THE RELATIONSHIP BETWEEN SUBTERRANEAN TERMITES AND SOILBORNE PATHOGENS

A Dissertation

by

CARLOS MCGEE AGUERO

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Chair of Committee,	Edward L. Vargo
Committee Members,	Michel A. Slotman
	Raul F. Medina
	Tawni L. Crippen
Head of Department,	Pete D. Teel

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ABSTRACT

Social insects are among some of the most ecologically successful species. However, social living can also carry severe costs, such as an increased risk of disease transmission. In response, social insects have evolved social immunity, which comprises a variety of defenses that provide colony-wide disease protection. Studies investigating the diversity and success of social immune defenses have largely focused on the social Hymenoptera (bees, wasps, and ants). Termites, however, have independently evolved social immunity and serve as an important counterpoint to studies with Hymenoptera. In this thesis, I investigate several facets of social immunity using the subterranean termite, *Reticulitermes flavipes*.

In my first two objectives, I tested if changes in genetic diversity influenced social immunity at both the individual and collective levels. In contrast to what has been found in several Hymenoptera species, increased genetic diversity does not appear to improve immunity in *R. flavipes*. Next, I determined if colonies of *R. flavipes* are able to manipulate the surrounding environmental microbial communities in order to create more favorable nest conditions. I found that microbial community diversity is significantly reduced in the foraging galleries and on the cuticle, compared to the surrounding soil. In addition, I found evidence that the foraging galleries of *R. flavipes* host beneficial bacterial associations and colonies may accumulate pathogen loads as they age. My final objective aimed to determine if infected individuals alter their immune behaviors in order to decrease the risk of disease transmission throughout the colony. Termites display shaking alarms in response to pathogens and may become less active as an infection becomes lethal, but I found that these changes in behavior were not influenced by the presence of nestmates. Additionally, instead of self-isolating from the colony, infected individuals congregated with large numbers of workers. Together, these results illustrate how social immunity can be

achieved through a variety of mechanisms and highlight the need for further research across different taxa.

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Contributors

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CHAPTER I

INTRODUCTION

The evolution of eusociality in insects has repeatedly led to remarkable ecological success (Wilson 1990). Species that are eusocial have overlapping generations, cooperative brood care, and a reproductive division of labor. This division of labor allows for the efficient allocation of a worker caste to accomplish tasks that benefit one or a few reproductives. Typically, workers forego their own reproduction and take on other responsibilities, such as brood care, foraging, nest homeostasis, and colony defense. As most eusocial colonies are headed by a small number of reproductives, there is high relatedness among nestmate workers, which elevates indirect fitness benefits and increases their inclusive fitness (Hamilton 1964). However, eusociality also carries severe costs. High worker density, high relatedness, and closed nests strongly increase the chance of pathogen transmission, suggesting that these species are vulnerable to disease outbreaks (Hamilton 1987). In response, social insects have evolved various social mechanisms to protect themselves against pathogens and parasites, collectively known as social immunity.

Social immunity is a term that encompasses a wide variety of defenses that provide colony-wide pathogen resistance (Cremer et al. 2007, Cremer et al. 2018, Liu et al. 2019b). These defenses are physiological or behavioral responses that are exhibited by individuals, but collectively work together as an immune system for the colony. Grooming (Peng et al. 1987, Drees et al. 1992, Oi and Pereira 1993, Rosengaus et al. 1998b, Hughes et al. 2002, Wilson-Rich et al. 2007, Yanagawa and Shimizu 2007, Liu et al. 2019a), nest hygiene (Howard and Tschinkel 1976, Siebeneicher et al. 1992, Trumbo et al. 1997, Julian and Cahan 1999, Bot et al. 2001, Hart and Ratnieks 2002, Ballari et al. 2007, Sun and Zhou 2013), and the removal of diseased individuals (Heinze and Walter 2010, Rueppell et al. 2010, Davis et al. 2018, Pull et al. 2018) are just some examples of social immunity. The diversity and effectiveness of immune strategies that have

evolved in social insects have contributed greatly to their success by mitigating some of the costs of social living.

A majority of studies investigating social immunity have been focused on the insect order Hymenoptera. However, eusociality has evolved independently in other orders as well. The other major group of eusocial insects are termites in the order Blattodea. Although these orders share many similarities in their level of sociality, they actually differ sharply in key aspects of their biology, making termites an intriguing counterpoint to studies of Hymenoptera (Thorne 1997, Nalepa 2015). By analyzing the differences between both orders, we can deepen our understanding of the underlying mechanisms influencing social immunity.

One particular area of interest in social immunity is how species circumvent the consequences of low genetic diversity within colonies. Increased relatedness within colonies allows diseases to transmit to new hosts more easily (Shykoff and Schmid-Hempel 1991, Palmer and Oldroyd 2003, van Baalen and Beekman 2006, Bourgeois et al. 2012, Evison et al. 2013, Lee et al. 2013, Denier and Bulmer 2015). In social Hymenoptera, colonies may increase their level of genetic diversity by increasing the number of reproductives (Bourke and Franks 1995, Crozier and Fjerdingstad 2001) and in several species this results in improved pathogen resistance (Liersch and Schmid-Hempel 1998, Baer and Schmid-Hempel 1999, Baer and Schmid-Hempel 2001, Palmer and Oldroyd 2003, Tarpy 2003, Hughes and Boomsma 2004, Seeley and Tarpy 2006, Tarpy and Seeley 2006, Reber et al. 2008, Bourgeois et al. 2012, Mattila et al. 2012, Evison et al. 2013, Lee et al. 2013). Termites do not exhibit variation in the initial number of breeders within a colony and, instead, are generally founded by a single pair of reproductives (Vargo 2019). However, genetic diversity within a termite colony can change over time. Many termite species are able to develop new reproductives from workers within the colony, which leads to increased inbreeding.

There is also the possibility for separate colonies to merge, creating a more diverse workforce. Evidence in some termite species suggests that low genetic diversity can have negative impacts on colony health (Calleri et al. 2006, DeHeer and Vargo 2006). However, there still has not been a thorough investigation of how changes in colony breeding system can alter social immunity.

The conditions within social insect nests make them ideal for undesirable microbes to grow. This is especially true for species that live in subterranean environments, as soil harbors a diverse microbiota. Part of the success of ants inhabiting soil environments is due to their metapleural glands. This gland produces antimicrobial compounds and allows them to continually disinfect their nests (Brown Jr 1968, Hölldobler and Engel-Siegel 1984, Ortius-Lechner et al. 2000). Subterranean termites do not have metapleural glands, but may be able to achieve protection against harmful microbiota by using their feces and saliva, which have antimicrobial properties, as nest building materials (Rosengaus et al. 1998a, Bulmer and Crozier 2004, Bulmer et al. 2009, Hamilton et al. 2011). Additionally, some termite species can harbor beneficial bacteria within their nest structures, which provide additional disease protection (Visser et al. 2012, Benndorf et al. 2018, Chouvenc et al. 2018).

The success of many social immune defenses hinges on whether the reproductive ability of a colony is protected from disease. Many workers will inevitably be exposed to pathogens and these individuals risk further spreading the disease. Thus, social insect colonies should maintain limited interaction between infected individuals and sensitive castes (reproductives and brood). Social network analyses of ant colonies reveal that behavioral changes after pathogen exposure limit transmission throughout the colony (Stroeymeyt et al. 2018). Some termite species demonstrate clear individual behavioral changes in response to pathogens (Rosengaus et al. 1999, Myles 2002, Wilson-Rich et al. 2007, Yanagawa et al. 2008, Yanagawa et al. 2011b, a, 2012,

Yanagawa et al. 2015, Davis et al. 2018). How these behaviors operate at the colony level is less clear, since some of these behaviors seem to be context dependent. For example, the odor from pathogenic spores typically repels termite workers, but can attract workers when combined with nestmate cues (Yanagawa et al. 2015). Termite colonies may also change their response to infected nestmates depending on the stage of infection. Workers that have been recently exposed to a pathogen are typically groomed of spores, but when the infection has reached lethal or near-lethal levels, infected individuals are more likely to be cannibalized (Davis et al. 2018).

I sought to investigate the relationship between social immunity in the eastern subterranean termite, *Reticulitermes flavipes*, and its pathogens. My first objective aims to determine if pathogen susceptibility for *R. flavipes* colonies is affected by the degree of inbreeding within the colony. The second objective expands on this question by testing if pathogen resistance is improved when genetic diversity is artificially increased by merging termites from different colonies. In my third objective, I characterize the microbial communities in the soil surrounding termite colonies, within the foraging galleries, and on the cuticular surface of the termites. By comparing the bacteria and fungi outside and within the termite nest and associated galleries, I sought to find indirect evidence that may suggest *R. flavipes* are able to somehow prevent harmful microbes from entering their nest. The final objective examines if infected individuals change their immune behaviors after being grouped with uninfected nestmates and whether they are excluded from sensitive areas in the nest. These objectives aim to provide further insight into termite social immunity and highlight future directions for research.

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CHAPTER II

NATURAL VARIATION IN A SUBTERRANEAN TERMITE'S INDIVIDUAL IMMUNITY IS NOT RELATED TO COLONY INBREEDING

Introduction

The loss of genetic diversity through inbreeding can have severely negative effects on the fitness of an organism. Inbreeding depression may result in a reduced immune response to pathogens, which has been a prominent area of concern in populations with exceptionally low genetic diversity, such as in agricultural monocultures (Zhu et al. 2000) and endangered species (O'Brien et al. 1985). The effects of inbreeding on disease dynamics in social species, however, may be more complex. On the one hand, social species may be more prone to disease outbreaks as group living increases the frequency of interactions between host organisms, and therefore, the risk of disease transmission. On the other hand, these species may benefit from herd immunity, whereby enough resistant individuals in a group may reduce the transmission of a disease (Anderson and May 1985). In most eusocial species, all members of a group (*i.e.*, colony) arise from the reproduction of a small number of breeders, sometimes only a single queen. Thus, in addition to living in densely packed groups, these species must cope with high relatedness among group members, which may hamper herd immunity if related individuals are more likely to succumb to the same disease.

To date, most social immunity studies have focused on social insects, and more specifically on eusocial Hymenoptera (*i.e.*, social bees, wasps and ants). In this group, colony resistance to pathogens is often associated with within-colony genetic diversity, as genetically distinct individuals may vary in their susceptibility to different disease strains (Shykoff and Schmid-Hempel 1991, Palmer and Oldroyd 2003, van Baalen and Beekman 2006, Bourgeois et al.

