most variable of all IC tests performed. We feel encapsulation response would not be adequate by itself to sufficiently document the ontogeny of honey bee immunity. This finding is potentially alarming considering the high number of papers that draw conclusions from this measurement of IC alone (including but not limited to König & Schmid-Hempel 1995; Schmid-Hempel and Schmid-Hempel 1998; Allander and Schmid-Hempel 2000; Zuk et al. 2004; Civantos et al. 2005; Kapari et al. 2006; Haviola et al. 2007; Sorvari et al. 2007).

Our results have potential application as standard IC parameters to evaluate colony health. While we only collected data from three field sites, additional data from multiple populations of honey bee colonies will enable a larger geographic mosaic

overview (Thompson 1994). Our results show that older adult bees have the greatest PO activity per unit of hemolymph protein. As such, if foraging bees are lost the disease resistance capacity of the colony is reduced. Because nurse bees are not as immunologically competent as foraging bees (Figure 15), they are not as well equipped to combat the increased pathogen exposure that older foragers encounter. In the absence of foragers, younger nurse bees prematurely transition to precocious foragers (Huang & Robinson 1996). This behavioral shift is likely to negatively impact colony fitness, regardless of the pathogen pressure. Examples of when foragers may be lost include the recent onset of Colony Collapse Disorder (Oldroyd 2007) or through present U.S. apicultural practices involving the transport of honey bee colonies for seasonal pollination, though this has not yet been empirically shown. In this light, foraging bees may play a similar role as vaccinated individuals in a population by providing a type of herd immunity, and in their absence the disease resistance capacity of the group is likely compromised.

In all, these results elucidate the patterns of cellular and molecular organization in the eusocial superorganism, *A. mellifera* (Page & Erber 2002; Amdam & Seehuus 2006). Further investigation should explore the relationship between genetic diversity, behavior, IC, and intercolonial variation. High genetic diversity has been shown to decrease variation in disease resistance across honey bee colonies (Tarpy 2003). Because disease susceptibility should increase with the number of mates (Schmid-Hempel 1998), there is a conflict between queen mating behavior (i.e., polyandry) and colony-level infection. However, the benefit of increased genetic diversity at disease resistance loci may provide an even greater benefit to the colony (Schmid-Hempel 1998; Tarpy and Seeley 2006; Seeley and Tarpy 2007; Reber et al. 2008), and warrants additional study.

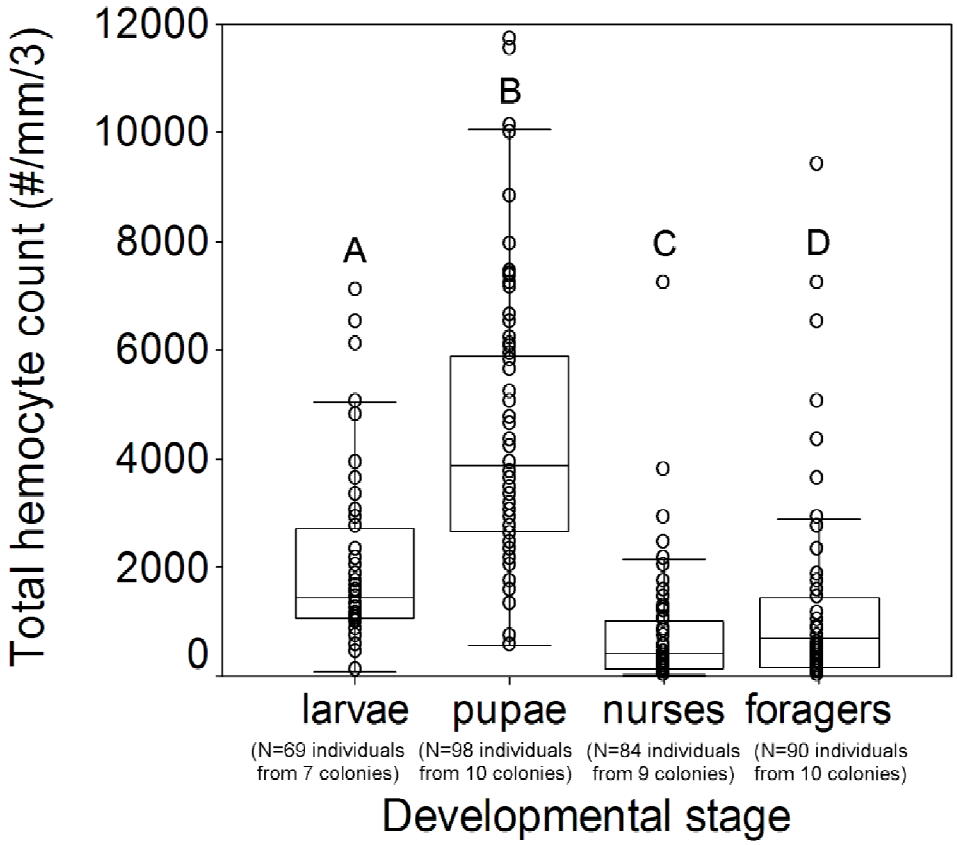

Figure 13. Hemocyte number varies with honey bee (*A. mellifera*) ontogeny. Circles represent individuals. Boxes show 1st and 3rd interquartile range with line denoting medians. Whiskers encompass 95% of the individuals, beyond which outliers reside. Statistics were calculated using a Kruskal-Wallace test with Mann-Whitney U pairwise comparisons. Significant differences indicated with letters.

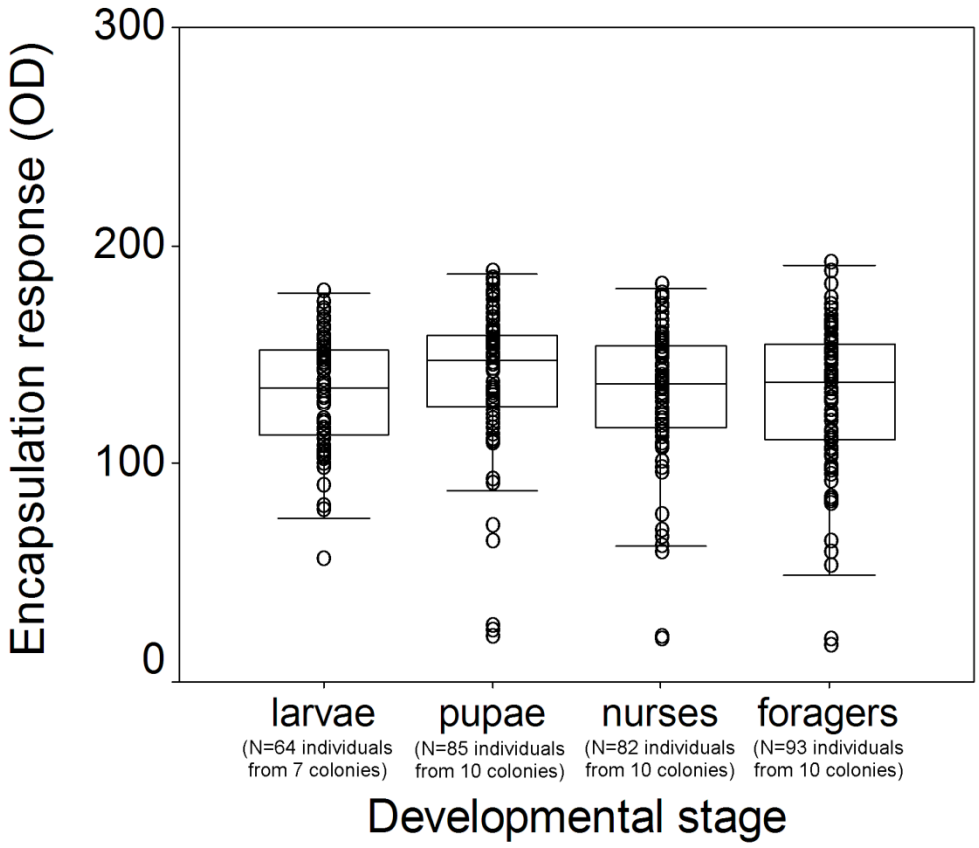

Figure 14. Encapsulation response does not vary with honey bee (*A. mellifera*) ontogeny. Circles represent individuals. Boxes show 1st and 3rd interquartile range with line denoting medians. Whiskers encompass 95% of the individuals, beyond which outliers reside. There were no significant differences in encapsulation rate between developmental stages. Statistics were calculated using a Kruskal-Wallace test with Mann-Whitney U pairwise comparisons.

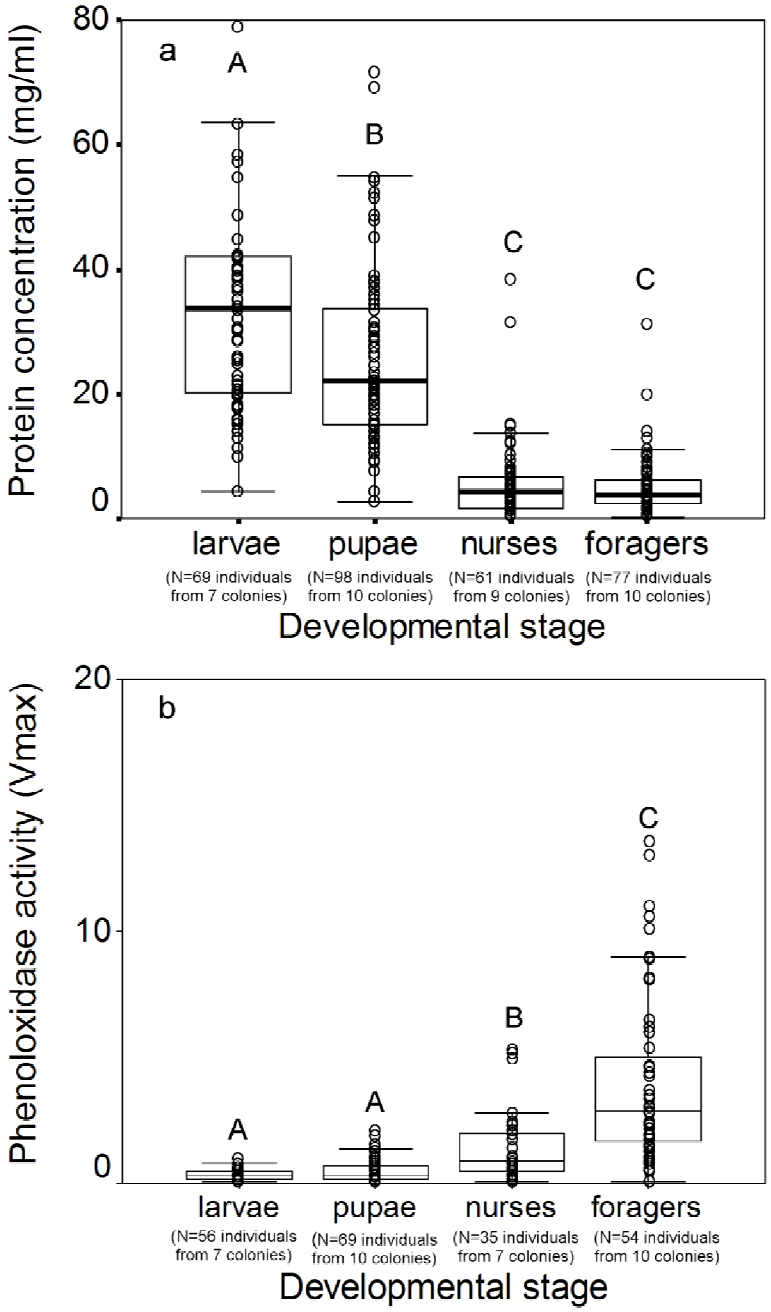

Figure 15. Hemolymph protein concentration (a) and phenoloxidase (PO) activity (b) vary with honey bee (*A. mellifera*) senescence. Circles represent individuals. Boxes show 1st and 3rd interquartile range with line denoting medians. Whiskers encompass 95% of the individuals, beyond which outliers reside. Statistics were calculated using a Kruskal-Wallace test with Mann-Whitney U pairwise comparisons. Significant differences indicated with letters.

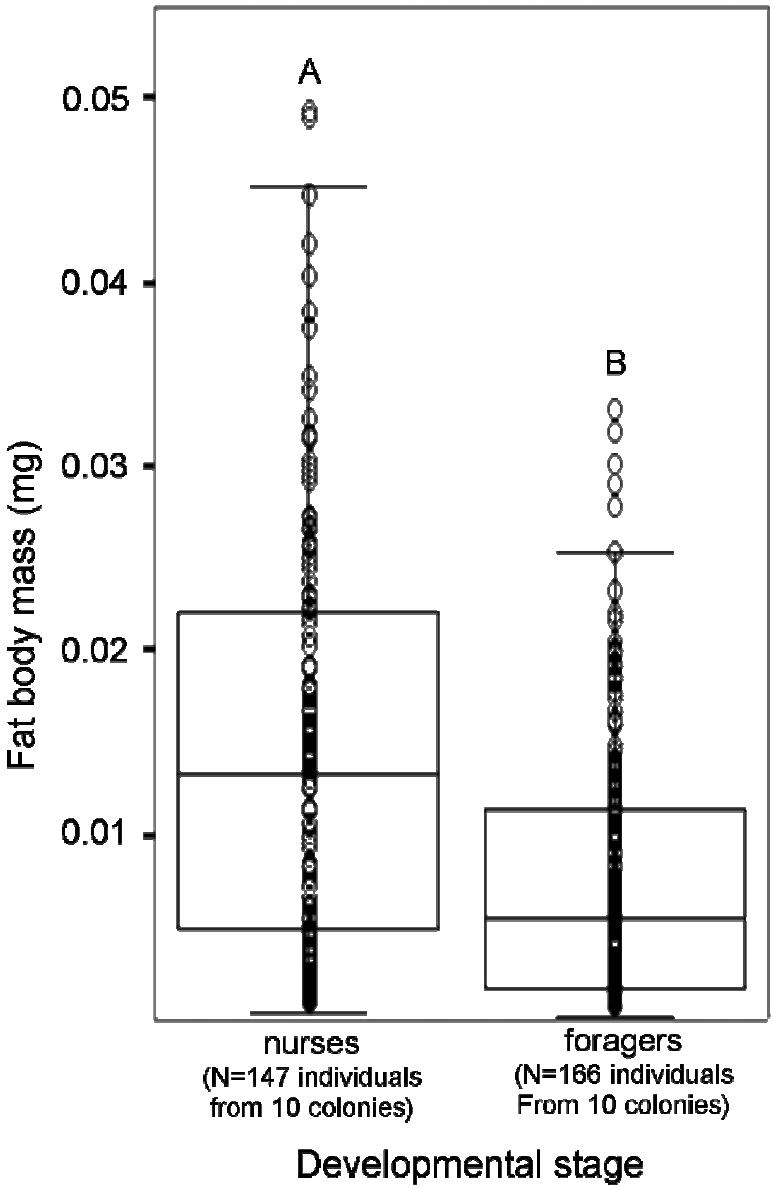

Figure 16. Fat body mass decreases as adult honey bees (*A. mellifera*) age. Circles represent individuals. Boxes show 1st and 3rd interquartile range with line denoting medians. Whiskers encompass 95% of the individuals, beyond which outliers reside. Statistics were calculated using a Kruskal-Wallace test with Mann-Whitney U pairwise comparisons. Significant differences indicated with letters.

CHAPTER 7

THE *Polistes* WAR:

WEAK IMMUNE FUNCTION

IN THE INVASIVE *P. dominulus*

RELATIVE TO THE NATIVE *P. fuscatus*

CHAPTER 7.
The *Polistes* War: Weak immune function in the invasive *P. dominulus* relative to the native *P. fuscatus*

Noah Wilson-Rich and Philip T. Starks

Biology Department, Tufts University, Medford, MA 02155, USA

Insectes Sociaux 2010, 57:47-52. doi: 10.1007/s00040-009-0049-6

ABSTRACT

Invasive species are of growing ecological concern, in part because of conflicts arising with native congeners. The European paper wasp *Polistes dominulus* was first introduced to North America in the 1970s, and may be displacing at least one native species, *P. fuscatus*. Previous reports indicate that in native territories over half of *P. dominulus* colonies are infected by Strepsipteran parasites, which decrease host fitness. In North America, *P. fuscatus* are parasitized to a lesser degree (approximately one-third), but no infected colonies of invasive *P. dominulus* have been reported. Because immune function is an indicator of susceptibility to parasitism, we quantified activated levels of immune function by measuring the encapsulation response and phenoloxidase activity and then compared these levels across species. Counter-intuitively, our results indicate that *P. dominulus* has lower levels of both forms of immunity. Additionally, *P. dominulus* displayed less self-grooming activity than *P. fuscatus*. We briefly discuss possible immunological explanations for this invasion success, including the selective expression of low immunocompetence.

INTRODUCTION

The ecological impact of exotic and invasive species is of growing importance. The rate at which species are transported to novel habitats is increasing, fueled by human activity (Levine and D'Antonio 2003) and global climate change (Simberloff 2000). Successful regional establishment may be facilitated by many factors, including a release from enemies (Porter et al. 1997; Holway and Suarez 1999; Hänfling and Kollmann 2002). Changing landscapes create opportunities for habitat expansions, forcing previously allopatric species into novel sympatric environments. For congeneric species that share a habitat, inevitable battles for niche occupation ensue. One recent example is the invasion of *Polistes dominulus* in North America. *P. dominulus* is currently expanding its range in North America from its native Europe and North Africa. Since their introduction, at least one native congener, *P. fuscatus*, has been outcompeted for nesting sites; its former nesting sites are now being occupied by *P. dominulus* (Gamboa et al. 2002, 2004).

Recently, Polistes wasps have been used as a model system to study invasion biology (see Cervo et al. 2000; Gamboa et al. 2002, 2004, 2005; Silagi et al. 2003; Johnson and Starks 2004; Liebert et al. 2006). *P. dominulus* was first documented in the US in 1978 in Cambridge, Massachusetts (Eickwort 1978; Hathaway 1981). Over the subsequent three decades, *P. dominulus* has been documented across the US (Cervo et al. 2000) and is established across the northern, eastern, and western portions of the country (reviewed in Liebert et al. 2006). The nesting ecology of *P. dominulus* overlaps directly with other native paper wasps, with multiple observations of displacement events of natives (Gamboa et al. 2002, 2004; Liebert et al. 2006). In contrast, *P. dominulus* is found living sympatrically with at least three congeners in its native Europe, with no reports of competitive displacement.

Multiple hypotheses exist for why *P. dominulus* may be so successful in North America (reviewed in Liebert et al. 2006). Cervo and colleagues (2000) proposed that in their invasive range, *P. dominulus* might be unaffected by their naturally associated European parasites. This putative release from enemies might improve their invasion success (Cervo et al. 2000). In 1998 and 1999, Pickett and Wenzel (2000) noted New York populations of *P. fuscatus* were infected by an obligate parasite within the genus *Xenos* (Order Strepsiptera; Kathirithamby 1998, 2009). Parasites from this genus commonly infect *P. dominulus* in its native Old World habitat; however, invasive *P. dominulus* populations were not infected (Pickett and Wenzel 2000). Gamboa and colleagues (2004) further tested this hypothesis in Michigan, USA by surveying 28 *P. fuscatus* and 30 *P. dominulus* colonies for Strepsipteran infection. Eleven *P. fuscatus* nests were infected, yet no *P. dominulus* nests were infected. The reason why invasive populations of *P. dominulus* appear to avoid infection by *X. vesparum* remains unclear. To our knowledge, no reports of the Old World species of *Xenos*, *X. vesparum*, exist documenting the parasite in the New World, neither in an infected host nor in free-living form.

In this study, we investigate the immune function of these two recently sympatric, congeneric, social insects. We hypothesized that there is a difference in the activated cellular and humoral immune response between invasive *P. dominulus* and native *P. fuscatus* populations, in conjunction with the differential pathogen pressure in North America. To test this hypothesis, we quantified two levels of innate immunocompetence (IC), defined as the ability of an organism to mount an immune response (Wilson-Rich et al. 2009).

METHODS

Specimen collection: All wasps were collected from August 9 – 16, 2006 from standard wooden nest boxes at the Cummings School of Veterinary Medicine at Tufts University in North Grafton, MA, USA. Wasp nests were transported to Tufts University in Medford, MA for analyses of immune function. We collected one to seven individual female *P. dominulus* (mean ± SD: 2.6 ± 2.4 wasps) per colony (N=26 *P. dominulus* individuals from 10 colonies). Because *P. fuscatus* were more prevalent at our collection site in 2006, we collected one individual female wasp from each colony (N=26 *P. fuscatus* individuals from 26 colonies). Importantly, no parasitism or outward sign of disease was noted on any wasp or nest collected.

Encapsulation response: The ability to encapsulation a novel foreign body is effective against relatively larger pathogens, including parasitoids (Carton and David 1983; Kraaijeveld et al. 2001a, 2001b), parasites (Doums and Schmid-Hempel 2000), and host cells infected with viruses (Washburn et al. 1996; Trudeau et al. 2001). A modified encapsulation response assay (König and Schmid-Hempel 1995) was performed to quantify the innate cellular immune response.

Each wasp was ice anesthetized and implanted with a sterile 1-2mm nylon monofilament ventrally between 4^{th} and 5^{th} abdominal sternites. A very tiny portion of the monofilament remains outside the abdomen to facilitate its removal for later analysis. The monofilament approximates the presence of a parasite protruding through the intersegmental membrane, as in female *Xenos spp*. After implantation, specimens were placed individually in 1.5ml microcentrifuge tubes (Fisherbrand USA) to protect the implant from grooming activity. After four hours, the monofilament was removed (now termed ‘explant') and mounted in glycerol on a glass slide. Digital images of explants

were captured at 40x magnification using an Olympus VX40 fluorescence-detecting microscope and image-capturing software (Optronics Magna Fire-SP v1.0 x5). The autofluorescent properties of melanin enabled us to capture images through a multi-wavelength filter and detect only particles emitting within a confined spectra and control for non-melanized debris accumulated on the monofilament.

Melanin deposition was assessed using digital images captured from three focal depths (top, mid-section, and bottom). The mean gray value (MGV) of each experimental and one unimplanted control monofilament was quantified using ImageJ (v. 1.34s, NIH, USA). The control MGV was subtracted from each experimental implant, and then converted to optical density (OD) units using a step-function calibration curve generated through ImageJ. Two explants from *P. fuscatus* were not different from control were not used for further analysis, as these likely did not puncture the intersegmental membrane (Wilson-Rich et al. 2008).

Morphometric analyses: Digital images of a forewing, a proleg, and the head were taken for each specimen under 10X magnification. The following measurements were quantified using ImageJ: first discoid cell in wing length, total forewing length, proleg tarsus and tibia length, and head width (Field et al. 1998; Cervo et al. 2004; Tibbetts and Curtis 2007). Whole weight and abdominal weight were recorded for each specimen.

Hemolymph isolation: Hemolymph samples are very difficult to collect from *Polistes*; the conventional 'poke-and-bleed' techniques commonly performed in other insects is not reliably carried out, presumably do to low hydration states of wasps. Instead, we employed a reliable method of hemolymph collection by modifying a technique described by Korner and Schmid-Hempel (2004). First, we thawed frozen

abdomens and then homogenized each by hand in phosphate-buffered saline (PBS) using sterile 1.5ml pellet pestles (Kontes). Next, we centrifuged the homogenate 4˚C for three minutes at 2,000 x gravity, to pull soluble hemolymph proteins to the supernatant and apart from the cuticle. Finally, we transferred supernatant samples to fresh PBS and re-froze each at -20˚C for later use in the phenoloxidase assay.

Phenoloxidase (PO) activity: PO is an enzyme of vital importance to the invertebrate immune response (Hoffman 2003). This enzyme is effective against relatively small-scale pathogens, including viruses (Wilson et al. 2001; Beck and Strand 2007), bacteria (Pye 1974; Ashida and Brey 1997), fungi (Ochiai and Ashida 1988), and parasites (Leonard et al. 1985; Paskewitz and Riehle 1994; Gorman et al. 1996; Siva-Jothy 2000). PO plays an important role in both cellular and humoral immune defense; however, in this test, we quantified PO activity in a cell-free environment so as to measure its humoral activity (Rantala et al. 2003; Wilson-Rich et al. 2009).

To quantify activity of the PO enzyme, we first added a tyrosine-derived substrate, L-dopa (Thermo Sci Acros Organics, Fair Lawn, NJ, USA) to thawed hemolymph samples, to reach a final concentration of 0.03M. Next, we measured the rate of melanin production spectrophotometer (Bio-Rad microplate reader, model 450) at 490nm every two minutes for 30 minutes. Last, we graphically determined the linear reaction phase for each sample, which typically occurred during the first 10 minutes after substrate addition. The slope of this line was used to determine the maximum reaction velocity (V_{max}).

Total protein concentration was determined for each individual using a standard Bradford protein assay (see methods described in Wilson-Rich et al. 2008). PO activity

was divided by total protein concentration for final data analysis. This additional step allowed us to control for differences in hydration state between individuals.

Statistical analyses: Encapsulation response and PO activity were compared between species using a univariate general linear model (GLM; SPSS for Windows, v.16.0). The original GLM incorporated species as the independent variable, immunity (either encapsulation response or PO activity) as discrete dependent variables, colony of origin nested under species as a random factor, and size (head width) as a covariate fixed factor.

RESULTS

Encapsulation response: The ability to encapsulate a novel foreign object was quantified by calculating the optical density (OD) of removed implants (now termed 'explants'). The net, mean OD of explants was low in *P. dominulus* compared to *P. fuscatus* (Figure 17; 1.02 OD and 1.62 OD, for each species respectively). Colony of origin was not a significant factor influencing encapsulation response ($F=1.710$, $df=21$, $p=0.160$). Likewise, size (head width) was not a significant covariate with encapsulation response ($F=0.361$, $df=1$, $p=0.259$). As such, both were removed from the final GLM. After ensuring that differences were not driven by colony of origin or size, results showed the difference in encapsulation response between species was significant ($F=8.621$, $df=1$, $p=0.006$).

Putative grooming response: An unexpected observation occurred during the encapsulation response experiment. After four hours, all monofilaments implanted in *P. dominulus* remained *in situ*. Surprisingly, nine monofilaments (34.6%) were completely removed from *P. fuscatus* abdomens and found at the bottom of the isolation tubes. The

difference in implant displacement frequency between species was significant (χ^2=8.33, df=1, p<0.01).

Phenoloxidase (PO) activity: The ability to inactivate pathogens through the PO cascade was quantified by determining the linear phase of PO activity, which occurred during the first 10 minutes of reaction (*P. dominulus*, Vmax=11.57uM/min; *P. fuscatus*: Vmax=15.73uM/min). PO activity (Vmax) was then divided by total protein concentration (mg/ml). The mean PO : total protein concentration ratio was low in *P. dominulus* compared to *P. fuscatus* (Figure 18; median ± SE: 8.68 ± 1.03 Vmax*ml/mg and 13.95 ± 2.18 Vmax*ml/mg, for each species respectively). Colony of origin was not a significant factor influencing PO activity (F=1.082, df=31, p=0.558). Likewise, size (head width) was not a significant covariate with PO activity (F=0.361, df=1, p=0.558). As such, both were removed from the final GLM. After ensuring that differences were not driven by colony of origin and size, results showed the difference in PO activity between species was significant (F=6.540, df=1, p=0.014).