2012, Evison et al. 2013, Lee et al. 2013, Denier and Bulmer 2015). Genetic diversity within colonies disrupts genotype x genotype interactions (*i.e.*, restores herd immunity), as a pathogen that can infect one host genotype, may be unable to spread if its next host is resistant. Also, genetically distinct individuals may differ in their propensities to detect, survive and respond to different pathogens, theoretically making genetically diverse colonies better protected against a diverse array of disease agents (Hamilton 1987, Sherman et al. 1988, Schmid-Hempel 1998, van Baalen and Beekman 2006). Inversely, increased genetic diversity within a colony may also facilitate infections from a broader range of pathogens (Anderson and May 1985, van Baalen and Beekman 2006). However, as only a fraction of a genetically diverse colony will be susceptible to a single pathogen genotype, the cost per infection is reduced and may not reach the point of endangering the overall survival of the colony (van Baalen and Beekman 2006). In social Hymenoptera, several species are able to increase genetic diversity within a colony by increasing the number of breeders. Empirical evidence in honeybees (Palmer and Oldroyd 2003, Tarpy 2003, Seeley and Tarpy 2006, Tarpy and Seeley 2006, Bourgeois et al. 2012, Mattila et al. 2012, Evison et al. 2013, Lee et al. 2013), bumblebees (Liersch and Schmid-Hempel 1998, Baer and Schmid-Hempel 1999, Baer and Schmid-Hempel 2001), and ants (Hughes and Boomsma 2004, Reber et al. 2008) shows that increased genetic diversity does indeed improve pathogen resistance.

In termites, the relationship between genetic diversity and immunity is less clear. Unlike many Hymenoptera, termites do not exhibit variation in their initial number of breeders within a colony. Instead, colonies are typically founded by a single pair of reproductives—a single primary king and queen (simple family)—and the relatedness between the founders determines the overall level of inbreeding within the colony (Nutting 1969, Eggleton 2010). Yet, genetic diversity within colonies may be altered afterward by changes in colony breeding system (Vargo 2019). Many

termite species may exhibit extended family colonies, where secondary reproductives (*i.e.*, neotenic) develop from the colony's offspring when one or both of the founding primary reproductives dies (Myles 1999). Although secondary reproductives reach sexual maturity, they never develop functional wings and do not leave the colony. Therefore, the reproduction of neotenics extends the life of a colony that would otherwise collapse, at the expense of the colony becoming more inbred over time. Mixed families colonies occur when two separate termite colonies fuse together (Deheer and Vargo 2004, Fisher et al. 2004, Deheer and Kamble 2008, Perdereau et al. 2010, Aguero et al. 2020). Genetic diversity usually increases in mixed families, proportionally to the relatedness of the two original colonies. Potentially, the reproductives of both colonies can interbreed and therefore create new genotypic combinations in the worker force. Thus, genetic diversity in termite colonies is initially limited by having only two founders, but diversity can either decrease (in extended families) or increase (in mixed families) over time. However, the degree to which genetic diversity within a colony affects immunity in termites has still not been thoroughly investigated. Colonies of the dampwood termite *Zootermopsis angusticollis* founded by sibling reproductives carried higher microbial loads on their cuticle compared to outbred colonies, presumably due to reduced grooming (Calleri et al. 2006). In the subterranean termite *Reticulitermes flavipes*, colonies can vary dramatically in their susceptibility to different pathogens (Denier and Bulmer 2015). However, social immunity is not improved when genetic diversity is artificially increased by creating mixed families in the lab (Aguero et al. 2020). In one field site of this species, about 25% of reproductives pairing after a mating swarm were siblings; however, the proportion of sibling-founded mature colonies in the site was significantly lower, suggesting that inbred colonies did not survive over time (DeHeer and Vargo 2006). Yet, despite indirect evidence suggesting a lower survival of inbred colonies, the factors driving inbred

colonies to collapse and the mechanisms underlying improved pathogen resistance through increased within-colony genetic diversity remain unclear.

The difficulty in determining the mechanisms influencing pathogen resistance in these species may stem from their complex 'social immunity', whereby overall colony survival is influenced by physiological and behavioral factors at both individual and collective levels (Cremer et al. 2007, Cremer et al. 2018, Liu et al. 2019b). Individual-level defenses of social insects are the same as those exhibited by solitary insects, like the production of defensive compounds (Brown Jr 1968, Hölldobler and Engel-Siegel 1984, Rosengaus et al. 1998a, Ortius-Lechner et al. 2000, Bulmer and Crozier 2004, 2006, Turillazzi et al. 2006, Rosengaus et al. 2013) and pathogen avoidance (Marikovsky 1962, Epsky and Capinera 1988, Yanagawa et al. 2012). In addition, social insects display collective immune responses based on interactions between at least two individuals, such as allogrooming (Peng et al. 1987, Drees et al. 1992, Oi and Pereira 1993, Rosengaus et al. 1998b, Hughes et al. 2002, Wilson-Rich et al. 2007, Yanagawa and Shimizu 2007, Chouvenc et al. 2009, Liu et al. 2019a), the transfer of antimicrobial substances through trophallaxis (Hamilton et al. 2011b), and nest hygiene (Howard and Tschinkel 1976, Siebeneicher et al. 1992, Trumbo et al. 1997, Julian and Cahan 1999, Bot et al. 2001, Hart and Ratnieks 2002, Ballari et al. 2007, Sun and Zhou 2013). Likely, immunity of social insects relies on complex interactions between individual and collective-level responses. Understanding how genetic diversity influences colony survival requires individual and collective levels to be studied separately so that their contributions can be disentangled.

In this study, we aimed to determine whether genetic diversity affects individual immunity in *R. flavipes*. We sampled termite workers from mature colonies across eight sites distributed throughout four states in the eastern US, where this species exhibits variation in the proportion of

family types and the level of within-colony inbreeding found within populations (Jenkins et al. 1999, Bulmer et al. 2001, DeHeer and Kamble 2008, Vargo et al. 2013, Majid et al. 2018). We challenged individual immune responses of workers from each colony with two strains of a fungal pathogen and used molecular markers to determine the family type and level of inbreeding within each colony.

Methods

Termite sampling

Groups of termite workers were collected from 68 colonies spread among eight sites from Texas (TX1 & TX2), North Carolina (NC1 & NC2), Maryland (MD1 & MD2) and Massachusetts (MA1 & MA2). All collections were made during the summer of 2015. From each colony, 24 workers were kept alive for pathogen bioassays and 20 were directly stored in 100% ethanol for subsequent genetic analyses. The location of sites and the number of nests collected in each site are summarized in Figure II-1 and Appendix A-1. Within each site, all nests were separated from each other by at least 15 m, as this distance is sufficient to ensure that each nest represents a distinct colony (Vargo 2003, DeHeer and Vargo 2004, DeHeer et al. 2005).

Pathogen sampling and bioassay

The immune response of each colony was determined by testing the individual immunity of termite workers against two entomopathogenic fungal strains and a control solution. Both pathogens used were strains of *Metarhizium* collected in this study. Soil samples were collected and pathogens were isolated from soil using a mealworm baiting assay (Hughes et al. 2004, Denier and Bulmer 2015). Fungal isolates were identified following the molecular methodology of Denier and Bulmer (2015). One of these strains was isolated from soil collected at a site from which termites were sampled (site MD1), and is referred to in this study as the "local" strain. The other strain was isolated from soil collected in Huntly, Virginia at elevations where *R. flavipes* is not found, and is referred to as "naïve" in our study. Both pathogen strains were prepared at the concentration of 1×10^7 conidia/mL in a 0.1% TWEEN®80 (Sigma-Aldrich Chemie N.V, The Netherlands) solution.

The control treatment was the 0.1% TWEEN®80 solution by itself. 24 termite workers from each colony (n = 68 colonies) were individually placed in 60mm petri dishes that were lined with filter paper moistened with 300 μL dH_2O for two days prior to the start of the experiment. Then, 300 μL of either the pathogen or the control solution was applied to the filter paper. After 24 hours of exposure, the treated filter paper was replaced with filter paper that had been moistened with 300 μL dH_2O and termite survival was monitored for 21 days.

Genetic Analyses

For each colony, family type and level of inbreeding were determined using DNA from 20 termite workers extracted by a modified PureGene extraction protocol (Appendix A-2). Extracted DNA was amplified at nine microsatellite loci that have been previously developed for this species (Vargo 2000, Dronnet et al. 2004). Microsatellite markers, PCR conditions, and multiplex arrangements are described in Appendix A-2. Amplicons were visualized on an ABI 3500 capillary sequencer against a LIZ500 internal standard (Applied Biosystems) and scored using the software Geneious v.9.1 (Kearse et al. 2012). The inbreeding coefficient F_{IC} for each colony was calculated using the software FSTAT (Goudet 1995). F_{IC} estimates the homozygosity of individuals within a social insect colony and is analogous to F_{IS} (Thorne et al. 1999, Bulmer et al. 2001, Vargo 2003). To account for genetic differences between sites, F_{IC} was calculated separately for each site. The family type of each colony was determined by observing the number and frequency of alleles within each colony. Colonies with more than four alleles at a locus were classified as mixed families, as more than two unrelated reproductives would be necessary to produce this result. Colonies that had no more than four alleles at a locus, but had genotypic combinations that were inconsistent with a monogamous pair of reproductives (for example, an allele paired with itself

and two others) were classified as an extended family. When colonies had no more than four alleles at a locus, and genotypic combinations typical of a simple family, a G-test was used to determine if the frequency of genotypes observed was significantly different from what would be expected from a simple family (Vargo 2003). Colonies that differed significantly were categorized as extended families, and those that did not were labeled as simple families.

Statistical Analyses

Hazard ratios of both pathogen strains were calculated for each colony using a Cox proportional-hazards model, which compared the survival of workers exposed to a pathogen with those treated with a control solution. A linear regression was performed to determine the relationship between F_{IC} and hazard ratio for both tested pathogens. The distribution of hazard ratio values for both pathogens was visualized on histograms. A generalized linear model was used to determine whether all of the recorded variables (F_{IC}, family type, and sampling site) individually influenced the hazard ratios or if there were any interaction effects. Also, the effects of site and family type on F_{IC} were determined with an analysis of variance (ANOVA), separately. We also compared the hazard ratios of both pathogen strains with a linear regression and an ANOVA to determine if colonies were consistent in their susceptibility to both pathogen strains and if they were more susceptible to one over the other. All analyses were performed in the statistical software R 3.5.0 (R Core Team 2018).