Morphometric analyses: All seven morphometric measurements (length of first discoid wing cell, total wing length, femur length, tibia length, whole weight, abdominal weight, and head width) correlated with one another (1-tailed Pearson correlation; p<0.001 for all pairs). Overall, *P. dominulus* was smaller than *P. fuscatus* for all morphometric measurements (Kruskal-Wallace test: p<0.001 for all seven measurements). Despite the smaller body size of *P. dominulus*, morphometric differences between species were not significant covariates with either measure of immune function (see *Statistical analyses* in Methods).

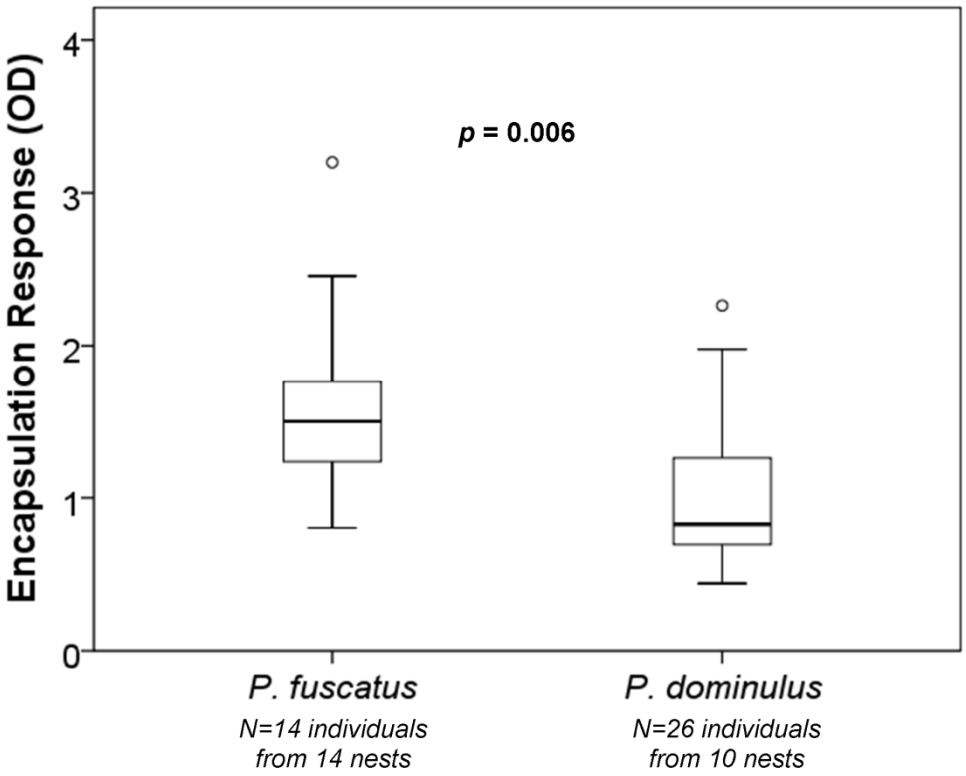

Figure 17. Native *P. fuscatus* have a stronger encapsulation response than invasive *P. dominulus* (Chapter 7). A standard encapsulation response assay was used as a direct measure of the innate, cellular immune response. Boxes show 1st and 3rd interquartile range (middle 50% of individuals); lines in middle of boxes indicate median values. Whiskers extending from boxes encompass 95% of individuals, beyond which outliers reside. Statistics were calculated using a general linear model (see methods).

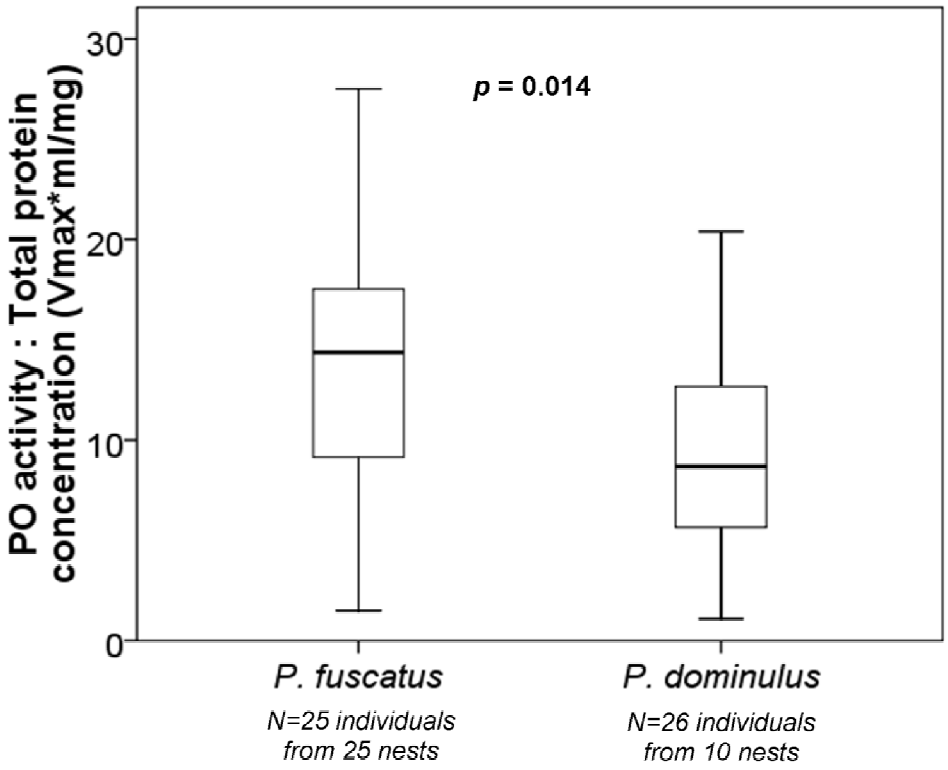

Figure 18. Native *P. fuscatus* have greater phenoloxidase (PO) activity than invasive *P. dominulus*. A standard PO activity assay was performed as a direct measure of the innate, humoral immune response. Total hemolymph protein concentration was corrected for in post-hoc analysis to control for variation in individual hydration state. Boxes show 1st and 3rd interquartile range (middle 50% of individuals); lines in middle of boxes indicate median values. Whiskers extending from boxes encompass 95% of individuals, beyond which outliers reside. Statistics were calculated using a general linear model (see methods).

DISCUSSION

We report two direct measures of IC in a strong invasive species (Liebert et al. 2006). Our data indicate the cellular and humoral innate immune response of an invasive population of *P. dominulus* is significantly lower than that of a native sympatric population of *P. fuscatus*. Subsequent data from implant displacement rates suggest *P.*

dominulus groomed out a foreign body from the ventral abdomen significantly less frequently than *P. fuscatus*. This observation is unexpected and surprising, given that *P. fuscatus* have a larger body size and therefore less room to maneuver within the confined chamber than did the smaller *P. dominulus*. Although direct grooming out of these implants was not observed, this finding warrants further investigation into differential grooming rates between these species as a measure of behavioral IC.

Data collected from seven morphometric measures showed that *P. dominulus* is significantly smaller than *P. fuscatus*. These findings are consistent with previous reports of size differences between these recently sympatric congeners (reviewed in Liebert et al. 2006). Our data show that size was not a significant covariate of either encapsulation response or phenoloxidase activity, indicating differences in immunity are not due to size.

Immunological study of native *P. dominulus* populations is crucial before solid expectations of the factors influencing relatively low IC can be postulated. Even without these data, at least three mutually-exclusive hypotheses explaining low IC in invasive *P. dominulus* are conceivable: 1) there has been no change in IC from native to invasive, 2) IC in *P. dominulus* is a phenotypically plastic trait, or 3) IC in *P. dominulus* has evolved to lower levels. The first prediction, that native populations may have undergone no change in immune investment and IC subsequent to North American invasions, is the most parsimonious explanation. A key assumption of this hypothesis is that IC is a trait with little variation between environments. This assumption is not likely because of noted differences in pathogen pressure between Old and New World habitats. Variation in pathogen pressure should drive variation in immune function.

The second and third hypotheses are more plausible given the high invasion success and evidence of competitive ability of North American *P. dominulus*. The second hypothesis, that IC is a phenotypically plastic trait, does not make any assumptions about variation in alleles coding for IC. Instead, this hypothesis assumes variable phenotypic expression of IC, dependent upon environmental cues. Assuming mounting an immune response is costly, low IC should be expressed when high IC is wasteful. And indeed, there is likely a fitness cost for maintaining high levels of immunity (Hughes and Cremer 2007).

The third hypothesis -- that IC in *P. dominulus* evolved to lower levels in invasive populations -- assumes variation in alleles coding for IC function. Low IC may occur in at least two ways: either through a genetic bottleneck in IC alleles in the invasive population that by happenstance rendered the new population with alleles for low IC (hereafter, the 'non-selective hypothesis'), or if individuals with alleles for low IC had a selective advantage (the 'selective hypothesis'). The non-selective hypothesis is unlikely in *P. dominulus* because this species has undergone multiple invasions with no evidence of a genetic bottleneck (Johnson and Starks 2004; Liebert et al. 2006). Of these two hypotheses, the selective hypothesis seems more likely, given the degree of genetic diversity in the population. Natural selection should favor individuals with inherently low IC when it is advantageous. Assuming IC is energetically costly, resources formerly invested in IC might be diverted to competitive life-history traits (Blossey and Nötzold 1995; Hänfling and Kollmann 2002; Lee and Klasing 2003). The North American landscape may provide such an environment, with relatively low pathogen pressure and observed competition for nesting sites. Clearly, IC data from native *P. dominulus* populations are needed in order to discriminate between these three major hypotheses.

The enemy-release hypothesis suggests the establishment and spread of an introduced species is fueled in part by a release from the parasites, pathogens, and predators that were major ecological constraints in the native habitat (Porter et al. 1997). In the northern United States, between 12-39% of *P. fuscatus* are infected by *Xenos spp.* (Pickett & Wenzel 2000; Gamboa et al. 2002, 2004). In Tuscany, Italy, 58% of *P. dominulus* are infected by *Xenos spp.* (Hughes et al. 2003); there have been no reports of the parasite in North American populations to date. Future study should investigate the identity of enemy populations and whether or not these enemies recognize *P. dominulus* as a host.

Invasive species are of growing ecological importance. Data relating to these animals provide researchers with valuable insight into what makes a non-native species successful. With the case of the *P. dominulus* invasion, our results demonstrate weak cellular and humoral IC (vis-à-vis encapsulation response phenoloxidase activity), and low rates of hygienic behavioral acts (self-grooming), compared to the native *P. fuscatus*. Counter-intuitively, release from predators may be driving low IC, resulting in a highly successful, but immunocompromized, invasion.

CHAPTER 8:

CONCLUSIONS AND FUTURE DIRECTIONS

CHAPTER 8.
Conclusions and future directions

Insects make up approximately 80% of all the animal species known to date (Otvos 2000). A major driver of their remarkable evolutionary success is partly due to their highly efficient and effective innate immune response (Otvos 2000). Like social insects, humans are social animals. We live in groups, with overlapping generations, reproductive division of labor, and cooperative brood care. Granted, there is natural variation across human societies with the degree to which any given population conforms to these categories. The argument has been made that humans are facultatively eusocial (Foster and Ratnieks 2005). Knowledge gained through the study of social insects – their behavior, their sociality, their ecology – may be scaled up to provide valuable insights into all social animals, insects and humans alike. How do individuals evolve such complex forms of living in the face of disease? With sociality also comes increased interaction with related individuals, shared food sources, and cohabitation. Each of these factors facilitates disease spread.

The main goal of this dissertation was to answer the question, how do social insects resist disease? We achieved this goal by using multiple methods of quantifying immune function in honey bees (*Apis mellifera*) and two species of paper wasp (*Polistes dominulus* and *P. fuscatus*). We relied on two primary measures of immune function: phenoloxidase activity and encapsulation response. We complemented these direct measures with as many as three indirect measures of immune function: total hemolymph protein concentration, total hemocyte count, and fat body mass. These indirect measures serve as direct assessments of body condition. The concentration of proteins and cells in an insect's hemolymph informs us about the hydration state of each insect and can be used as dependent variables in their own right. Both these measures can

further be used as preliminary steps in laboratory tests to control protein concentration and hemolymph concentration evenly across samples. Likewise, fat body mass provides useful information about energy stores. Each of these measures was published in the literature before I began this dissertation work in 2005; however, none were used with honey bees or paper wasps, until now.

Of the many conclusions and take-home messages in this dissertation, I would most like readers to acknowledge the importance of incorporating multiple approaches to measuring immune function concurrently in ecological immunology studies. Incorporating multiple methods into ecological immunology studies is important (Adamo 2004a). Adamo (2004a) argues that single method approaches provide a nearsighted result, and ignore real-life phenomena such as immunoredistribution – when one immune mechanism is downregulated while another is selectively upregulated. The single-method approach may inhibit researchers from gaining an understanding of what occurs during co-infection, and also prevents thorough exploration of trade-offs occurring during the life history of study organisms, such as between defense, growth, and reproduction. Along with my terrific coworkers, we showed suggestive evidence that there are differences in the honey bee immune response to different types of pathogens (Ch. 2).

The immune system is dynamic and multifaceted, with behavioral, cellular, chemical, and humoral aspects to it. A limited amount of energy is likely partitioned for investment into immunity and defense (König & Schmid-Hempel 1995; Doums and Schmid-Hempel 2000; Amdam et al. 2005; Behrends et al. 2007), which is assumed to be divided amongst the different flavors of immunity. When exploring questions relating to ecological immunology, it is important that researchers measure different modes of immunity concurrently. How does the immune system develop in a social animal? What

role do certain individuals play in the disease resistance capacity of a colony? What trade-offs exist between defense, growth, and reproduction? The only way to achieve thorough and objective data sets to answer these questions and test related hypotheses is by using a multiple measures approach.