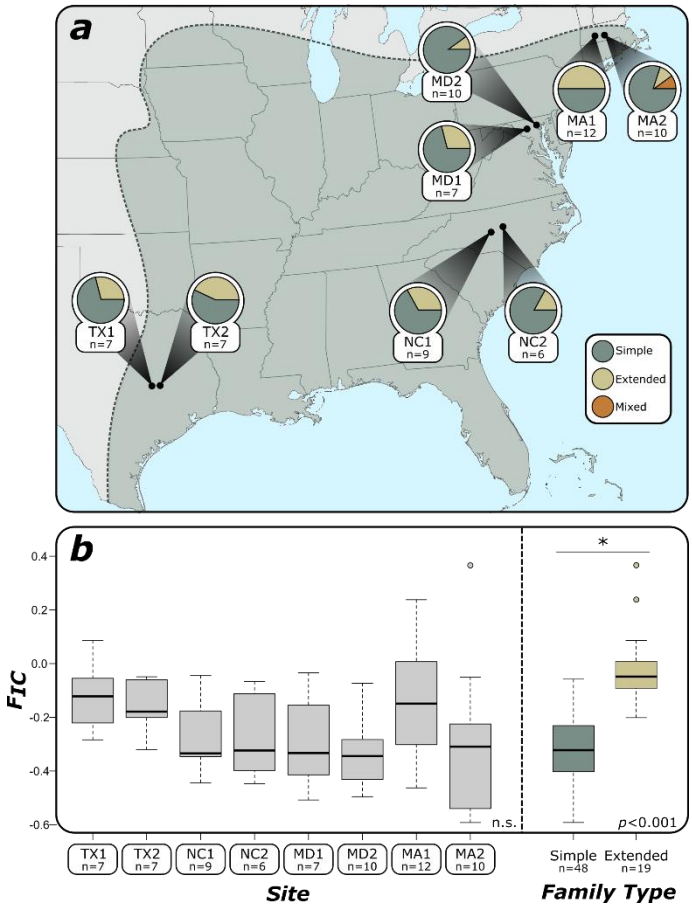

Figure II-1 Distribution of colonies and comparison of the level of inbreeding

(a) Location of termite sampling sites located within the native distribution of *Reticulitermes flavipes* (shaded area of map). Pie charts indicate the proportion of different family types identified from the colonies (n = 68 colonies) collected from each site. (b) There was no significant difference found in colony levels of inbreeding (F_{IC}) between sites (n = 68 colonies, $p < 0.134$). Extended families had significantly higher F_{IC} than simple families (n = 67 colonies, $p < 0.001$). Mixed families were not included in the analysis as only one was found.

Results

Simple and extended families were found in every site that was sampled, but a majority of the collected colonies were simple families. The proportion of simple families found within each site ranged from 50% (site MA2) to 90% (site MD2) (Figure II-1a). Only a single mixed family was found (site MA2). Overall, the level of inbreeding within each colony (F_{IC}) ranged between -0.592 to 0.366 (both from site MA2). There was no significant difference in F_{IC} between sites ($p < 0.134$; Figure II-1b), but F_{IC} did differ between simple families ($F_{IC} \pm SD = -0.320 \pm 0.131$) and extended families ($F_{IC} \pm SD = -0.027 \pm 0.137$) ($p < 0.001$; Figure II-1b).

No significant correlation was found between F_{IC} and the susceptibility of colonies to either pathogen (Local: $p < 0.587$; Naïve: $p < 0.758$; Figure II-2a). The results of a generalized linear model show that there was also no significant effect from family type, level of inbreeding, or sampling site (Appendix A-3). There was a double interaction effect found between simple families and the site TX2, but only for the local strain hazard ratio ($p < 0.043$). Although there was no clear main effects of any one variable we recorded, we do report a bimodal distribution of hazard ratios for both strains of pathogens that were tested, separating colonies into either high or low susceptible groups (Figure II-2b). We also found that colony hazard ratios to both pathogens correlated with each other, such that a colony that was susceptible to one pathogen was also susceptible to the other, and *vice versa* ($p < 0.001$; Figure II-3a). Interestingly, we found that the naïve strain of *Metarhizium* was significantly more lethal than the local strain at the same concentration ($p < 0.001$; Figure II-3b).

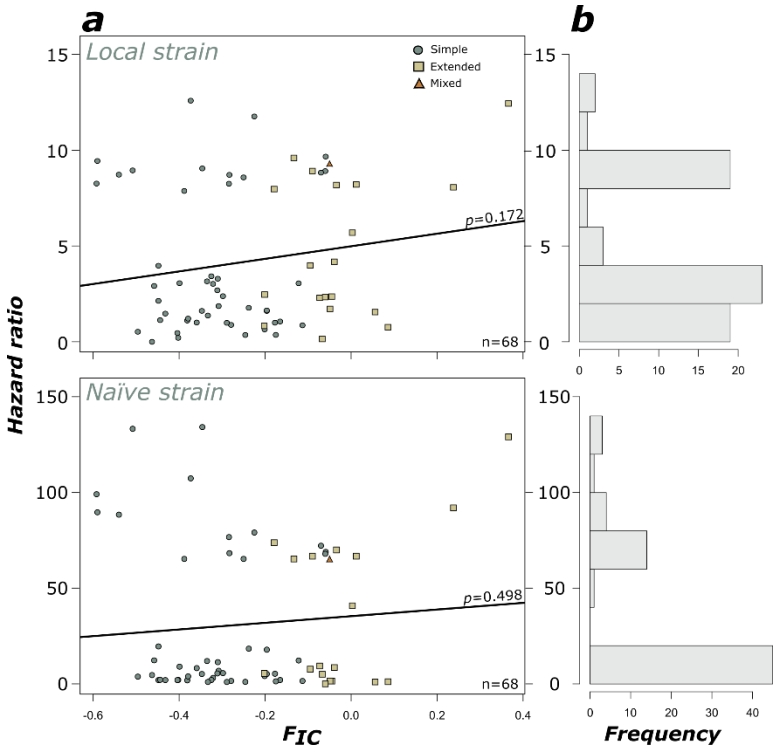

Figure II-2 Inbreeding does not influence pathogen susceptibility

(a) No significant correlation was found between the level of colony inbreeding (F_{IC}) and the hazard ratio for the local strain (n = 68 colonies, p = 0.172) or the naïve strain (n = 68 colonies, p = 0.498). (b) However, histograms reveal a bimodal distribution of hazard ratios for both pathogen strains.

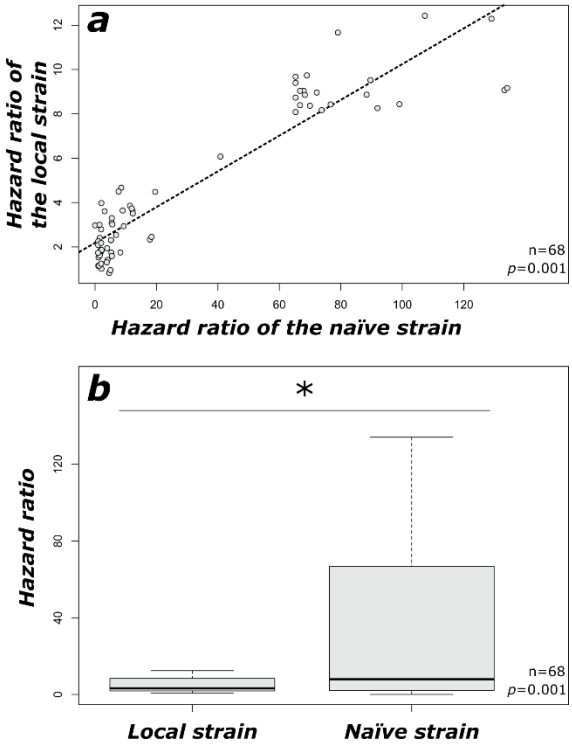

Figure II-3 Comparison between local and naïve pathogen strains

(a) Workers from different colonies were consistent in their hazard ratios for both pathogen strains, such that workers from a colony that was susceptible to the local strain were also susceptible to the naïve strain, and *vice versa* (n = 68 colonies, $p < 0.001$). (b) The naïve strain was significantly more hazardous to termite workers than the local strain (n = 68 colonies, $p < 0.001$).

Discussion

There is natural variation in the level of inbreeding among colonies of *R. flavipes*, which increases in extended families. However, the level of inbreeding did not influence the susceptibility of workers to two strains of a pathogenic fungus. Similarly, pathogen resistance to these strains was not influenced by family type or location. However, workers from different colonies showed either high or low susceptibility, which may suggest that additional factors, such as the genetic background of the colony or its immune priming through previous exposure, may influence survival (Rosengaus et al. 1998b, Rosengaus et al. 1999, Traniello et al. 2002, Hamilton et al. 2011a, Denier and Bulmer 2015, Cole et al. 2020).

Termite colonies are highly variable in their susceptibility to pathogens, such that a pathogen strain that negatively affects one colony may be harmless against another colony (Rosengaus et al. 1998b, Denier and Bulmer 2015). In *R. flavipes*, the difference in virulence in colonies correlates with the genetic distance between strains of *Metarhizium*, suggesting that genetically similar pathogens have similar virulence depending on the workers' colony of origin (Denier and Bulmer 2015). This variation in susceptibility may be explained by genotype x genotype interactions, where some colonies consist of more resistant genotypes towards specific pathogen strains. This may explain the bimodal distribution of worker survival observed in the colonies we studied, whereby individuals exhibited either high or low susceptibility regardless of their level of inbreeding. This finding suggests that pathogen resistance may rely on specific genetic combinations rather than solely on genetic diversity, as workers from low susceptibility colonies may have 100% of the most resistant genotype, despite being highly inbred (van Baalen and Beekman 2006). If pathogen resistance is genetically based, the clear separation between high and low susceptibility observed in our study may suggest that resistance is influenced only by a

few loci. A large number of loci involved (and their random inheritance) would translate into a random distribution of 'resistant' loci among colonies, and would therefore exhibit a more continuous response of worker survival. Additionally, alleles influencing pathogen susceptibility may be dominantly inherited, as recessive inheritance would have likely resulted in a correlation between pathogen susceptibility and inbreeding, which would allow the expression of recessive alleles. Potentially, the few loci involved in pathogen resistance may code for specific immune behaviors (social immunity) or for the production of defensive compounds (individual immunity). Genomic studies have shown that termites carry a full repertoire of immune genes, including all immune-related pathways present in *Drosophila melanogaster*, such as pattern recognition, signaling and gene regulation (Terrapon et al. 2014, Korb et al. 2015).