The methods used in this dissertation are not perfect. There are limitations to each test because the vast majority of prior investigates study them in a functional vacuum, with limited biological relevance in the sterile laboratory. We hypothesize that fat body mass correlates with immune protein production, but support for this prediction remains elusive. So much has yet to be done. However, what remains established is the fact that invertebrates are excellent study systems to use for precisely this type of investigation.

Invertebrates are excellent model systems with which to study ecological immunology because their immune systems are more basic than those of vertebrates. This simple nature allows for scientists to vary many ecological and environmental factors while monitoring just a handful of immune mechanisms rather than the many complex “adaptive” pathways of vertebrate immune systems. Several invertebrate genomes are now sequenced (e.g., *Drosophila melanogaster*, *Apis mellifera*, *Tribolium castaneum*) as well as some of their naturally associated pathogens (e.g., the bacterium *Buchnera aphidicola* hosted by most species of aphid, *Phytoplasma asteris* hosted by leafhopper insects, *Photorhabdus luminescens* hosted by entomopathogenic nematodes that infect many insect species, *Pseudomanas aeruginosa*, four species of *Rickettsia* hosted by ticks and fleas and transmitted to humans, *Wigglesworthia glossinidia* hosted by the tsetse fly, *Wolbachia pipientis* hosted by many insects, *Xylella fastidiosa* hosted by sharpshooter insects that are vectors of plant disease, and *Yersinia pestis* hosted by

fleas). Invertebrate immunity is destined to grow in its usefulness, applicability, and versatility of results and subsequent contributions to science.

Invertebrate study systems are easy to rear in the laboratory, more often are inexpensive to maintain than not, have fewer regulations than vertebrates for laboratory use, and typically higher available sample size by many folds. Results from invertebrate systems can be scaled up to test ecological and evolutionary theories. Despite the differences between invertebrates and vertebrates, similarities abound that allow results to be applied across a diverse group of animal model systems. On the one hand, these similarities between vertebrate and invertebrate immunology are fundamental, including phagocytosis, antioxidants, and free radicals (reactive oxygen and nitrogen species). On the other hand, invertebrates do not possess adaptive immunity, whereby they cannot produce antibodies in response to specific antigens.

There are unique characteristics associated with invertebrate systems that allow for uniquely controlled experiments against broad spectrums of pathogens. When immunology was a budding field of research in the 19th century, pioneers such as Louis Pasteur and Eli Metchnikoff studied invertebrates to gain a basic understanding of these mechanisms. Over a century later, researchers continue to use very basic methods of assaying immunocompetence (such as those in this dissertation). Looking forward, the future of ecological immunology research will inevitably involve a blending of invertebrate and vertebrate model systems and their methods.

So what does the future hold for this field of research? Following the assumption that immunity is costly, and this energy must be divided between different modes of immune function, the multiple methods approach will continue to be invaluable. Complementary measures of genetic, cellular, humoral, biochemical, structural, and behavioral immunity will each provide valuable results with which to answer questions related to ecological immunology. Genetic methods include making use of genomes for

bioinformatics study, and also continued use of gene arrays that enable researchers to visualize which immune genes are actively undergoing transcription in response to a given stimuli (e.g., Johnson et al. 2009; Simone et al. 2009).

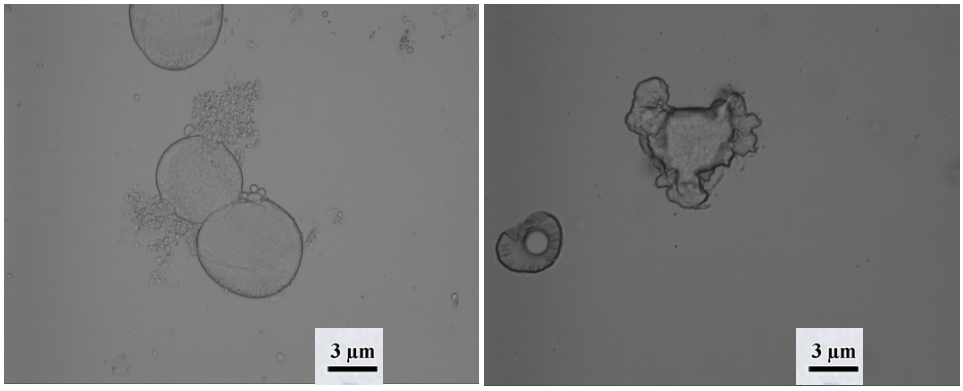

Figure 19. Honey bee (*A. mellifera*) hemocytes visualized through a light-contract microscope. Size standard bars are approximate measure inserted post hoc. Three cell types were visualized based on morphology. Granular cells, or granulocytes, appear to be dumping granules after colliding (left). Prohemoctyes (right image, left side) appear with a defined nucleus, while plasmatocytes (right image, right side) have visible pseudopodia. Each sample was prepared by mixing 1µL hemolymph with 3µL grace's insect medium between a glass slide and cover slip; no dye was used in the samples shown here.

Cellular analyses are only beginning to advance their methods as reported in the literature (see Manfredini et al. 2008). We still do not even know what types of hemocytes honey bees have! Together with Jon Snow, we gathered preliminary data to investigate the honey bee hemocytes using multiple cell staining techniques including DAPI and May-Gruenwald-Giemsa to visualize nucleated cells and cell wall morphologies, respectively (Figure 19). We believe we visualized granulocytes, plasmatocytes, and prohemocytes. Jon and I also used advanced techniques to separate cell types, including fluorescent-activated cell sorting (FACS) and flow cytometry. Our preliminary FACS analyses may have identified cells containing reactive oxygen species. These methods are commonly used in vertebrate studies, and are ready for incorporation into the invertebrate literature. Likewise, humoral (protein-based)

factors in the hemolymph may be studied using techniques already established in vertebrate studies, including SDS-PAGE and western blot techniques. Rosengaus and coworkers (2007) went a step farther and harvested proteins of specific sizes from gels and tested their antimicrobial activity. This example of linking immune function with disease resistance is most definitely a peek into the future of ecological immunology methods.

Scaling further up the levels of biological organization – beyond DNA, cells, and proteins – broadens the lens onto a world we currently know little about. How have social insects resisted disease successfully for millions of years? Behavioral ecologists have studied individual behavior for decades, but what about other individual processes? Like vertebrates, insects produce other chemical products in their blood that may become harmful to their own bodies if not carefully regulated. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are harmful to pathogens, but also to nearby tissues. Anti-oxidants are either produced by the body or taken in through the diet, and help reduce the risk of autoimmunity. Kalevi Trontti presented novel methods for assaying anti-oxidant activity in ants at the IUSSU XVI Congress in Copenhagen (2010) by measuring expression of anti-oxidant genes, specifically *sod1*, *sod2*, *sod3*, and *gst*, using quantitative real-time PCR. Unlike vertebrates, insects possess unique membrane-bound structures that contain defensive chemicals and substances, including metapleural, cephalic, poison, sternal, salivary glands (see Eisner 2003). Results from these studies not only elucidate the insects' fascinating mechanisms of enemy resistance, but also open up a world of opportunity for humans to explore novel defenses, some of which may be applicable to human medicine through the discovery of novel antiseptic compounds.

At the whole-body level of organization, structures such as the cuticular wall and blocks over spiracle openings are the first lines of defense against infection. Empirical methods for examining variation in these defenses include tests of cuticle strength using weights or puncture tests, and advanced microcopy for observation of orifices, such as the spiracles, mouth, and anus. Observations of individual behavior provide important insight to how invertebrates groom themselves, avoid or move away from septic locations, scratch, raise abdominal tips, or other like actions (see Wilson-Rich et al. 2007). These observations can also be scaled up to visualize the group facilitation of disease resistance. Videotape analyses of behavior in response to controlled infections also provide ample opportunity for undergraduate researchers to get involved in the scientific process.

Ultimately, tests of immune function are meaningless without data linking them to actual disease resistance (see Luster et al. 1993). There are many ways to do this, both *in vitro* and *in vivo*. Both approaches involve exposing naturally-associated pathogens to host hemolymph and measuring survival. *In vitro* tests typically collect hemolymph, transfer it to a sterile container, and add to it a set concentration of pathogens. After a brief incubation period, variation in the opacity of samples collected from different cohorts becomes apparent, and can be measured using a spectrophotometer. Samples from each solution can further be plated onto agar, and the resulting colonies can be counted and their numbers statistically compared. (This latter assay is often referred to as CFU, or colony forming units.) Another commonly used *in vitro* disease resistance test involves adding hemolymph of either controlled volume or protein concentration to a Petri dish pre-inoculated with naturally-associated microbial pathogens. The zones of inhibited microbial growth can be measured with a ruler and then statistically compared across independent variable cohorts. *In vivo* tests require inoculation of hosts with a

known concentration of pathogens, and then measuring either host survival, pathogen load, or both.

These methods are visions for the future of ecological immunology. I see them as in addition to the techniques used in my dissertation (PO activity, encapsulation response, fat body mass, total hemocyte count, total hemolymph protein concentration). The methods used by my fabulous team of coworkers and me will, in all likelihood, continue to be used because they are well established and provide valuable, relevant results. It will be interesting to watch, and to be a part of, the progression of this field of research. I look forward to continuing to contribute to advancing the field, and all of the fascinating things about the natural world, and specifically about what enables insects to be so successful, that we have yet to learn.

BIBLIOGRAPHY

1. Adamo S. 2004a. How should behavioural ecologists interpret measurements of immunity? *Anim. Behav.* 68:1443-1449
2. Adamo S. 2004b. Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *J. Insect Phys.* 50:209-216
3. Adamo S, Parsons N. 2006. The emergency life-history stage and immunity in the cricket, *Gryllus texensis*. *Anim. Behav.* 72:235-244
4. Ahtiainen J, Alatalo R, Rantala M. 2004. Sexual advertisement and immune function in an arachnid species (Lycosidae). *Beh. Ecol*. 15:602-606
5. Alcock J. 1993. *Animal Behavior. An Evolutionary Approach.* Sunderland, MA: Sinauer. 525 pp.
6. Allander K, Schmid-Hempel P. 2000. Immune defence reaction in bumble-bee workers after a previous challenge and parasitic coinfection. *Funct. Ecol.* 14:711-717
7. Amdam G, Simões Z, Hagen A, Norberg K, Schrøder K, Mikkelsen Ø, et al. 2004. Hormonal control of the yolk precursor vitellogenin regulates immune function and longevity in honeybees. *Exp. Gerontol.* 39:767-773
8. Amdam G, Aase A, Seehuus S-C, Fondrk M, Norberg K, Hartfelder K. 2005. Social reversal of immunosenescence in honey bee workers. *Exp. Gerontol.* 40:939-947
9. Amdam G, Seehuus S-C. 2006. Order, disorder, death: Lessons from a superorganism. *Adv. Cancer Res.* 95:31-60
10. Arathi HS, Burns I, Spivak M. 2000. Ethology of hygienic behaviour in the honey bee, *Apis mellifera* (Hymenoptera: Apidae): behavioural repertoire of hygienic bees. *Ethology* 106:1–15
11. Arathi HS, Ho G, Spivak M. 2006. Inefficient task partitioning among nonhygienic honeybees, *Apis mellifera* L., and implications for disease transmission. *Anim. Behav.* 72:431–38

12. Arathi HS, Spivak M. 2001. Influence of colony genotypic composition on the performance of hygienic behavior in the honey bee (*Apis mellifera* L). *Anim. Behav.* 62:57–664.
13. Ashida M, Brey P. 1997. Recent advances in research on the insect prophenoloxidase cascade. In: *Molecular mechanisms of immune responses in insects*, ed. P Brey, D Hultmark, pp. 135–172. London, UK: Chapman & Hall
14. Aubert, A., Richard, F.-J., 2008. Social management of LPS-induced inflammation in *Formica polyctena* ants. *Brain, Behav., Immun.* 22:833-837
15. Baer B, Krug A, Boomsma J, Hughes W. 2005. Examination of the immune responses of males and workers of the leaf-cutting ant *Acromyrmex echinatior* and the effect of infection. *Insectes Soc.* 52:298–303
16. Baer B, Schmid-Hempel P. 1999. Experimental variation in polyandry affects parasite loads in a bumblebee. *Nature* 397:151–54
17. Baer B, Schmid-Hempel P. 2001. Unexpected consequences of polyandry for parasitism and fitness in the bumblebee, *Bombus terrestris*. *Evolution* 55(8):1639–43
18. Baer B, Schmid-Hempel P. 2003. Bumblebee workers from different sire groups vary in susceptibility to parasite infection. *Ecol. Lett.* 6:106–10
19. Baer B, Schmid-Hempel P. 2006. Phenotypic variation in male and worker encapsulation response in the bumblebee *Bombus terrestris*. *Ecol. Entomol.* 31:591-596
20. Behrends A, Scheiner R, Baker N, Amdam G. 2007. Cognitive aging is linking to social role in honey bees (*Apis mellifera*). *Exp. Gerontol.* 42:1146-1153
21. Bailey L. 1968a. Honey bee pathology. *Ann. Rev. Entomol.* 13:191-212