Individual physiological responses of termites also include the production of gram-negative binding proteins (GNBPs), some of them being termite-specific, and different antimicrobial peptides (AMPs), such as attacin, diptericin, termicins and β-1,3-glucanases (Lamberty et al. 2001, Da Silva et al. 2003, Bulmer et al. 2009). In our study, the bimodal distribution of hazard ratios may illustrate differences in the quantity of defensive compounds produced between colonies. This bimodal distribution may also be explained by differences in the type of defensive compound produced, and their differential effectiveness toward specific strains of pathogens. In our study, we used two strains of the same genus, *Metarhizium*, and found a strong correlation between the hazard ratios of the two strains, meaning that workers from colonies resistant to the 'local' strain also had resistance against the 'naïve' strain. Therefore, colonies with individuals producing a high dose of an effective defensive compound toward this fungal genus would have greater resistance than those with individuals producing a high dose of an ineffective compound or a broad variety of compounds. Interestingly, the genes coding for antimicrobial

peptides (*i.e.*, termicin) show unusually strong signatures of adaptive evolution in *Reticulitermes* species, while they have mostly evolved toward neutrality in species of the genus *Nasutitermes* (Bulmer and Crozier 2004, 2006, Bulmer et al. 2010). However, GNBP genes followed the opposite pattern, as they evolved neutrally in *Reticulitermes* species but under strong positive selection in *Nasutitermes* species (Bulmer and Crozier 2004, 2006, Bulmer et al. 2010). Overall, this suggests that GNBPs and termicin may play complementary roles, potentially targeting different pathogenic agents. Different species, especially those with different habitats, may face distinct selective pressures from fungal pathogens and have therefore evolved distinct antifungal strategies (Terrapon et al. 2014, Korb et al. 2015).

Several termite species may be able to prime their immune defenses against pathogens. Notably, immune priming does not function in the same way as an adaptive immune system, which acts as an immune memory protecting the organism from subsequent exposures to the same pathogen (Janeway et al. 1999). Immune priming prepares the insect's immune system to be more responsive against any imminent pathogenic threat. Activating innate immune responses can carry high fitness costs, so it is crucial that immune priming only occurs when the host organism is under threat (Schmid-Hempel 2005). In our study, the immune priming of some colonies (*i.e.*, highly resistant) shortly before we performed pathogen assays may account for the bimodal distribution of colony survival. The previous exposure of these colonies before collection may have allowed them to anticipate future pathogenic threats, such as the ones we applied in our experiments. In termites, the efficiency of immune priming relies on how well individuals can detect nearby pathogens. In *Z. angusticollis*, individuals that have been previously challenged with non-living pathogenic bacterial cells show improved resistance to live pathogen treatments, which lasts for several days (Rosengaus et al. 1999). Immune priming can also be triggered in individuals that

have never been exposed to the pathogen and have only come into contact with pathogen-challenged nestmates (Traniello et al. 2002). Additionally, *Z. angusticollis* offspring show increased transcription of immune genes when parents have been previously challenged with a pathogen (Cole et al. 2020). In *R. flavipes*, the presence of pathogen components within nests' gallery walls after their degradation may prime nearby termites against subsequent infection attempts (Hamilton et al. 2011a). In this species, variation in colony survival can be explained by differences in detection abilities, as the strength of termite alarm behaviors correlates to colony survival against pathogens (Bulmer et al. 2019). At the collective-level, pathogen detection alerts nestmates to begin social immune responses, such as grooming or cannibalism, whereas at the individual-level, detecting nearby pathogens may initiate immune priming. In this study, we do not have any measure of the termites' ability to detect pathogens. Potentially, though, the increased susceptibility to the naïve strain could be due to reduced immune priming to that specific strain, rather than the shortcomings of defensive compounds.

Beyond immune priming, pathogen detection ability also influences behavioral defenses. When termites detect pathogens, they avoid infected areas (Epsky and Capinera 1988, Yanagawa et al. 2012, Yanagawa et al. 2015). The experiment in this study was designed so that termites must walk on a substrate that has been treated with pathogenic spores. By reducing their own movement on the substrate, they can effectively reduce the number of spores to which they are exposed. In addition, individual termites can also groom themselves, although spores that accumulate in difficult-to-reach parts of the body are most effectively removed through allogrooming (Rosengaus et al. 1998b, Shimizu and Yamaji 2003, Yanagawa and Shimizu 2007, Yanagawa et al. 2008, Chouvenc et al. 2009, Davis et al. 2018, Bulmer et al. 2019).

Our results showed no relationship between genetic diversity and individual immunity in *R. flavipes* colonies. This result is consistent with the absence of improved group immunity through increased genetic diversity in artificially mixed colonies of this species (Aguero et al. 2020). However, these results were found when colonies were challenged with only two specific pathogen strains within the same genus. Together, with the clear separation between resistant and vulnerable colonies observed in this study, these findings suggest that colony survival, at least toward a single pathogen, may rely more on a specific genetic background, rather than be due solely to overall genetic diversity of the colony. How genetic diversity affects overall colony survival against the broad range of pathogens that termite colonies naturally face awaits further investigation bridging genes to collective social behavior.

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CHAPTER III*

INCREASED GENETIC DIVERSITY FROM COLONY MERGING IN TERMITES DOES NOT IMPROVE SURVIVAL AGAINST A FUNGAL PATHOGEN

Introduction

Social insects are among the most abundant and ecologically successful species (Wilson 1990). Their success is inextricably linked to their division of labor where workers engage in different tasks to benefit one or a few reproductives at the expense of their own reproduction. The low number of reproductives in colonies of most species results in high relatedness among nestmate workers, elevating indirect fitness benefits (Hamilton 1964). Paradoxically, their social life also entails severe costs, as high worker densities, high relatedness, and closed nests strongly increase the chance of pathogen transmission, which would suggest that these species are vulnerable to disease outbreaks (Hamilton 1987). Owing to such pathogenic pressure, social insects have evolved social immunity, whereby individual immune functions and behaviors collectively provide colony-wide disease protection (Cremer et al. 2007, Cremer et al. 2018, Liu et al. 2019b). Social immunity includes self/allogrooming (Peng et al. 1987, Drees et al. 1992, Oi and Pereira 1993, Rosengaus et al. 1998b, Hughes et al. 2002, Wilson-Rich et al. 2007, Yanagawa and Shimizu 2007, Liu et al. 2019a), nest hygiene (Howard and Tschinkel 1976, Siebeneicher et al. 1992, Trumbo et al. 1997, Julian and Cahan 1999, Bot et al. 2001, Hart and Ratnieks 2002, Ballari et al. 2007, Sun and Zhou 2013), removal of diseased individuals (Heinze and Walter 2010, Rueppell et al. 2010, Davis et al. 2018, Pull et al. 2018), and the use of antimicrobial compounds either produced by individuals or from materials incorporated into the nest (Brown Jr 1968, Hölldobler

and Engel-Siegel 1984, Gilliam et al. 1988, Ortius-Lechner et al. 2000, Christe et al. 2003, Turillazzi et al. 2006, Chapuisat et al. 2007, Simone et al. 2009, Visser et al. 2012, Rosengaus et al. 2013, Arango et al. 2016, Chouvenc et al. 2018). These diverse immune strategies have undoubtedly reduced the costs of social living, facilitating the success of social insects.

Colony resistance to pathogens is also associated with within-colony genetic diversity, as genetically distinct individuals may vary in their susceptibility to different disease strains (Shykoff and Schmid-Hempel 1991, Palmer and Oldroyd 2003, van Baalen and Beekman 2006, Bourgeois et al. 2012, Evison et al. 2013, Lee et al. 2013, Denier and Bulmer 2015). Therefore, a mix of distinct genotypes within a colony interferes with genotype x genotype interactions, such that a pathogen able to infect one genotype may fail to transmit to new hosts if it encounters host genotypes that it cannot infect. Consequently, a genetically diverse colony may reduce the overall spread of a pathogen. Although variation in susceptibility may increase the likelihood of an infection, it may also prevent the risk that an outbreak of a single strain of pathogen wipes out all individuals, as a diverse colony will only lose a fraction of its population (van Baalen and Beekman 2006). In addition, the efficiency of social immunity may increase with within-colony genetic diversity whereby genetically distinct individuals differ in their propensities to detect, survive and respond to different pathogens. Therefore, genetically diverse colonies may be better protected against the threat of a variety of disease agents (Hamilton 1987, Sherman et al. 1988, Schmid-Hempel 1998, van Baalen and Beekman 2006).

In social Hymenoptera, colonies may increase their level of genetic diversity by the presence of several reproductive queens (*i.e.*, polygyny) and queens mated with several males (*i.e.*, polyandry) (Bourke and Franks 1995, Crozier and Fjerdingstad 2001). Despite these strategies being associated with a reduction of relatedness within colonies (*i.e.*, the indirect fitness of

workers), they have evolved several times in social Hymenoptera (Hughes et al. 2008). One of the main hypotheses suggests that the increased genetic diversity that results from the high number of reproductives in a colony can strengthen their resistance toward pathogens (Hamilton 1987, Sherman et al. 1988, Shykoff and Schmid-Hempel 1991). Empirical studies have shown improved pathogen resistance from polyandry in bumblebees (Liersch and Schmid-Hempel 1998, Baer and Schmid-Hempel 1999, Baer and Schmid-Hempel 2001), honeybees (Palmer and Oldroyd 2003, Tarpy 2003, Seeley and Tarpy 2006, Tarpy and Seeley 2006, Bourgeois et al. 2012, Mattila et al. 2012, Evison et al. 2013, Lee et al. 2013), and ants (Hughes and Boomsma 2004), as well as from polygyny in ants (Reber et al. 2008). The beneficial effect of genetic diversity on social immunity has been widely examined in social Hymenoptera, but its evidence in termites, the other major group of social insects, is scarce.