22. Bailey L. 1968b. The multiplication of sacbrood virus in the adult honeybee. *Virology* 36:312-313
23. Bailey L, Ball BV. 1991. *Honey Bee Pathology*. London: Academic. 208 pp. 2nd ed.
24. Bakonyi T, Grabensteiner E, Kolodziejek J, Rusvai M, Topolska G, Ritter W, Nowotny N. 2002. Phylogenetic analysis of acute bee paralysis virus strains. *Appl. Environ. Microb.* 68:6446-6450
25. Ball BV, Allen MF. 1988. The prevalence of pathogens in honey bee (*Apis mellifera*) colonies infested with the parasitic mite *Varroa jacobsoni*. *Ann. Appl. Biol.* 113(2):237–44
26. Bankova V, de Castro S, Marcucci M. 2000. Propolis: recent advances in chemistry and plant origin. *Apidologie* 31:3–15
27. Bastos E, Simone M, Jorge D, Soares A, Spivak M. 2008. In vitro study of the antimicrobial activity of Brazilian propolis against *Paenibacillus larvae*. *J. Invertebr. Pathol.* 97:273–81
28. Beani L. 2006. Crazy wasps: When parasites manipulate the *Polistes* phenotype. *Ann. Zool. Fenn.* 43:564-574
29. Beck M, Strand M. 2007. A novel polydnavirus protein inhibits the insect prophenoloxidase activation pathway. *P. Natl. Acad. Sci. USA* 104:19267-19272
30. Bedick J, Tunaz H, Nor Aliza A, Putnam S, Ellis M, Stanley D, 2001. Eicosanoids act in nodulation reactions to bacterial infections in newly emerged adult honey bees, *Apis mellifera*, but not in older foragers. *Comp. Biochem. Phys. C* 130:107-117
31. Beshers S, Fewell J. 2001. Models of division of labor in social insects. *Annu. Rev. Entomol.* 46:413–40
32. Bilikova K, Wu G, Simuth J. 2002. Isolation of a peptide fraction from honeybee royal jelly as a potential antifoulbrood factor. *Apidologie* 32:275–83

33. Blossey B, Nötzold R. 1995. Evolution of increased competitive ability in invasive nonindigenous plants: A hypothesis. *J. Ecol.* 83:887-889
34. Bocher A, Tirard C, Doums C. 2007. Phenotypic plasticity of immune defence linked with foraging activity in the ant *Cataglyphis velox*. *J. Evol. Biol.* 20:2228–34
35. Boecking O, Spivak M. 1999. Behavioral defenses of honey bees against *Varroa jacobsoni* Oud. *Apidologie* 30:141–58
36. Boëte C, Paul R, Koella J. 2002. Reduced efficacy of the immune melanization response in mosquitoes infected by malaria parasites. *Parasitology* 125:93-98
37. Bonabeau E, Theraulaz G. 1999. Role and variability of response thresholds in the regulation of division of labor in insect societies. In *Information Processing in Social Insects*, ed. C Detrain, JL Deneubourg, JM Pasteels, pp. 141–63. Basel: Birkhäuser
38. Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254
39. Braude S, Tang-Martinez Z, Taylor G. 1999. Stress, testosterone, and the immunoredistribution hypothesis. *Behav. Ecol.* 10:345-350
40. Bronstein S, Connor W. 1984. Endotoxin-induced behavioural fever in the Madagascar cockroach, *Gromphadorhina portentosa*. *J. Insect Physiol.* 30(4):327–30
41. Brown M, Moret Y, Schmid-Hempel P. 2003. Activation of host constitutive immune defence by an intestinal trypanosome parasite of bumble bees. *Parasitology* 126:253-260
42. Bujok B, Kleinhenz M, Fuchs S, Tautz J. 2002. Hot spots in the bee hive. *Naturwissenschaften* 8:229–301
43. Calderone N, Page R. 1988. Genotypic variability in age polyethism and task specialization in the honey bee, *Apis mellifera* (Hymenoptera: Apidae). *Behav. Ecol. Sociobiol.* 22:17-25

44. Calleri II D, Reid E, Rosengaus R, Vargo E, Traniello J. 2006. Inbreeding and disease resistance in a social insect: effects of heterozygosity on immunocompetence in the termite *Zootermopsis angusticollis*. *P. Roy. Soc. Lond. B Bio.* 273:2633-2540
45. Calleri II D, Rosengaus R, Traniello J. 2007. Immunity and reproductionduring colony foundation in the dampwood termite, *Zootermopsis angusticollis*. *Physiol. Entomol.* 32:136-142
46. Carton Y, David J. 1983. Reduction of fitness in *Drosophila* adults surviving parasitization by a cynipid wasp. *Experientia* 39:231-233
47. Castella G, Chapuisat M, Christe P. 2008. Prophylaxis with resin in wood ants. *Anim. Behav.* 75:1591–96
48. Cerenius L, Söderhäll K. 2004. The prophenoloxidase-activating system in invertebrates. *Immunol. Rev.* 198:116–26
49. Cervo R, Zacchi F, Turillazzi, S. 2000 *Polistes dominulus* (Hymenoptera: Vespidae) invading North America; some hypotheses for its rapid spread. *Insect. Soc.* 47:155-157
50. Cervo R, Macinai V, Dechigi F, Turilazzi S. 2004. Fast growth of immature brood in a social parasite wasp: a convergent evolution between avian and insects cuckoos. Am. Nat. 164:814-820
51. Chan Q, Howes C, Foster L. 2006. Quantitative comparison of caste differences in honeybee hemolymph. *Mol. Cell. Proteomics.* 5(12):2252–62
52. Chapman R. 1971. *The Insects: Structure and Function*. New York: Elsevier. 819 pp. 2nd ed.
53. Christe P, Oppliger A, Bancala F, Castella G, Chapuisat M. 2003. Evidence for collective medication in ants. *Ecol. Lett.* 6(1):19–22
54. Civantos E, Ahnesjö J, Forsman A. 2005. Immune function, parasitization and extended phenotypes in colour polymorphic pygmy grasshoppers. *Biol. J. Linn. Soc.* 85:373-383
55. Cole B. 1983. Multiple mating and the evolution of social behavior in the hymenoptera. *Behav. Ecol. Sociobiol.* 12(3):191–201

56. Contreras-Garduño J, Canales-Lazcano J, Córdoba-Aguilar A. 2006. Wing pigmentation, immune ability, fat reserves and territorial status in males of the rubyspot damselfly, *Hetaerina americana*. *J. Ethol.* 24:165-173
57. Cotter S, Wilson K. 2002. Heritability of immune function in the caterpillar *Spodoptera littoralis*. *Heredity* 88:229-234
58. Cotter S, Kruuk L, Wilson K. 2004. Costs of resistance: genetic correlations and potential trade-offs in an insect immune system. *J. Evol. Biol.* 17:421–29
59. Cox-Foster D, Conlan S, Holmes E, Palacios G, Evans J, Moran N, et al. 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. *Science* 318:283-287
60. Cox-Foster D, Stehr J. 1994. Induction and localization of FAD-glucose dehydrogenase (GLD) during encapsulation of abiotic implants in *Manduca sexta* larvae. *J. Insect Physiol.* 40:235-249
61. Cremer S, Armitage S, Schmid-Hempel P. 2007. Social immunity. *Curr. Biol.* 17:R693–R702
62. Da Silva, J. 2002. Role of the phagocytes on embryos: Some morphological aspects. *Microsc. Res. Tech.* 57:498-506
63. Darwin C. 1859. *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Existence.* London, UK: John Murray
64. de Souza D, Van Vlaenderen J, Moret Y, Lenoir A. 2008. Immune response affects ant trophallactic behaviour. *J. Insect Physiol.* 54:828-832
65. de Verno P, Chadwick J, Aston W, Dunphy G. 1984. The in vitro generation of an antibacterial activity from the fat body and hemolymph of non-immunized larvae of *Galleria mellonella*. *Dev. Comp. Immunol.* 8:537-546

66. Diniz-Filho J, Malaspina O. 1995. Evolution and population structure of Africanized honey bees in Brazil: Evidence from spatial analysis of morphometric data. *Evolution* 49:1172-1179
67. Doums C, Schmid-Hempel P. 2000. Immunocompetence in workers of a social insect, *Bombus terrestris* L., in relation to foraging activity and parasitic infection. *Can. J. Zool.* 78:1060-1066
68. Doums C, Moret Y, Benelli E, Schmid-Hempel P. 2002. Senescence of immune defence in Bombus workers. *Ecol. Entomol.* 27:138-144
69. Dudley R, Milton K. 1990. Parasite deterrence and the energetic costs of slapping in howler monkeys, *Alouatta palliata*. *J. Mammal.* 71(3):463–65
70. Eickwort G. 1978 *Polistes dominulus* discovered near Boston. *Polistine Inform. Bull. Newslett.*
71. Eisner T. 2003. *For love of insects*. Cambridge, MA: Belknap. 448 pp.
72. Ellers J. 1996. Fat and eggs: an alternative method to measure the trade-off between survival and reproduction in insect parasitoids. *Neth. J. Zool.* 46:227-235
73. Eshete F, LoVerde P. 1993. Characteristics of phenol oxidase of *Schistosoma mansoni* and its functional implications in eggshell synthesis. *J. Parasitol.* 79:309-317
74. Eslin P, Prévost G. 1998. Hemocyte load and immune response to *Asobara tabida* are correlated in species of the *Drosophila melanogaster* subgroup. *J. Insect Physiol.* 44:807-816
75. Estoup A, Garnery L, Solignac M, Cornuett J.-M. 1995. Microsatellite variation in honey bee (*Apis mellifera* L.) populations: Hierarchical genetic structure and tests of the infinite allele and stepwise mutation models. *Genetics* 140:679-695

76. Evans J, Aronstein K, Chen Y, Hetru C, Imler J-L, et al. 2006. Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect. Mol. Biol.* 15(5):645–56
77. Evans J, Pettis J. 2005. Colony-level impacts of immune responsiveness in honey bees, *Apis mellifera*. *Evolution* 59:2270-2274
78. Evans J, Pettis J, Smith I. 2007. A diagnostic genetics test for the honey bee tracheal mite, *Acarapis woodi*. *J. Apicult. Res.* 46:195-197
79. Faye I, Wyatt G. 1980. The synthesis of antibacterial proteins in isolated fat body from *Cecropia* silkmoth pupae. *Experientia* 36:1325-1326
80. Fefferman N, Traniello J, Rosengaus R, Calleri D II. 2007. Disease prevention and resistance in social insects: modeling the survival consequences of immunity, hygienic behavior, and colony organization. *Behav. Ecol. Sociobiol.* 61(4):565–77
81. Field J, Solis C, Queller D, Strassmann J. 1998 Social and genetic structure of paper wasp cofoundress associations: Tests of reproductive skew models. *Am. Nat.* 151:545-563
82. Foster K, Ratnieks F. 2005. A new eusocial vertebrate? *Trends Ecol. Evol.* 20:363-364
83. Freitak D, Ots I, Vanatoa A, Hõrak P. 2003. Immune response is energetically costly in white cabbage butterfly pupae. *P. Roy. Soc. Lond. B Bio.* 270:S220-S222
84. Frumhoff P, Baker J. 1988. A genetic component to division of labour within honey bee colonies. *Nature*. 333:358-361
85. Fuchs S, Moritz R. 1998. Evolution of extreme polyandry in the honeybee *Apis mellifera* L. *Behav. Ecol. Sociobiol.* 9:269-275
86. Fukuda H, Sakagami SF. 1968.Worker brood survival in honeybees. *Res. Popul. Ecol.* 10:31–39

87. Gamboa G, Greig E, Thom M. 2002. The comparative biology of two sympatric paper wasps, the native *Polistes fuscatus* and the invasive *Polistes dominulus* (Hymenoptera, Vespidae). *Insect. Soc.* 49:45-49
88. Gamboa G, Noble M, Thorn M, Togal J, Srinivasan R, Murphy B 2004. The comparative biology of two sympatric paper wasps in Michigan, the native *Polistes fuscatus* and the invasive *Polistes dominulus* (Hymenoptera, Vespidae). Insect. Soc. 51:153-157
89. Gamboa G, Austin J, Monnet K. 2005. Effects of different habitats on the productivity of the native paper wasp *Polistes fuscatus* and the invasive, exotic paper wasp *P. dominulus* (Hymenoptera: Vespidae). *Great Lakes Entomol.* 38:170-176
90. Gatehouse H, Malone L. 1998. The ribosomal RNA gene region of *Nosema apis* (Microspora): DNA sequence for small and large subunit rRNA genes and evidence of a large tandem repeat unit size. *J. Invert. Pathol.* 71:97-105
91. Gerloff C, Ottmer B, Schmid-Hempel P. 2003. Effects of inbreeding on immune response and body size in a social insect, *Bombus terrestris*. *Funct. Ecol.* 17:582–89
92. Gershman S. 2008. Sex-specific differences in immunological costs of multiple mating in *Gryllus vocalis* field crickets. *Behav. Ecol.* 19:810-815
93. Ghosh R, Ball B, Willcocks M, Carter M. 1999. The nucleotide sequence of sacbrood virus of the honey bee: An insect picorna-like virus. *J. Gen. Virol.* 80:1541-1549
94. Gillespie J, Kanost M, Trenczek T. 1997. Biological mediators of insect immunity. *Ann. Rev. Entomol.* 42:611-643
95. Gilliam M, Shimanuki H. 1970. Coagulation of hemolymph of the larval honey bee (*Apis mellifera* L.). *Experientia* 26:908-909
96. Gilliam M, Taber S, Richardson G. 1983. Hygienic behavior of honey bees in relation to chalkbrood disease. *Apidologie* 14:29–39

97. Giorgi M, Arlettaz R, Christe P, Vogel P. 2001. The energetic grooming costs imposed by a parasitic mite (*Spinturnix myoti*) upon its bat host (*Myotis myotis*). *P. Roy. Soc. Lond. B Bio.* 268:2071–75
98. Giraud T, Pedersen J, Keller L. 2002. Evolution of supercolonies: the Argentine ants of southern Europe. *Proc. Natl. Acad. Sci. USA* 99(9):6075–79
99. Godfrey S, Bull C, Murray K, Gardner M. 2006. Transmission mode and distribution of parasites among groups of the social lizard *Egernia stokesii*. *Parasitol. Res.* 99:223–30
100. Gorman M, Cornel A, Collins F, Paskewitz S. 1996. A shared genetic mechanism for melanotic encapsulation of CM-sephadex beads and a malaria parasite, *Plasmodium cynomolgi* B, in the mosquito, *Anopheles gambiae*. *Exp. Parasitol.* 84:380-386
101. Govan V, Brözel V, Allsopp M, Davison S. 1998. A PCR detection method for rapid identification of *Melissococcus pluton* in honeybee larvae. *Appl. Environ. Microbiol.* 64:1983-1985
102. Govan V, Allsopp M, Davison S. 1999. A PCR detection method for rapid identification of *Paenibacillus larvae*. Appl. Environ. Microbiol. 65:2243-2245
103. Gramacho K. 1999. *Fatores que interferem no comportamento higiênico das abelhas* Apis mellifera. PhD thesis. Univ. São Paulo. 225 pp.
104. Gramacho K, Spivak M. 2003. Differences in olfactory sensitivity and behavioral responses among honey bees bred for hygienic behavior. *Behav. Ecol. Sociobiol.* 54:472–79
105. Grozinger C, Fan Y, Hoover S, Winston M. 2007. Genome-wide analysis reveals differences in brain gene expression patterns associated with caste and reproductive status in honey bees (*Apis mellifera*). *Mol. Ecol.* 16:4837–48

106. Hänfling B, Kollmann J. 2002. An evolutionary perspective of biological invasions. *Trends Ecol. Evol.* 17:545-546
107. Hamilton W. 1964a. The Genetical Evolution of Social Behaviour. I. *J. Theor. Biol.* 7:1-16
108. Hamilton W. 1964b. The Genetical Evolution of Social Behaviour. II. *J. Theor. Biol.* 7:17-52
109. Hamilton W. 1987. Kinship, recognition, disease, and intelligence: constraints of social evolution. In *Animal Societies: Theory and Facts*, ed. Y Ito, J Brown, J Kikkawa, pp. 81–102. Tokyo: Jpn. Sci. Soc.
110. Hammer M. 1993. An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* 366:59–63
111. Hammer M, Menzel R. 1995. Learning and memory in the honeybee. *J. Neurobiol.* 15:1617–30
112. Harris JW. 2007. Bees with *Varroa*-sensitive hygienic preferentially remove mite infested pupae aged ≤ five days post capping. *J. Apic. Res.* 46:134–39
113. Hathaway M. 1981. *Polistes gallicus* in Massachusetts (Hymenoptera: Vespidae). *Psyche* 88:169-173
114. Haviola S, Kapari L, Ossipov V, Rantala M, Ruuhola T, Haukioja E. 2007. Foliar phenolics are differently associated with *Epirrita autumnata* growth and immunocompetence. *J. Chem. Ecol.* 33:1013-1023
115. Heinrich B. 1980. Mechanisms of body-temperature regulation in honeybees, *Apis mellifera*. *J. Exp. Biol.* 85:61–87
116. Heinrich B. 1985. The social physiology of temperature regulation in honeybees. In *Experimental Behavioral Ecology and Sociobiology*, ed. B Hölldobler, M Lindauer, pp. 393–406. Sunderland, MA: Sinauer

117. Henshaw M. 2000. Microsatellite loci for the social wasp *Polistes dominulus* and their application in other Polistine wasps. *Mol. Ecol.* 9:2155-2157
118. Hetru C, Hoffmann D, Bulet P. 1998. Antimicrobial peptides from insects. In *Molecular Mechanisms of Immune Responses in Insects*, ed. P Brey, D Hultmark, pp. 40–66. Chapman & Hall: London
119. Hildebrandt H, Muller U. 1995. Octopamine mediates rapid stimulation of protein kinase A in the antennal lobe of honeybees. *J. Neurobiol.* 27:44–50
120. Hoffmann J. 2003. The immune response of *Drosophila*. *Nature* 426(6):33–38
121. Holway D, Suarez A. 1999. Animal behavior: an essential component of invasion biology. *Trends Ecol. Evol.* 14(8):328–30
122. Huang Z-Y, Robinson G. 1996. Regulation of honey bee division of labor by colony age demography. *Beh. Ecol. Sociobiol.* 39:147-158
123. Hughes D, Beani L, Turillazi S, Kathirithamby J. 2003. Prevalence of the parasite *Strepsiptera* in *Polistes* as detected by dissection of immatures. *Insect. Soc.* 50:62-68
124. Hughes D, Cremer S. 2007. Plasticity in antiparasite behaviours and its suggested role in invasion biology. *Anim. Behav.* 74:1593–99
125. Hughes W, Boomsma J. 2004. Genetic diversity and disease resistance in leaf-cutting ant societies. *Evolution* 58(6):1251–60
126. Hughes W, Boomsma J. 2006. Does genetic diversity hinder parasite evolution in social inset colonies? *J. Evol. Biol.* 19:132-143
127. Hultmark D. 1993. Immune reactions in *Drosophila* and other insects: a model for innate immunity. *Trends Genet.* 9:178-183
128. Ibrahim A, Spivak M. 2006. The relationship between hygienic behavior and suppression of mite reproduction as honey bee

mechanisms of resistance to *Varroa destructor*. *Apidologie* 37:31–40

129. Jacot A, Scheuber H, Kurtz J, Brinkhof M. 2005. Juvenile immune system activation induces a costly upregulation of adult immunity in field crickets *Gryllus campestris*. *P. Roy. Soc. Lond. B Bio.* 272:63-69
130. Johnson R, Evans J, Robinson G, Berenbaum M. 2009. Changes in transcript abundance relating to colony collapse disorder in honey bees (*Apis mellifera*). *P. Roy. Soc. Lond. B Bio.* 106:14790-14795
131. Johnson R, Starks P. 2004. A surprising level of genetic diversity in an invasive wasp: *Polistes dominulus* in the northeastern United States. *Ann. Entomol. Soc. Am.* 97:732–737
132. Johnson R, Zaman M, Decelle M, Siegel A, Tarpy D, Siegel E, Starks P. 2005. Multiple micro-organisms in chalkbrood mummies: evidence and implications. *J. Apicult. Res.* 44:29-32
133. Jones JC, Hellweill P, Beekman M, Maleszka R, Oldroyd BP. 2005. The effects of rearing temperature on developmental stability and learning and memory in the honey bee, *Apis mellifera*. *J. Comp. Physiol.* 191(12):1121–29
134. Jones R, Rothenbuhler W. 1964. Behaviour genetics of nest cleaning in honey bees. II. Responses of two inbred lines to various amounts of cyanide-killed brood. *Anim. Behav.* 12:584–88
135. Kapari L, Haukioja E, Rantala M, Ruuhola, T. 2006. Defoliating insect immune defense interacts with induced plant defense during a population outbreak. *Ecology* 87:291-296
136. Kathirithamby J. 1998. Host-parasitoid associations of *Strepsiptera*: Anatomical and developmental consequences. *Int. J. Insect Morphol.* 27:39-51
137. Kathirithamby J. 2009. Host-parasitoid associations in *Strepsiptera*. *Ann. Rev. Entomol.* 54:227-249

138. Keil D, Luebke R, Pruett S. 2001. Quantifying the relationship between multiple immunological parameters and host resistance: Probing the limits of reductionism. *J. Immunol.* 167:4543-4552.
139. Keller L, Reeve H. 1994. Partitioning of reproduction in animal societies. *Trends Ecol. Evol.* 9:98-102
140. Klaudiny J, Albert T, Bachanova K, Koperick J, Simuth J. 2005. Two structurally different defensin genes, one of them encoding a novel defensin isoform, are expressed in honeybee *Apis mellifera*. *Insect Biochem. Mol. Biol.* 35:11–22
141. Kleinhenz M, Bujok B, Fuchs S, Tautz H. 2003. Hot bees in empty broodnest cells: heating from within. *J. Exp. Biol.* 206:4217–31
142. Klemola N, Klemola T, Rantala M, Ruuhola T. 2007. Natural host-plant quality affects immune defence of an insect herbivore. *Entomol. Exp. Appl.* 123:167-176
143. Kluger M. 1979. *Fever, its Biology, Evolution, and Function*. Princeton, NJ: Princeton Univ. Press. 196 pp.
144. König C, Schmid-Hempel P. 1995. Foraging activity and immunocompetence in workers of the bumble bee *Bombus terrestris* L. *P. Roy. Soc. Lond. B Bio.* 260:225–27
145. Korner P, Schmid-Hempel P. 2004. In vivo dynamics of an immune response in the bumble bee *Bombus terrestris*. *J. Invertebr. Pathol.* 87:59-66
146. Koskimäki J, Rantala M, Taskinen J, Tynkknynen K, Suhonen J. 2004. Immunocompetence and resource holding potential in the damselfly, *Calopteryx virgo* L. Beh. Ecol. 15:169–173
147. Kozikowski S, Wolfram L, Alfano R. 1984. Fluorescence spectroscopy of eumelanins. *IEEE J. Quantum Elect.* 20:1379-1382
148. Kraaijeveld A, Limentani E, Godfray H. 2001a. Basis of the trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. *P. Roy. Soc. Lond. B Bio.* 268:259-261

149. Kraaijeveld A, Hutcheson K, Limentani E, Godfray H. 2001b. Costs of counterdefenses to host resistance in a parasitoid of Drosophila. *Evolution* 55:1815-723
150. Lapidge KL, Oldroyd BP, Spivak M. 2002. Seven suggestive quantitative trait loci influence hygienic behavior of honey bees. *Naturwissenschaften* 89:565–68
151. Lavine M, Strand M. 2002. Insect hemocytes and their role in immunity. *Insect Biochem. Mol.* 32:1295–309
152. Leclerc V, Pelte N, El Chamy L, Martinelli C, Ligoxygakisz P, Hoffmann J, Reichhart J.-M. 2006. Prophenoloxidase activation is not required for survival to microbial infections in *Drosophila*. *EMBO Rep.* 7:231-235
153. Lee K, Cory J, Wilson K, Raubenheimer D, Simpson, S. 2006. Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. *P. Roy. Soc. Lond. B Bio.* 273:823-829
154. Lee K, Klasing K. 2004. A role for immunology in biological invasions. *Trends Ecol. Evol.* 19:523-529
155. Leonard C, Ratcliffe N, Rowley A. 1985. The role of prophenoloxidase activation in non-self recognition and phagocytosis by insect blood cells. *J. Insect Physiol.* 31:789-799
156. Levine J, D'Antonio C. 2003. Forecasting biological invasions with increasing international trade. *Conserv. Biol.* 17:322-326
157. Liebert A, Gamboa G, Stamp N, Curtis T, Monnet K, et al. 2006. Genetics, behavior and ecology of a paper wasp invasion: *Polistes dominulus* in North America. *Ann. Zool. Fenn.* 43(5–6):595–624
158. Liebert A, Johnson R, Switz G, Starks PT. 2004. Triploid females and diploid males: underreported phenomena in *Polistes* wasps? *Insectes Soc.* 51:205–11
159. Liebert A, Starks P. 2006. Taming of the skew: transactional models fail to predict reproductive partitioning in the paper wasp *Polistes dominulus*. *Anim. Behav.* 71:913-923

160. Liebert A, Sumana A, Starks PT. 2005. Diploid males and their triploid offspring in the paper wasp *Polistes dominulus*. *Biol. Lett.* 1:200–3
161. Liersch S, Schmid-Hempel P. 1998. Genetic variation within social insect colonies reduces parasite load. *P. Roy. Soc. Lond. B Bio.* 265:221–25
162. Lobo J, Del Lama M, Mestriner M. 1989. Population differentiation and racial admixture in the Africanized honeybee (*Apis mellifera* L.). *Evolution* 43:794-802
163. Lourenço A, Zufelato M, Bitondi M, Simoes Z. 2005. Molecular characterization of a cDNA encoding prophenoloxidase and its expression in *Apis mellifera*. *Insect Biochem. Mol.* 35:541–52
164. Lu Z, Jiang H. 2007. Regulation of phenoloxidase activity by high- and low-molecular-weight inhibitors from the larval hemolymph of *Manduca sexta*. *Insect Biochem. Mol.* 37:478–85
165. Luster M, Portier C, Pait D, Rosenthal G, Germolec D, Corsini E, et al. 1993. Risk assessment in immunotoxicology. *Fund. Appl. Toxicol.* 21:71-82
166. Magasanik D, Metchnikoff E, Coons A. 1984. Concerning the relationship between phagocytes and *Anthrax bacilli*. *Rev. Infect. Dis.* 1:761-770
167. Mallon E, Loosli R, Schmid-Hempel P. 2003. Specific versus nonspecific immune defense in the bumblebee, *Bombus terrestris* L. *Evolution* 57(6):1444–47
168. Manfredini F, Beani L, Taormina M, Vannini L. 2010a. Parasitic infection protects wasp larvae against a bacterial challenge. *Microbes Infec*. 12:727-735
169. Manfredini F, Benati D, Beani L. 2010b. The strepsipteran endoparasite *Xenos vesparum* alters the immunocompetence of its host, the paper wasp *Polistes dominulus*. *J. Insect Physiol.* 56:253-259