Termite colonies share several features with Hymenopteran colonies even though sociality evolved independently in this group (Thorne 1997, Nalepa 2015). However, they differ from social Hymenoptera in that their colonies are founded by a single pair of primary reproductives, the queen and the king. Consequently, as termites have a relatedness initially locked at 0.5, they lack the flexibility to potentially buffer variable pathogen pressure with increased genetic diversity by varying the initial number of reproductives. After the founding stage, though, many termite species are able to merge colonies, which can result in a genetically diverse, yet cohesive, workforce (Vargo 2019). The factors underlying colony merging are not well understood. Discrimination between nestmates and non-nestmates may play an important role in colony merging. In the subterranean termite, *Reticulitermes flavipes*, merged colonies had strong similarities in their mitochondrial DNA. The close relatedness between merging colonies, associated with a shared maternally inherited factor, may decrease nestmate recognition and favor colony fusion (DeHeer

and Vargo 2008). Potentially, nestmate recognition could also be related to the gut microbiota, as the microbial communities of termite guts are colony-specific (Minkley et al. 2006). In the species *R. speratus*, colonies can be made to accept foreign workers by experimentally altering the microbial gut communities (Matsuura 2001). In this species, colonies are also more likely to merge if the introduced colony has low proportions of nymphs (Matsuura and Nishida 2001a). Termite nymphs have high resource demands (Thorne 1983), which makes them energetically expensive for the receiving colony. Thus, colony merging may be influenced by the current status of the colonies involved. The costs and benefits of taking in additional, unrelated workers likely vary across different colonies and species. It is also possible that colony merging could not be a specific behavior that is selected for, but a byproduct of distinct colonies expanding their foraging galleries in the same food source. As they consume more wood and expand their foraging galleries, then those colonies would not be able to remain separate.

Colony merging occurs naturally in *R. flavipes* (Jenkins et al. 1999, Bulmer et al. 2001, DeHeer and Kamble 2008, Perdereau et al. 2010, Majid et al. 2018) and this species shows a lack of intercolonial aggression in laboratory assays (Polizzi and Forschler 1999, Fisher et al. 2004). In this study, we investigated whether the increased group diversity through colony merging benefits social immunity and pathogen resistance in *R. flavipes*. We first confirmed the ability of *R. flavipes* to fuse colonies by investigating merging rate and aggression between pairs of colonies through a behavioral assay. Potentially, merged colonies could preferentially groom their relatives, so we also assessed whether social immunity was symmetrical in artificially merged colonies by comparing the amount of grooming between nestmates and non-nestmates. Finally, we tested the hypothesis that increased group diversity enhances pathogen resistance by determining whether the survival of artificially merged colonies was higher than that of single colonies against an

entomopathogenic fungus. Further, we determined if single colonies benefited from specific pairings by comparing the survival of each merged colony pairing to that of its two constituent colonies. Finally, we discuss the ecological and evolutionary factors underlying termite colony merging from the perspective of social immunity.

Methods

Termite and pathogen collection

We collected groups of termites from 20 colonies of *R. flavipes* and one colony of the related species *R. virginicus*. The colonies were sampled from wood debris found in College Station, Texas from October 2018 to February 2019. Based on previous studies, all collections were made at least 15m apart to ensure that each colony was unique (Vargo 2003, DeHeer and Vargo 2004, DeHeer et al. 2005). In *R. flavipes*, mate pairing is random during large, synchronous nuptial flights, leading to an absence of isolation-by-distance patterns at short distances, meaning that two geographically close colonies are not genetically more similar than two geographically distant colonies (Bulmer et al. 2001, Vargo 2003, Vargo and Carlson 2006, Vargo et al. 2006). As a consequence, our sampling colonies likely represent a spectrum of varying levels of relatedness, regardless of their sampling distances. The termites were separated from wood in the lab, assigned an identifying letter (A-T) and maintained in rearing chambers at 85% relative humidity and 27°C. All termites were used in experiments within 2 weeks of collection. The DNA of one worker per colony was extracted and sequenced at the 16S mitochondrial locus to confirm the species identification of the colonies sampled (Appendix B-1).

A field-collected strain of the fungus *Metarhizium robertsii* was used in the pathogenic treatment for this study. This strain was isolated from soil collected from the Sam Houston National Forest, TX using a mealworm baiting method, then cultured on a medium of potato dextrose agar (Hughes et al. 2004). The identity of the strain was confirmed through sequencing analysis of the ITS region (Appendix B-1). Fungal conidia were collected from the medium and suspended in a 0.1% TWEEN80 solution. This solution was concentrated at $1x10^6$ conidia/mL using a hemocytometer (Bulldog Bio, Inc. Portsmouth, NH, USA) and used as a pathogen

treatment. The 0.1% TWEEN80 solution without fungal conidia was used as a control. These pathogen and control solutions were used for all immune challenges in this study.

Colony merging

We performed an agonism assay using the arena described in Chouvenc & Su 2017. Ten workers and one soldier from two different colonies were introduced into opposite sides of a 3x10 cm arena and were allowed to tunnel through sand along a preformed path, until the two groups encountered each other (Chouvenc and Su 2017). We tested every combination of eight different *R. flavipes* colonies (colonies A-H; N = 28) and recorded any signs of aggression between colonies (biting, tunnel blocking, casualties, etc.). As controls, we also tested all eight of these colonies against themselves (N = 8), as well as against the colony of *R. virginicus* (N = 8). All pairings were monitored for behavior every 15 minutes for 3 hours after introduction, which was enough time for termites from both sides to connect the tunnels and interact with each other. Pairings were denoted as aggressive if any signs of aggression between the two groups was observed. The two groups were considered to have merged if there were no signs of aggression and workers freely moved between both sides of the assay.

Grooming behavior

Six colonies (colonies I-N) were used to determine the amount of grooming in the presence of pathogens, as well as to compare the amount of grooming between nestmates and non-nestmates within artificially merged colonies. At the time of collection, a subset of workers from each colony were fed cellulose material containing Nile blue (a fat-soluble stain used to mark termites) so that workers from different colonies could be identified when mixed with another

colony (Figure III-1b). Nile blue has been used in a number of studies to mark termites and has not been reported to affect termite behavior (Traniello et al. 2002, Chouvenc et al. 2008, Yanagawa et al. 2011, Davis et al. 2018). Groups of 20 workers were set up with either 20 workers from the same colony (*i.e.*, single colony), or by combining 10 dyed workers from one colony with 10 undyed workers from another colony to create six merged colonies (**J**xK, Jx**L**, **K**xL, **M**xN, Mx**O** and **N**xO; bolded letters indicate the dyed colony). Groups were isolated in 60mm petri dishes with moist filter paper and allowed to acclimate for 1 week before exposure. Two identical replicates were performed for all single colony (N=6) and merged colony (N=6) groups. Pathogen and control solutions were applied by pipetting 200 µL of solution onto the filter paper in each petri dish. Five-minute videos were recorded for each petri dish 15 minutes after the solution application. The time spent grooming other termites was recorded for every individual and then totaled for each replicate. In merged colonies, the direction of grooming (towards nestmates or non-nestmates) was also recorded for each sub-colony within a merged colony (N = 6).

Survival of single and merged colonies

From the remaining six colonies (colonies O-T), groups of 20 workers were set up with either 20 workers from the same colony (*i.e.*, single colony), or by combining 10 workers each from two different colonies into the same dish (*i.e.*, merged colony; PxQ, PxR, QxR, SxT, SxU and TxU). Groups were isolated in 60mm petri dishes with moist filter paper and allowed to acclimate for 1 week before exposure. All of these combinations (N=6) were simultaneously replicated four times with the same pathogen and control solutions. Treatments were applied by pipetting 200

μL of solution onto the filter paper in each petri dish. Mortality was recorded daily for 2 weeks following the solution application.

Statistical analysis

A Pearson's χ^2 test of independence was used to determine if aggressive behavior was associated with pairings between species, within species, or within colonies. Grooming time was compared between single and merged colonies using a nested ANOVA, with colony status (single colony or merged colony) nested within treatment group. We also used a nested ANOVA to determine if grooming in merged colonies was directed more towards nestmates or non-nestmates, with grooming direction nested within treatment group. Using the *coxph* function implemented in the *survival* package in R (Therneau and Lumley 2015), termite mortality in the survival assay was analyzed using a Cox proportional hazard survival model with the factor of colony status nested within treatment group. Pairwise comparisons using a log-rank test were performed for the survival distributions of every merged colony and its two corresponding single colonies. To avoid inflation of Type I errors, the Benjamini-Hochberg procedure for adjusting p-values was used. All analyses were performed in the statistical software R 3.5.0 (R Core Team 2018).

Results

Intercolonial aggression and colony merging was determined using a behavioral assay designed to test agonism between groups of termites (Chouvenc and Su 2017). We recorded aggressive behaviors exhibited between termites from a colony of *R. flavipes* that were either paired with termites from another *R. flavipes* colony, a group of termites from the same colony, or termites from a colony of the related species, *R. virginicus*. Aggressive behavior was strongly associated with the type of pairing that was tested ($\chi^2 = 38.973$, $p < 0.0005$; Figure III-1a). Out of the 28 pairings between groups of *R. flavipes* originating from two different colonies, 27 merged without any evidence of aggression. Only one pairing (colonies E and H) resulted in aggression. In the positive control, no aggression was found in pairings between two groups of the same colony. In the negative control, all pairings between colonies of *R. flavipes* and *R. virginicus* displayed aggressive behaviors, showing the ability of *R. flavipes* to exhibit agonistic behaviors while in this experimental setting.

We measured grooming in groups of termite workers that either all originated from the same colony or were artificially merged from two different colonies. In these merged groups, one colony was previously fed a blue dye, so that both colonies could be distinguished from each other (Figure III-1b). These single and merged colony groups were exposed to either a control solution or a solution containing conidia of the fungal pathogen, *Metarhizium robertsii*, at a concentration of $1x10^6$ conidia/mL. Then, we recorded the time spent grooming, as well as whether the grooming was directed towards nestmates or non-nestmates. We observed a significant increase in grooming within termite groups exposed to a pathogen, in both single and artificially merged colonies ($p < 0.05$; Figure III-1c). However, the total duration of grooming did not differ significantly between single and merged colonies, in both control and pathogen

groups ($p = 0.8849$; Figure III-1c). In merged colonies, termites did not invest significantly more time in grooming nestmates or non-nestmates, whether they were exposed to a control or pathogen solution ($p = 0.90711$; Figure III-1d).