170. Manfredini F, Dallai R, Ottaviani E. 2008. Circulating hemocytes from larvae of the paper wasp *Polistes dominulus* (Hymenoptera, Vespidae). *Tissue Cell* 40(2):103–12
171. Mattila H, Burke K, Seeley T. 2008. Genetic diversity within honeybee colonies increases signal production by waggle-dancing foragers. *P. Roy. Soc. Lon. B.* 275:809-816
172. Mattila H, Seeley T. 2010. Promiscuous honeybee queens generate colonies with a critical minority of waggle-dancing foragers. *Behav. Ecol. Sociobiol.* 64:875-889
173. Masterman R, Ross R, Mesce K, Spivak M. 2001. Olfactory and behavioral response thresholds to odors of diseased brood differ between hygienic and nonhygienic honey bees (*Apis mellifera* L.). *J. Comp. Physiol*. 187:441–52
174. Masterman R, Smith BH, Spivak M. 2000. Brood odor discrimination abilities in honey bees (*Apis mellifera* L.) using proboscis extension reflex conditioning. *J. Insect Behav.* 13:87–101
175. McMullan J, Brown M. 2006. The influence of small-cell brood combs on the morphometry of honeybees (*Apis mellifera*). *Apidologie* 37:665-672
176. Mead F, Pratte M, Gabouriaut G. 1990. Influence of a difference in temperature and day-time conditions on the progression of the society life in *Polistes dominulus* Christ reared at the laboratory (Hymenoptera: Vespidae). *Insect. Soc.* 37:236-250
177. Mercer AR, Menzel R. 1982. The effects of biogenic amines on conditioned and unconditioned responses to olfactory stimuli in the honeybee *Apis mellifera*. *J. Comp. Physiol.* 145:363–68
178. Meredith P, Sarna T. 2006. The physical and chemical properties of eumelanin. *Pigm. Cell Res.* 19:572-594
179. Metchnikoff E. 1884. Concerning the relationship between phagocytes and *Anthrax bacilli*. *Virchows Arch. A.* 97:502-506

180. Moller H. 1996. Lessons for invasion theory from social insects. *Biol. Conserv.* 78:125–42
181. Momot JP, Rothenbuhler WC. 1971. Behaviour genetics of nest cleaning in honey bees. VI. Interactions of age and genotype of bees, and nectar flow. *J. Apic. Res.* 10:11–21
182. Mondragon L, Spivak M, Vandame R. 2005. A multifactorial study of the resistance of Africanized and hybrid honey bees *Apis mellifera* to the mite *Varroa destructor* over one year in Mexico. *Apidologie* 36: 345–58
183. Moret Y, Schmid-Hempel P. 2001. Immune defence in bumble-bee offspring. *Nature* 414:506
184. Moritz RFA. 1988. A reevaluation of the two-locus model for hygienic behavior in honey bees (*Apis mellifera* L.). *J. Hered.* 79:257–62
185. Mostajeran M, Edriss M, Basiri M. 2006. Analysis of colony and morphological characters in honey bees (*Apis mellifera meda*). *Pak. J. Biol. Sci.* 9:2685-2688
186. Mullen L, Goldsworthy G. 2006. Immune responses of locusts to challenge with the pathogenic fungus *Metarhizium* or high doses of laminarin. *J. Insect Phys.* 52:389-398
187. Naug D, Camazine S. 2002. The role of colony organization on pathogen transmission in social insects. *J. Theor. Biol.* 215:427–39
188. Nesse R, Williams G. 1995. *Why We Get Sick: The New Science of Darwinian Medicine*. New York: Times Books. 291 pp.
189. Nigam Y, Maudlin I, Welburn S, Ratcliffe N. 1997. Detection of phenoloxidase activity in the hemolymph of tsetse flies, refractory and susceptible to infection with *Trypanosoma brucei rhodesiense*. *J. Invert. Pathol.* 69:279-281
190. Nonacs P, Liebert A, Starks P. 2006. Transactional skew and assured fitness return models fail to predict patterns of cooperation in wasps. *Am. Nat.* 167:467-480

191. Nürnberger T, Brunner F, Kemmerling B, Piater L. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. *Immun. Rev.* 198:249-66
192. Ochiai M, Ashida M. 1988. Purification of a β-1,3-glucan recognition protein in the prophenoloxidase activating system from hemolymph of the silkworm, *Bombyx mori*. *J. Biol. Chem.* 263:12056-12062
193. O'Donnell S, Beshers S. 2004. The role of male disease susceptibility in the evolution of haplodiploid insect societies. *P. Roy. Soc. Lond. B Bio.* 271:979–83
194. Oldroyd B. 2007. What's killing American honey bees? *PLoS Biology* 5:1195-1199
195. Oldroyd B, Fewell J. 2007. Genetic diversity promotes homeostasis in insect colonies. *Trends Ecol. Evol.* 22:408-413
196. Oldroyd B, Fewell J. 2008. Large fitness benefits from polyandry in the honey bee, *Apis mellifera*. *Trends Ecol. Evol.* 23:59-60
197. Otvos L. 2000. Antibacterial peptides isolates from insects. *J. Pept. Sci.* 6:497–511
198. Page R. 1980. The evolution of multiple mating behavior by honey bee queens (*Apis mellifera*). *Genetics* 96:263-273
199. Page R, Erber J. 2002. Levels of behavioral organization and the evolution of division of labor. *Naturwissenschaften* 89:91-106
200. Page R, Robinson G, Fondrk M. 1989. Genetic specialists, kin recognition, and nepotism in honey-bee colonies. *Nature* 338:576-579
201. Palmer K, Oldroyd B. 2000. Evolution of multiple mating in the genus *Apis*. *Apidologie* 31:235-248
202. Park O. 1937. Testing for resistance to American foulbrood in honeybees. *J. Econ. Entomol.* 30:504–12
203. Park O, Pellet F, Paddock F. 1937. Disease resistance and American foulbrood. *Am. Bee J.* 77:20–25

204. Park O, Pellet F, Paddock F. 1939. Results of Iowa's 1937 and 1938 honeybee disease resistance program. *Trans. Iowa State Hortic. Soc.* 1939:401–9
205. Parkinson N, Weaver R. 1999. Noxious components of venom from the pupa specific parasitoid *Pimpla hypochondriaca*. *J. Invert. Pathol.* 73:74-83
206. Parker G. 1984. Sperm competition and the evolution of animal mating strategies. In *Sperm competition and the evolution of animal mating systems* ed. R Smith, pp. 1-60. Academic Press.
207. Paskewitz S, Riehle M. 1994. Response on *Plasmodium* refractory and susceptible strains of *Anopheles gambiae* to inoculated sephadex beads. *Dev. Comp. Immunol.* 18:369-375
208. Payne A, Starks P, Liebert A. 2010. Kin recognition and genetics. In *Encyclopedia of Animal Behavior*, ed. M Breed, J Moore. 2:237-246
209. Peng Y, Fang Y, Xu S, Ge L, Nasr M. 1987. Response of foster Asian honey bee (*Apis cerana* Fabr.) colonies to the brood of European honey bee (*Apis mellifera* L.) infested with parasitic mite *Varroa jacobsoni* Oudemanns. *J. Invertebr. Pathol.* 49:259–64
210. Pickett K, Wenzel J. 2000. High productivity in haplometrotic colonies of the introduced Paper wasp *Polistes dominulus* (Hymenoptera: Vespidae; Polistinae). *J. New York Entomol. S.* 108:314-325
211. Pie M, Rosengaus R, Traniello J. 2004. Nest architecture, activity pattern, worker density and the dynamics of disease transmission in social insects. *J. Theor. Biol.* 226:45–51
212. Pomfret J, Knell R. 2006. Immunity and the expression if secondary sexual traits in a horned beetle. *Behav. Ecol.* 17:466-472
213. Porter S, Williams D, Patterson R, Fowleri H. 1997. Intercontinental differences in the abundance of *Solenopsis* fire ants (Hymenoptera: Formicidae): Escape from natural enemies? *Environ. Entomol.* 26:373-384

214. Postel S, Kemmerling B. 2009. Plant systems for recognition of pathogen-associated molecular patterns. *Semin. Cell Dev. Biol.* 20:1025-1031
215. Pye A. 1974. Microbial activation of prophenoloxidase from immune insect larvae. *Nature* 251:610-613
216. Rantala M, Jokinen I, Kortet R, Vainikka A, Suhonen J. 2002. Do pheromones reveal male immunocompetence? *P. Roy. Soc. Lond. B Bio.* 269:1681–1685
217. Rantala M, Kortet R. 2003. Courtship song and immune function in the field cricket *Gryllus bimaculatus*. *Biol. J. Linn. Soc.* 79:503–510
218. Rantala M, Kortet R. 2004. Male dominance and immunocompetence in a field cricket. *Beh. Ecol.* 15:187-191
219. Rantala M, Kortet R, Kotiaho J, Vainikka A, Suhonen J. 2003. Condition dependence of pheromones and immune function in the grain beetle *Tenebrio molitor*. *Funct. Ecol.* 17:534-540
220. Rantala M, Koskimäki J, Taskinen J, Tynkkynen K, Suhonen J. 2000. Immunocompetence, developmental stability and wingspot size in the damselfly *Calopteryx splendens* L. *P. Roy. Soc. Lond. B Bio.* 267:2453-2457
221. Rantala M, Roff D. 2005. An analysis of trade-offs in immune function, body size and development time in the Mediterranean Field Cricket, *Gryllus bimaculatus*. *Func. Ecol.* 19:323-330
222. Rantala M, Vainikka A, Kortet R. 2003. The role of juvenile hormone in immune function and pheromone production trade-offs: a test of the immunocompetence handicap principle. *P. Roy. Soc. Lond. B Bio.* 270:2257-2261
223. Rasband W. 2007. ImageJ. U. S. National Institutes of Health. Bethesda, Maryland, USA. http://rsb.info.nih.gov/ij/
224. Ratnieks F, Miller D. 1993. Division of honey bee drones during swarming. *Anim. Behav.* 46(4):803–5

225. Reber A, Castella G, Christe P, Chapuisat M. 2008. Experimentally increased group diversity improves disease resistance in an ant species. *Ecol. Lett.* 11:682-689
226. Reeve H, Starks P, Peters J, Nonacs P. 2000. Genetic support for the evolutionary theory of reproductive transactions in social wasps. *P. Roy. Soc. Lond. B Bio.* 267:75-79
227. Ribeiro C, Brehélin M. 2006. Insect haemocytes: What type of cell is that? *J. Insect Physiol.* 52:417–429
228. Ribière M, Triboulot C, Mathieu L, Aurières C, Faucon J-P, Pépin M. 2002. Molecular diagnosis of chronic bee paralysis virus infection. *Apidologie* 33:339-351
229. Richards E, Manderyck B, Edwards J. 2005. Partial amino acid sequence and physiological effects of a 27 kDa parasitism-specific protein present in the plasm of parasitized *Lacanobia oleracea* (Noctuidae). *Physiol. Entomol.* 30:362-371
230. Riddell C, Mallon E. 2005. Insect psychoneuroimmunology: Immune response reduces learning in protein starved bumblebees (*Bombus terrestris*). *Brain Behav. Immun.* 20:135–38
231. Robb T, Forbes M, Jamieson I. 2003. Greater cuticular melanism is not associated with greater immunogenic response in adults of the polymorphic mountain stone weta, *Hemideina maori*. Ecol. Entomol. 28:738-746
232. Robinson G. 1992. Regulation of division of labor in insect societies. *Annu. Rev. Entomol.* 37:637–65
233. Robinson G, Page R. 1988. Genetic determination of guarding and undertaking in honey-bee colonies. *Nature* 333:356-358
234. Rolff J, Reynolds S. (eds.) 2009. *Insect infection and immunity: evolution, ecology, and mechanisms.* Oxford University Press, Oxford, 272pp.

235. Rolff J, Siva-Jothy M. 2002. Copulation corrupts immunity: A mechanism for a cost of mating in insects. *P. Natl. Acad. Sci. USA* 99:9916-9918
236. Rosengaus R, Cornelisse T, Guschanski K, Traniello J. 2007. Inducible immune proteins in the dampwood termite *Zootermopsis angusticollis*. *Naturwissenschaften* 94:25–33
237. Rosengaus R, Maxmen A, Coates L, Traniello J. 1998. Disease resistance: a benefit of sociality in the dampwood termite *Zootermopsis angusticollis* (Isoptera: Termopsidae). *Behav. Ecol. Sociobiol.* 44:125–34
238. Rosengaus R, Traniello J, Chen T, Brown J, Karp R. 1999. Immunity in a social insect. *Naturwissenschaften* 86:588-591
239. Rothenbuhler WC. 1964. Behaviour genetics of nest cleaning in honey bees. I. Responses of four inbred lines to disease-killed brood. *Anim. Behav.* 12:578–83
240. Rothenbuhler WC. 1964. Behaviour genetics of nest cleaning in honey bees. IV. Responses of F1 and backcross generations to disease-killed brood. *Am. Zool.* 4:111–23
241. Ruiz-González M, Brown M. 2006. Males vs workers: testing the assumptions of the haploid susceptibility hypothesis in bumblebees. *Behav. Ecol. Sociobiol.* 60:501–9
242. Rutrecht S, Brown M. 2008. Within colony dynamics of *Nosema bombi* infections: disease establishment, epidemiology and potential vertical transmission. *Apidologie* 39:504-514
243. Ryder J, Siva-Jothy M. 2000. Male calling song provides a reliable signal of immune function in cricket. *P. Roy. Soc. Lond. B Bio.* 267:1171-1175
244. Sadd B, Schmid-Hempel P. 2007. Facultative but persistent transgenerational immunity via the mother's eggs in bumblebees. *Curr. Biol.* 17(24):R1046–47

245. Sadd B, Siva-Jothy M. 2006. Self-harm caused by an insect's innate immunity. *P. Roy. Soc. Lond. B Bio.* 273:2571-2574
246. Sammataro D, Gerson U, Needham G. 2000. Parasitic mites of honey bees: Life history, implications, and impact. *Ann. Rev. Entomol.* 45:519-548
247. Saito A, Ueda K, Imamura M, Miura N, Atsumi S, et al. 2004. Purification and cDNA cloning of a novel antibacterial peptide with a cysteine-stabilized ab motif from the longicorn beetle, *Acalolepta luxuriosa*. *Dev. Comp. Immunol.* 28:1–7
248. Santillán-Galicia M, Otero-Colina G, Romero-Vera C, Cibrián-Tovar J. 2002. *Varroa destructor* (Acari: Varroidae) infestation in queen, worker, and drone brood of *Apis mellifera* (Hymenoptera: Apidae). *Can. Entomol.* 134:381–90
249. Schmid M, Brockmann A, Pirk C, Stanley D, Tautz J. 2008. Adult honeybees (*Apis mellifera* L.) abandon hemocytic, but not phenoloxidase-based immunity. *J. Insect Physiol.* 54:439-444
250. Schmid-Hempel P. 1998. *Parasites in Social Insects*. Princeton, NJ: Princeton Univ. Press. insect systems. 409 pp.
251. Schmid-Hempel R, Crozier R. 1999. Polyandry versus polygyny versus parasites. *Philos. Trans. R. Soc. London Sci. B* 354:507–15
252. Schmid-Hempel P, Ebert D. 2003. On the evolutionary ecology of specific immune defence. *Trends Ecol. Evol.* 18:27-32
253. Schmid-Hempel R, Schmid-Hempel P. 1998. Colony performance and immunocompetence of a social insect, *Bombus terrestris*, in poor and variable environments. *Funct. Ecol.* 12(1):22–30
254. Seeley T. 1985. *Honey Bee Ecology: A Study of Adaptation in Social Life*. Princeton, NJ: Princeton Univ. Press. 201 pp.
255. Seeley T, Tarpy D. 2007. Queen promiscuity lowers disease within honeybee colonies. *P. Roy. Soc. Lond. B Bio.* 274:67–72
256. Sheppard W, Meixner M. 2003. *Apis mellifera pomonella*, a new honeybee subspecies from Central Asia. *Apidologie* 34:367-375

257. Sherman P, Seeley T, Reeve H. 1988. Parasites, pathogens, and polyandry in social Hymenoptera. *Am. Nat.* 131:602–10
258. Siegel AJ, Hui J, Johnson RN, Starks PT. 2005. Honey bee workers as mobile insulating units. *Insectes Soc.* 52:242–46
259. Silagi S, Gamboa G, Klein C, Noble M. 2003. Behavioral differences between two recently sympatric paper wasps, the native *Polistes fuscatus* and the invasive *Polistes dominulus*. *Great Lakes Entomol*. 36:99-104
260. Simberloff D (2000) Global climate change and introduced species in United States forests. Sci Total Environ 262:253-261
261. Simone M, Evans J, Spivak M. 2009. Resin collection and social immunity in honey bees. *Evolution* 63:3016-3022
262. Siva-Jothy M. 1995. 'Immunocompetence': Conspicuous by its absence. *Trends Ecol. Evol.* 10:205-206
263. Siva-Jothy M. 2000. A mechanistic link between parasite resistance and expression of a sexually selected trait in a damselfly. *P. Roy. Soc. Lond. B Bio.* 267:2523-2527
264. Smilanich A, Dyer L, Gentry G. 2009. The insect immune response and other putative defenses as effective predictors of parasites. *Ecology* 90:1434-1440
265. Söderhäll K, Cerenius L. 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. *Curr. Biol.* 10:23-28
266. Sorvari J, Hakkarainen H, Rantala M. 2008. Immune defense of ants is associated with changes in habitat characteristics. *Environ. Ecol.* 37:51-56
267. Sorvari J, Rantala L, Rantala M, Hakkarainen H, Eeva T. 2007. Heavy metal pollution disturbs immune response in wild ant populations. *Environ. Pollut.* 145:324–328
268. Spivak M. 1996. Honey bee hygienic behavior and defense against *Varroa jacobsoni*. *Apidologie* 27:245–60

269. Spivak M, Gilliam M. 1998. Hygienic behaviour of honey bees and its application for control of brood diseases and *Varroa* mites. Part I: hygienic behaviour and resistance to American foulbrood. *Bee World* 79:124–34
270. Spivak M, Gilliam M. 1998. Hygienic behaviour of honey bees and its application for control of brood diseases and *Varroa* mites. Part II: studies on hygienic behaviour since the Rothenbuhler era. *Bee World* 79:165–82
271. Spivak M, Masterman R, Ross R, Mesce KA. 2003. Hygienic behavior in the honey bee (*Apis mellifera* L.) and the modulatory role of octopamine. *J. Neurobiol.* 55:341–54
272. Spivak M, Reuter GS. 1998. Performance of hygienic honey bee colonies in a commercial apiary. *Apidologie* 29:291–302
273. Spivak M, Reuter GS. 2001. *Varroa jacobsoni* infestation in untreated honey bee (Hymenoptera: Apidae) colonies selected for hygienic behavior. *J. Econ. Entomol.* 94:326–31
274. Starks PT. 2003. Selection for uniformity: xenophobia and invasion success. *Trends Ecol. Evol.* 18(4):159–62
275. Starks PT, Blackie C, Seeley T. 2000. Fever in honeybee colonies. *Naturwissenschaften* 87(5):229–31
276. Starks PT, Gilley DC. 1999. Heat shielding: a novel method of colonial thermoregulation in honeybees, *Apis mellifera*. *Naturwissenschaften.* 86:438–40
277. Starks PT, Johnson RN, Siegel AJ, Decelle MM. 2005. Heat-shielding: a task for youngsters. *Behav. Ecol.* 16:128–32
278. Starks PT, Poe E. 1997. Male-stuffing in wasp societies. *Nature* 389:450–50
279. Strassman J. 2001. The rarity of multiple mating by females in the social Hymenoptera. *Insect. Soc.* 48:1-13

280. Stow A, Briscoe D, Gillings M, Holley M, Smith S, Leys R, et al. 2007. Antimicrobial defences increase with sociality in bees. *Biol. Lett.* 3(4):422–24
281. Sumana A, Starks PT. 2004. Grooming patterns in the primitively eusocial wasp *Polistes dominulus*. *Ethology* 110:825–33
282. Sumpter D, Martin S. 2004. The dynamics of virus epidemics in *Varroa*-infested honey bee colonies. *J. Animal. Ecol.* 73(1):51–63
283. Tanji T, Xiaodi H, Weber A, Ip Y. 2007. Toll and IMD pathways synergistically activate an innate immune response in *Drosophila melanogaster*. *Mol. Cell. Bio.* 27:4578-4588
284. Tarpy D. 2003. Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. *P. Roy. Soc. Lond. B Bio.* 270:99–103
285. Tarpy D, Caren J, Delaney D, Sammataro D, Finley J, Loper G, DeGrandi-Hoffman G. 2010. Mating frequencies of Africanized honey bees in the south western USA. *J. Apicult. Res.* 49:302-310
286. Tarpy D, Nielson D. 2002. Sampling error, effective paternity, and estimating the genetic structure of honey bee colonies (Hymenoptera: Apidae). *Ann. Entomol. Soc. Am.* 95:513-528
287. Tarpy D, Nielsen R, Nielsen D. 2004. A scientific note on the revised estimates of effective paternity frequency in *Apis*. *Insect. Soc*. 51:203-204
288. Tarpy D, Page R. 2001. The curious promiscuity of queen honey bees (*Apis mellifera*): evolutionary and behavioral mechanisms. *Ann. Zool. Fennici.* 38:255-265
289. Tarpy D, Page R. 2002. Sex determination and the evolution of polyandry in honey bees (*Apis mellifera*). *Behav. Ecol. Sociobiol.* 52:143-150
290. Tarpy D, Seeley T. 2006. Lower disease infections in honeybee (*Apis mellifera*) colonies headed by polyandrous vs monandrous queens. *Naturwissenschaften* 93:195-199

291. The Honeybee Genome Sequencing Consortium. 2006. Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* 443:931-949
292. Thompson J. 1994. The geographic mosaic theory of coevolution. In: *The coevolutionary process*. University of Chicago Press: Chicago, pp. 219-238
293. Thompson V. 1964. Behaviour genetics of nest cleaning in honey bees. III. Effect of age of bees of a resistant line on their response to disease-killed brood. *J. Apic. Res.* 3:25–30
294. Tibbetts E, Curtis T. 2007. Rearing conditions influence quality signals but not individual identity signals in *Polistes* wasps. *Behav. Ecol.* 18:602-607
295. Traniello J, Rosengaus R, Savoie K. 2002. The development of immunity in a social insect: evidence for the group facilitation of disease. *Proc. Natl. Acad. Sci. USA* 99(10):6838–42
296. Trivers R, Hare H. 1976. Haplodiploidy and the evolution of the social insects. *Science* 191:249-263
297. Trudeau D, Washburn J, Volkman L. 2001. Central role of hemocytes in *Autographa californica* M nucleopolyhedrovirus pathogenesis in *Heliothis virescens* and *Helicoverpa zea*. *J. Virol*. 75:996-1003
298. Trump RF, Thompson VC, Rothenbuhler WC. 1967. Behaviour genetics of nest cleaning in honey bees. V. Effect of previous experience and composition of mixed colonies on response to disease-killed brood. *J. Apic. Res.* 6:127–31
299. Tyler E, Adams S, Mallon E. 2006. An immune response in the bumblebee, *Bombus terrestris* leads to increased food consumption. *BMC Physiol.* 6:6
300. Tzou P, Reichhart J-M, Lemaitre B. 2002. Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to

infection in immunodeficient *Drosophila* mutants. *Proc. Natl. Acad. Sci.USA.* 99:2152–57

301. Vainikka A, Rantala M, Seppälä O, Suhonen J. 2007. Do male mealworm beetles, *Tenebrio molitor*, sustain the honesty of pheromone signals under immune challenge? *Acta Ethol.* 10:63-72
302. Vainio L, Hakkarainen H, Rantala M, Sorvari J. 2004. Individual variation in immune function in the ant *Formica exsecta*; effects of the nest, body size and sex. *Evol. Ecol.* 18:75–84
303. van Zon A, Overmeet W, Helle W. 1964. Resistance to tedion in haploid and diploid offspring of *Tetranychus urticae* Koch. *Entomol. Exp. Appl.* 7:270-276
304. Vaughn L, Bernheim H, Kluger M. 1974. Fever in the lizard *Dipsosaurus dorsalis*. *Nature* 252:473–74
305. Visscher PK. 1980. Adaptations of honey bees (*Apis mellifera*) to problems of nest hygiene. *Sociobiol.* 5:249–60
306. Washburn J, Kirkpatrick B, Volkman L. 1996. Insect protection against viruses. *Nature* 383:767
307. Weinstock G, Robinson G, Gibbs R, Worley K, Evans J, Maleszka R, et al. 2006. Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* 443:931–49
308. Wheeler, W. 1928. *The Social Insects: Their origin and evolution*. London: Paul, Trench, Trubner and Co. 378 pp.
309. Whiteman N, Parker P. 2004. Effects of host sociality on ectoparasite population biology. *J. Parasitol.* 90(5):939–47
310. Wilson D, Sober E. 1989. Reviving the superorganism. *J. Theor. Biol.* 136:337–56
311. Wilson E. 1971. *The Insect Societies*. Cambridge, MA: Harvard University Press
312. Wilson E. 1975. *Sociobiology: The New Synthesis*. Cambridge, MA: Belknap. 697 pp.

313. Wilson K, Cotter S, Reeson A, Pell J. 2001. Melanism and disease resistance in insects. *Biol. Lett.* 4:637-649
314. Wilson K, Thomas M, Blanford S, Doggett M, Simpson S, Moore S. 2002. Coping with crowds: density dependent disease resistance in desert locusts. *Proc. Natl. Acad. Sci. USA* 99:5471–75
315. Wilson-Rich N, Dres S, Starks P. 2008. The ontogeny of immunity: development of innate immune strength in the honey bee (*Apis mellifera*). *J. Insect. Physiol.* In press
316. Wilson-Rich N, Rosengaus R, Starks P. 2010. Book Review: Rolff, J. & Reynolds, S.E. (Eds.) 2009: Insect infection and immunity: evolution, ecology, and mechanisms. *Myrmecol. News* 13:80
317. Wilson-Rich N, Spivak M, Fefferman N, Starks P. 2009. Genetic, individual, and group facilitation of disease resistance in insect societies. *Ann. Rev. Entomol.* 54:405-423
318. Wilson-Rich N, Starks P. 2010. The *Polistes* war: weak immune function in the invasive *P. dominulus* relative to the native *P. fuscatus*. *Insect. Soc.* 57:47-52
319. Wilson-Rich N, Stuart R, Rosengaus R. 2007. Susceptibility and behavioral responses of the dampwood termite *Zootermopsis angusticollis* to the entomopathogenic nematode *Steinernema carpocapsae*. *J. Invertebr. Pathol.* 95:17–25
320. Winston M. 1987. *The Biology of the Honey Bee*. Cambridge, MA: Harvard Univ. Press. 281 pp.
321. Woodrow A, Holst E. 1942. The mechanism of colony resistance to American foulbrood. *J. Econ.* 35:327–30
322. Wootton E, Dyrynda E, Ratcliffe N. 2006. Interaction between non-specific electrostatic forces and humoral factors in haemocyte attachment and encapsulation in the edible cockle, *Cerastoderma edule*. *J. Exp. Biol.* 209:1326-1335
323. Woyke J. 1963. What happens to diploid drone larvae in a honeybee colony. *J. Apicul. Res.* 2:73–76

324. Woyke J. 1969. A method of rearing diploid drones in a honeybee colony. *J. Apicul. Res.* 8:65–74
325. Woyke J. 1973. Reproductive organs of haploid and diploid drone honeybees. *J. Apicul. Res.* 12:35–51
326. Zachary D, Hoffman D. 1984. Lysozyme is stored in the granules of certain haemocyte types in *Locusta*. *J. Insect Physiol.* 30:405-411
327. Zettervall C-J, Anderl I, Williams M, Palmer R, Kurucz E, Ando I, Hultmark D. 2004. A directed screen for genes involved in *Drosophila* blood cell activation. *Proc. Natl. Acad. Sci.* 101:14192-14197
328. Zipfel C, Felix G. 2005. Plants and animals: A different taste for microbes? *Curr. Opin. Plant Biol.* 8:353-360
329. Zuk M, Simmons L, Rotenberry J, Stoehr A. 2004. Sex differences in immunity in two species of field crickets. *Can. J. Zool.* 82:627-634