Colonies exposed to the pathogen treatment had 20-25% lower survival ($p < 0.001$; Figure III-2). We also found that the survival of merged colonies exposed to a pathogen was slightly lower than the survival of single colonies ($p < 0.05$; Figure III-2). When we examined each specific merged colony pairing, we found that in five out of the six pairing combinations, the two single colonies used to build the artificially merged colony had significantly different survivals. In four of these pairings, the merged colony survival aligned with the survival of the more susceptible of the two single colonies (Figures III-3a-d). In the fifth pairing, the merged colony survival matched the more resistant of the two single colonies (Figure III-3e). In the last pairing, where the survival distributions of the two single colonies were not different from each other, the survival of the merged colony did not differ from either of the two single colonies (Figure III-3f). The results of the pairwise comparisons, including those made between controls are provided in Appendix B-2.

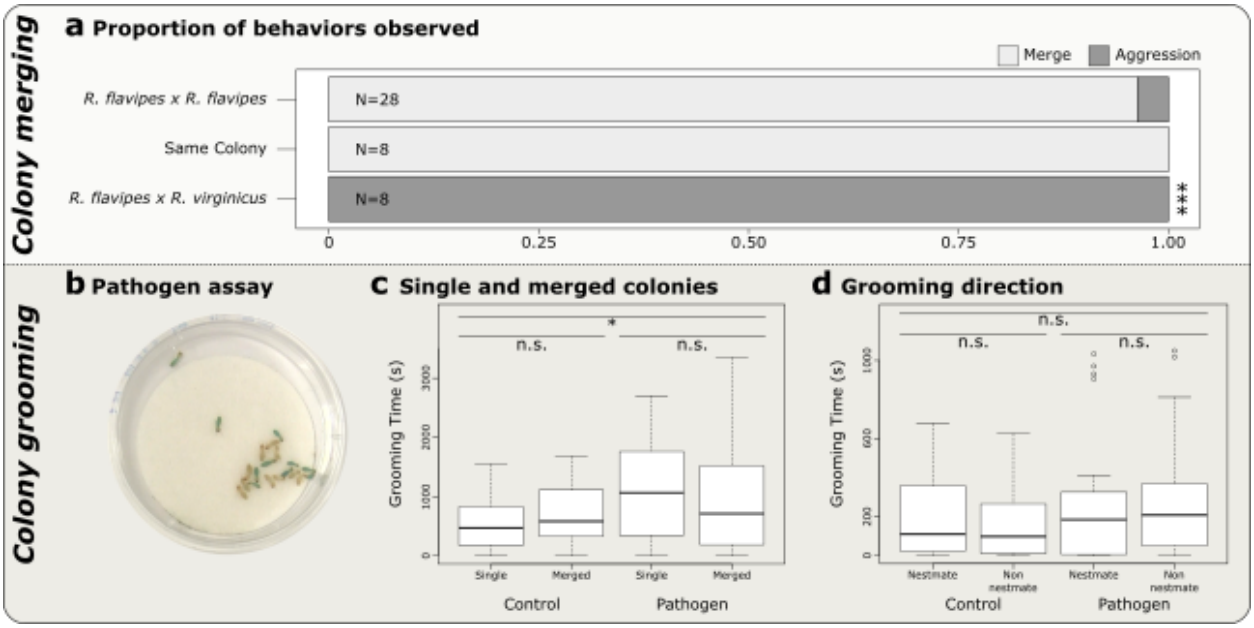

Figure III-1 Colony merging and grooming

(a) The proportion of behaviors observed in each type of pairing in the agonism assay. The asterisks indicates that aggressive behavior was highly associated with interspecific pairings between *R. flavipes* and *R. virginicus* (χ2-test, $p < 0.0005$). (b) The pathogen assay arena with examples of mutual grooming between dyed and undyed termites, originally from different colonies. (c) Colonies exposed to a pathogen treatment spent more time grooming (Nested ANOVA, $p < 0.05$), but there was no difference in grooming between single (N = 6) and merged colonies (N = 6) (Nested ANOVA, $p = 0.8849$). (d) There was no difference in the time that termites from merged colonies spent grooming nestmates or non-nestmates (Nested ANOVA, $p = 0.90711$, N = 6). All analyses were performed in the statistical software R 3.5.0 (https://www.r-project.org/).

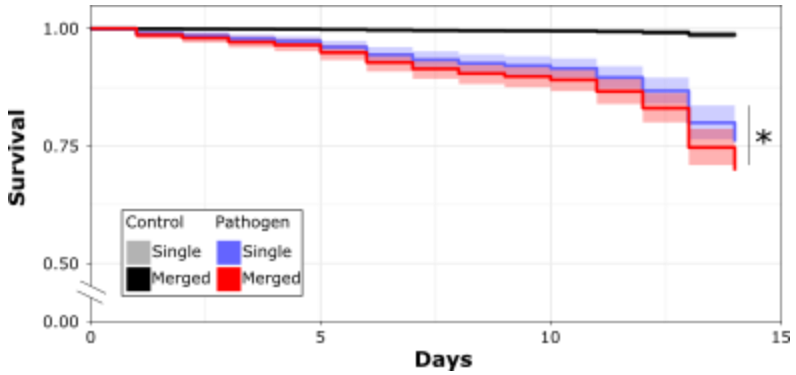

Figure III-2 Survival of single and merged colonies

Kaplan-Meier survival distributions of all single (N = 6) and merged colony (N = 6) groups that were exposed to either a control or pathogen solution. Termites exposed to a pathogen had significantly lower survival than termites which received a control solution ($p < 0.001$). The asterisk indicates that merged colonies were found to have slightly lower survival than single colonies ($p < 0.05$). All analyses were performed in the statistical software R 3.5.0 (https://www.r-project.org/).

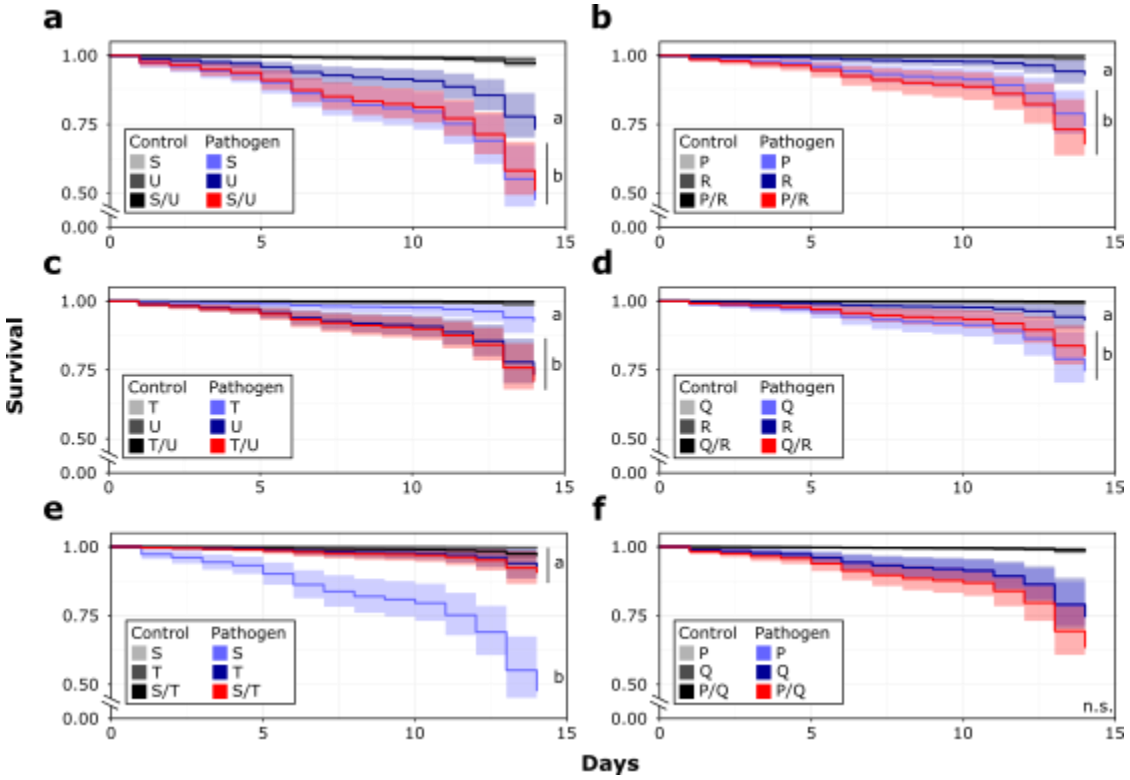

Figure III-3 Survival of merged colony pairs

Kaplan-Meier survival distributions of each merged colony pair (Red, N = 1) are plotted with the survival distributions of their corresponding single colonies (Light and dark blue, N = 2). Bolded letters (a-f) correspond to each of the six groupings of merged and single colonies that were tested. Within each plot, letters denote significant groupings between pathogen treatment groups. The survival distributions of control groups are depicted in the plots, but are not included in groupings. Significance was determined by pairwise comparisons using a log-rank test ($p < 0.05$; Appendix B-2). All analyses were performed in the statistical software R 3.5.0 (https://www.r-project.org/).

Discussion

We confirmed that colonies of this species readily merge in the lab and showed that workers groom nestmates and non-nestmates equally after merging. These two results are prerequisites for colony merging to test for improved resistance to pathogens. However, the survival of merged colonies was not improved from that of single colonies when challenged with a pathogen. Instead, our results showed that the overall survival of each merged colony was heavily influenced by the survival of the colonies from which it was composed. In most cases, the survival of the merged colony was reduced to that of the more susceptible colony, but in one case the survival of the group was raised to that of the more resistant colony. Our study brings little support to the hypothesis that colony merging may improve immunity through an increase of genetic diversity in *R. flavipes*.

In *R. flavipes*, different colonies have been shown to vary considerably in their ability to resist different strains of pathogens (Denier and Bulmer 2015). This finding was supported by our study, as most of the single colonies differed in their survival against the entomopathogenic fungus studied (Figure III-3). However, most studies, including ours, used a single generalist pathogenic agent to challenge colonies. The use of a diverse pathogen cocktail may further test the hypothesis that increased diversity within a colony will provide protection against a broad range of pathogen pressures (van Baalen and Beekman 2006), while it also better represents natural pressures that colonies encounter. In addition, it would be interesting to test whether the use of generalist or specialist pathogen agents have distinct outcomes on colony survival. Termites can display a vibratory alarm to their nestmates in response to pathogens (Rosengaus et al. 1999, Myles 2002). In *R. flavipes* the strength of this alarm varies between colonies and is positively correlated with the time that nestmates spend grooming, which in turn predicts their survival (Bulmer et al. 2019). Thus, variation in survival may also be influenced by the variation among colonies to detect, and

therefore respond or avoid different pathogens. In our study, we ensured the exposure of individuals to the pathogen by flooding the substrate with a pathogen suspension in a small arena. This setup allows the infection of all termites to measure differential survival, but hampers their survival through avoidance. We also showed for the first time that workers equally groom nestmates and non-nestmates after merging (Figure III-1d). One would therefore expect merged colonies to survive at the level of the more resistant colony, as workers with greater detection ability should be able to groom the entire merged colony. However, our finding that survival is lower in merged colonies indicates that the more susceptible colony may determine the level of group susceptibility.

Several mechanisms of social immunity have been examined in termites. Nest structures are typically constructed from fecal material, which can inhibit the growth of harmful microbes (Rosengaus et al. 1998a). Termite nests have also been found to harbor beneficial Actinobacteria, which are known to possess antimicrobial activity (Visser et al. 2012, Chouvenc et al. 2018). Colonies can maintain hygienic conditions by cannibalizing nestmates that are infected or have recently succumbed to disease (Rosengaus and Traniello 2001, Chouvenc and Su 2012, Sun et al. 2013, Davis et al. 2018). Additionally, the 'social transfer' of disease resistance has been reported in the dampwood termite species, *Zootermopsis angusticollis*, where individuals have improved survival against a fungal pathogen after being grouped with individuals that have survived a challenge with the same pathogen (Traniello et al. 2002). However, grooming is one of the most effective mechanisms of social immunity that has been studied in termites. Workers kept in isolation have much greater mortality than those kept in groups after exposure to a fungal pathogen (Rosengaus et al. 1998b, Shimizu and Yamaji 2003, Yanagawa and Shimizu 2007). Termite salivary glands produce antimicrobial peptides which, when applied though grooming, effectively

inhibit the growth of fungal pathogens (Lamberty et al. 2001, Bulmer and Crozier 2004, Hamilton and Bulmer 2012). In addition, grooming allows termites to remove fungal spores attached to the cuticle of a nestmate. These spores are swallowed and ultimately end up in the gut of their nestmates, where they are unable to germinate (Yanagawa and Shimizu 2007, Chouvenc et al. 2009). Even once the conidia have penetrated the cuticle, and the infected termite can no longer be saved, nestmates still show a large grooming response (Davis et al. 2018). This intense grooming may also ultimately lead to cannibalism to prevent dying individuals from proliferating disease. The unyielding nature of termite grooming behavior may help provide constant protection from disease spreading throughout the colony.

Termite colonies undergo developmental changes throughout their lifespan, which may affect the role that genetic diversity plays in immunity. In many termite species, including *R. flavipes*, genetic diversity may decline over time due to the development of secondary reproductives in the colony (*i.e.*, transition to an extended-family) (Myles 1999, Vargo 2019). When the founding king and queen perish, the workers or nymphs of the colony may become secondary kings and queens that engage in repeated inbreeding over time. These secondary reproductives prolong the life of the colony at the expense of reduced genetic diversity within the colony. Thus, extended-family colonies may be more likely to merge in order to restore the level of genetic diversity within colonies. Indeed, naturally merged colonies of *R. flavipes* appear to merge shortly after the death of the founders of one of the constituent colonies (DeHeer and Vargo 2004). Genetic diversity may not only be important to older colonies that experience inbreeding, but also to those that are still young. In *R. flavipes* and *R. virginicus*, the proportion of mature colonies headed by inbred reproductives is lower than the proportion of inbred pairs found during the nuptial flight suggesting that inbreeding depression negatively affects developing colonies

(DeHeer and Vargo 2006). Colony foundation represents an important threshold for termite survival, as young colonies undergo strong selective pressure (Nutting 1969). In *Z. angusticollis,* inbreeding between founding kings and queens produces offspring with increased disease susceptibility in comparison to outbred mating (Calleri et al. 2006). It has also been shown that the effects of pathogen exposure during early colony foundation constrain the reproductive output and overall survival of the future colony in this species (Cole et al. 2018). The king and queen play pivotal roles in incipient colonies. Until the workforce is large enough to completely sustain the colony, the king and queen are responsible for protecting themselves and rearing the first brood. Thus, the survival of incipient colonies is largely dependent on the fitness of the founding pair (Matsuura and Nishida 2001b, Matsuura et al. 2002, Cole et al. 2018).

In social Hymenoptera, strong support for improved disease resistance from increased genetic diversity has been demonstrated in bumblebees (Liersch and Schmid-Hempel 1998, Baer and Schmid-Hempel 1999, Baer and Schmid-Hempel 2001) and honeybees (Palmer and Oldroyd 2003, Tarpy 2003, Seeley and Tarpy 2006, Tarpy and Seeley 2006, Bourgeois et al. 2012, Mattila et al. 2012, Evison et al. 2013, Lee et al. 2013), while similar experiments in ants have produced contrasting results. While several ant species show no relationship between genetic diversity and pathogen resistance (Briano et al. 1995, Pérez-Lachaud et al. 2011, Schmidt et al. 2011), there are some that do. In the leaf-cutting ant, *Acromrymex echinatior*, workers from different patrilines vary in their susceptibility to a fungal pathogen, suggesting that a genetically diverse worker force may increase the overall colony survival against a variety of pathogens (Hughes and Boomsma 2004). In *Formica selysi*, naturally polygynous colonies do not have higher survival than monogynous colonies, but artificially combined workers from different monogynous colonies do show improved disease resistance (Reber et al. 2008). While there is some indication that genetic

diversity could provide health benefits for both ants and termites, strong evidence is still lacking for its relevance in natural settings.

In *R. flavipes* colonies vary in their ability to detect and respond to disease, they will readily merge, and workers will groom each other equally in merged colonies. Despite this, we do not find evidence that genetic diversity improves pathogen resistance in *R. flavipes*. Instead we observed that after merging, one colony heavily influenced the survival of the group. The factors that determine which colony is more influential to immunity after merging remain unknown. Additionally, there are some caveats to this study that may warrant further investigation. While our method of testing merging rates among colony fragments is sufficient to ensure that our merged colonies would not attack each other during the experiment, termites may still maintain colony boundaries without aggression. A study examining whether colony fragments of *R. flavipes* would share foraging and nesting sites reported merging rates to be as low as 55% in laboratory assays (Fisher et al. 2004). In our study, we found balanced grooming within the merged colonies, but the interactions between colonies that have fused may be more complex. Potentially, by allowing mixed colonies to acclimate together, the two colonies may have developed a combined colony odor that may preclude any kin recognition in grooming. As with what has been found in ants, the relationship between genetic diversity and immunity in termites may vary among species. Further studies investigating the proximate mechanisms by which changes in the genetic architecture of merged colonies, such as an increase in allelic diversity, heterozygosity, or differential gene expression, would affect colony survival are clearly worth more attention. Investigations into the potential benefits of colony merging in this and other species are needed to determine whether fitness gains derived from increased genetic diversity drive colony merging in termites.

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CHAPTER IV

REDUCED ENVIRONMENTAL MICROBIAL DIVERSITY ON THE CUTICLE AND IN THE GALLERIES OF A SUBTERRANEAN TERMITE COMPARED TO SURROUNDING SOIL

Introduction

Within insects, the evolution of eusociality has inevitably lead to remarkable ecological success, as evidenced by the rapid diversification of social insect species and their adaptation to a variety of different habitats (Wilson 1990). However, eusociality inherently brings several costs to colony life. The dense aggregation of closely related individuals could promote the spread of disease epidemics. This is especially true for social insects that live in subterranean environments, as soil is teeming with microbial life. Many general pathogens that can infect a broad range of insect hosts are found in the soil and their impact on subterranean social insects, such as ants and termites, has been heavily studied (Keller et al. 1989, Oi and Pereira 1993, Schmid-Hempel 1998, Vega et al. 2009, Chouvenc et al. 2011b). There are also host-specific pathogens that have coevolved with social insects. Fungi in the *Ophiocordyceps unilateralis* species complex are able to manipulate their host ant's behavior to promote their spread throughout the colony, with each species in this group having adapted to a different species of ant (Evans et al. 2011, Hughes et al. 2011). The nest of social insect colonies, often constructed from organic material, can also be colonized by soil-dwelling microbes (Bignell 2006, Jouquet et al. 2006, Chouvenc et al. 2011a). Although many of these saprophytes are not insect pathogens, colonies must still protect themselves from microbes that can infest and consume the nest. In collapsed laboratory colonies of the Formosan subterranean termite, *Coptotermes formosanus*, the colony is no longer able to inhibit the growth of microbes in the nest and, as a result, the remaining resources of the nest are consumed by a succession of saprophytic organisms (Chouvenc et al. 2013a).

Social insects have evolved numerous defenses to contend with such microbial adversity, making them remarkably resilient to disease. These defenses are known as social immunity, the collective organization of individual behaviors and physiological defenses that protect the colony from harmful pathogens (Cremer et al. 2007, Cremer et al. 2018, Liu et al. 2019). Ants have become dominant in soil environments, in part, due to their metapleural glands, a structure that produces antimicrobial secretions (Brown Jr 1968, Hölldobler and Engel-Siegel 1984, Ortius-Lechner et al. 2000). The compounds produced by this gland allow ants to continually disinfect themselves, their nestmates, and their nest structure. Although subterranean termites do not have a metapleural gland, they may be able to similarly reinforce their nests with antimicrobial activity. Termite fecal material, which is used to construct nests, exhibits inhibitory activity on fungal growth (Rosengaus et al. 1998a). Additionally, termites produce antifungal peptides in their salivary glands that may also be incorporated into the nest walls (Bulmer and Crozier 2004, Bulmer et al. 2009, Hamilton et al. 2011).

The termite nest primarily serves the vital function of housing the colony and providing fortification from external threats. Termite nesting strategies can be classified into one of three categories based on the nest's location relative to the colony's food source (Higashi et al. 1992, Eggleton 2010). In a one-piece nesting strategy, termites nest and feed in the same substrate. A separate-piece nesting strategy entails a central nest located in a substrate separate from the food source, best characterized by mound-building termites that must leave their nest to forage. Subterranean termites (Subfamily Rhinotermitidae) utilize an intermediate-nesting strategy that, like one-piece nesting species, will house the colony in a food source, but will then forage through the soil for additional food.

The eastern subterranean termite, *Reticulitermes flavipes*, implements an intermediate-nesting strategy and the nest walls of this species are known to harbor a rich microbial community, although the taxonomic identity of this community remains unknown (Chouvenc et al. 2011a). If *R. flavipes* can selectively maintain the microbial communities in their nests, then they may also be able to limit the penetration of harmful soilborne pathogens. In this study, we characterized the bacterial and fungal communities of the soil surrounding colonies of *R. flavipes*, the substrate lining foraging galleries, and the cuticle of the termites themselves. We examined diversity patterns in these three substrates (*i.e.*, soil, gallery, and cuticle) and taxonomically identified the bacteria and fungi in the communities we sampled. Then, we compared the relative abundance of species within targeted genera of interest among soil, gallery, and termite cuticle samples.

Methods

Soil, gallery, and termite collections

Sampling was performed at the Sam Houston State University Center for Biological Field Studies, a heavily forested site within the Sam Houston National Forest (Huntsville, Texas). Samples were taken from 10 collection points that were separated from each other by at least 15 m. Based on previous studies, this was a sufficient distance to ensure that termites collected at each point represented distinct colonies (Vargo 2003, DeHeer and Vargo 2004, DeHeer et al. 2005). At each collection point, wood debris were opened and inspected for termite activity. In order to sample microbial communities on termites' cuticle, 20 workers and one soldier were collected into vials using forceps. Gallery substrate (a mixture of soil, feces, and wood particles lining the foraging galleries) was then scraped into sampling bags. Finally, a 10 cm deep soil sample was collected from directly beneath the wood debris which contained termites. All samples were collected in triplicate using sterilized tools, stored on ice, and then brought back to the lab for immediate processing. In the lab, each sampled group of termites was washed by 15 minutes of gentle rotation in 500 μL of 0.1% TWEEN®80 (Sigma-Aldrich Chemie N.V, The Netherlands) diluted in ddH_2O. The solution was decanted out and used for DNA extraction.

DNA extraction, barcode amplification, and sequencing

All sampled colonies were confirmed as *R. flavipes* by extracting DNA of one worker from each collection point and sequencing its mitochondrial 16S rRNA gene (Appendix C-1). Then, DNA was extracted from cuticle washes using a modified phenol:chloroform DNA extraction protocol (Appendix C-1). Gallery and soil samples were each homogenized by shaking and then we

extracted DNA from 0.25 g of each substrate using the DNeasy PowerSoil Kit (Qiagen). To identify bacterial communities present in each sample, extracted DNA was amplified at the v4 hypervariable region of the 16S rRNA gene using the primers 515f (5'-GTGCCAGCMGCCGCGGTAA-3') and 806r (5'-GGACTACHVGGGTWTCTAAT-3') (Kozich et al. 2013). Fungal communities were identified by amplifying the ITS2 gene region using the primers ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). (See Appendix C-1 for PCR protocols). Following PCR, amplified DNA was sent to the RTSF Genomics Core at Michigan State University for sequencing. PCR products were batch normalized using SequalPrep (Thermo Scientific). Pooled amplicons were cleaned using a 0.8X Ampure XP beads/pool ratio (Beckman Coulter). Quality was quantified using a combination of Qubit dsDNA HS (Invitrogen), Advanced Analytical Fragment Analyzer High Sensitivity DNA (Agilent) and Illumina Library Quantification qPCR assays (KAPA). Each pool was loaded onto an Illumina MiSeq Standard v2 flow cell (Illumina, San Diego, CA, USA) and sequenced in a 2x250bp paired end format using a MiSeq v2 500 cycle reagent cartridge. Base calling was performed by Illumina Real Time Analysis v1.18.54 and the output was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1.

Data analysis

All analyses were performed using the microbiome bioinformatics platform QIIME 2 (Bolyen et al. 2018). Paired-end reads were filtered for quality control and merged using the DADA2 pipeline (Callahan et al. 2016). With a target median quality score of 30, 16S and ITS sequences were each joined at 250bp and then identified as ASVs. Processing the 16S sequence data produced 32,804

ASVs from 5,592,120 reads across 90 samples. Based on a rarefaction curve of observed OTUs (Appendix C-2) a sampling depth of 19,000 reads was determined to provide sufficient coverage to estimate the diversity of the bacterial communities in soil, gallery, and cuticle samples. Ten samples were removed from the analysis to achieve this sequencing depth (N = 80). For fungal community analysis 5,803 ASVs were identified from 4,329,649 across 90 samples with the ITS sequences. Using the rarefaction curve of fungal observed OTUs (Appendix C-2) we determined that a sampling depth of 10,000 reads provided sufficient coverage to estimate fungal diversity. Two samples were removed from the analysis to achieve this sequencing depth (N = 88).

Phylogenetic trees were constructed for ASVs using MAFFT FastTree implemented in QIIME 2 (Katoh and Standley 2013). The following metrics were calculated to estimate diversity within samples (*i.e.*, alpha diversity): Observed OTUs, ENS/PIE, Faith's Phylogenetic Diversity, Simpson Diversity, and Shannon Diversity. In order to estimate diversity between samples (*i.e.*, beta diversity) weighted and unweighted UniFrac distances were calculated between each sample and plotted using a Principal Coordinates Analysis (PCoA) (Hamady et al. 2010).

ASVs from all samples (N=90) were taxonomically identified using classifiers trained on either the 16S rRNA gene or ITS region. The 16S ASVs were classified using the SILVA v132 database with 99% sequence identity from the 515F/806R sequence region (Pruesse et al. 2007). The ITS ASVs were classified using the UNITE fungal database classifier trained on ITS sequences (Kõljalg et al. 2013). A Venn diagram was constructed showing the number of classified ASVs that were unique or shared among substrates. For each sample, barplots were created that show the taxonomic profile of bacteria and fungi that had been classified at the phylum-level, as this taxonomic level was the most informative for identifying differences between the three substrates. Heatmaps were created to show the relative abundance of species of interest.

Results

Bacterial communities assessed from 16S rRNA gene sequences showed significant differences in all alpha diversity metrics among substrates (Figure IV-1a). Diversity was highest in the soil, lower in the galleries and least on the cuticle. Fungal communities estimated from ITS gene sequences followed a similar pattern only when measuring observed OTUs and Faith's phylogenetic diversity (Figure IV-1b). No significant differences in Effective Number of Species/Probability of Intra- or Interspecific Encounter (ENS/PIE), Shannon, or Simpson diversity were found for fungal communities separated by substrate. To compare community similarity across samples a Principle Coordinates Analysis (PCoA) was used to plot unweighted and weighted UniFrac distances. Bacterial communities were largely separated according to the substrate from which they were sampled (Figure IV-2a). However, this pattern was less clear in fungal communities (Figure IV-2b). When plotting unweighted UniFrac values, soil and cuticle samples separated, but gallery samples did not. In the plot of weighted UniFrac scores, which accounts for abundance, there was no separation by substrate.

Amplicon sequence variants (ASVs) were taxonomically identified using classifiers trained on bacterial and fungal databases. We found a majority of these identified taxa (Bacteria: 83.19%; Fungi: 85.47%) to be exclusive to a single substrate, soil (Figure IV-3). Of the taxa shared between two substrates, the highest numbers were those shared between soil and gallery (Bacteria: 9.39%; Fungi: 5.82%) and the lowest were those shared between soil and cuticle (Bacteria: 0.53%; Fungi: 1.36%). Barplots of bacterial phyla diversity show that the communities sampled from the cuticle are distinct from soil and gallery samples, largely due to the ~50% increase in relative abundance of Spirochaetes (Figure IV-4a). However, while the phylum-level taxonomic profiles of soil and gallery samples are similar, one notable difference is the greater proportion of

Spirochaetes in the galleries. In fungal communities phyla diversity is largely dominated by high proportions (>60%) of Basidiomycota in each sample (Figure IV-4b). However, cuticle samples are distinct from the soil and galleries in that most cuticle communities have higher proportions of other phyla, including more unassigned or unidentified fungi. Patterns in phyla diversity may not reflect the changes that occur in microbes that are in low abundance, but are of ecological importance. Heatmaps were generated to examine the relative abundance of bacterial and fungal species identified in genera of interest (*Streptomyces*, methanotrophic bacteria, and pathogens). Of the three *Streptomyces* species identified, the most diversity and highest relative abundance was found in the gallery communities (Figure IV-5a). The two methanotrophic groups identified were Methylomonas and an uncultured strain of Methylacidiphilales (Figure IV-5b). Methylacidiphilales was the only reported methanotrophic microbe found in the gallery substrate, and it was found in relatively high abundance. Methylomonas was present in low abundance in soil and cuticle samples but was absent from the gallery samples.

A high degree of variation among the relative abundance patterns of harmful microbes was identified (Figure IV-5c). Several known entomopathogenic genera were identified. Some of these genera have been directly investigated as termite pathogens, such as *Bacillus*, *Pseudomonas*, *Serratia*, *Aspergillus*, and *Metarhizium* (Chouvenc et al. 2011b). *Metacordyceps chlamydosporia* and *Lecanicillium antillanum*, although not normally associated with termites, have been investigated as pathogens of other insect pests (Sung et al. 2007, Vega et al. 2009). The fungal genus *Trichoderma*, which does not infect termites, but can colonize their nest structures was found in all three substrates (Chouvenc et al. 2013a). The unclassified *Bacillus* sp. and *Trichoderma harnatum* had high relative abundance in the soil that decreased in gallery and cuticle samples. *Lecanicillium antillanum* and the unclassified *Serratia* sp. were present in highest

proportions on termite cuticle, while unclassified *Pseudomonas* sp. was relatively abundant in all three substrates. There were many differences in relative abundance among the classified species of *Aspergillus*. However, the cuticle communities harbored high proportions of the unclassified *Aspergillus* sp. All *Metarhizium* taxa identified were in the soil, but varied in relative abundance in the galleries and on the cuticles. Interestingly, *Metarhizium robertsii*, which was present in all three substrates, was in lowest relative abundance in the galleries.

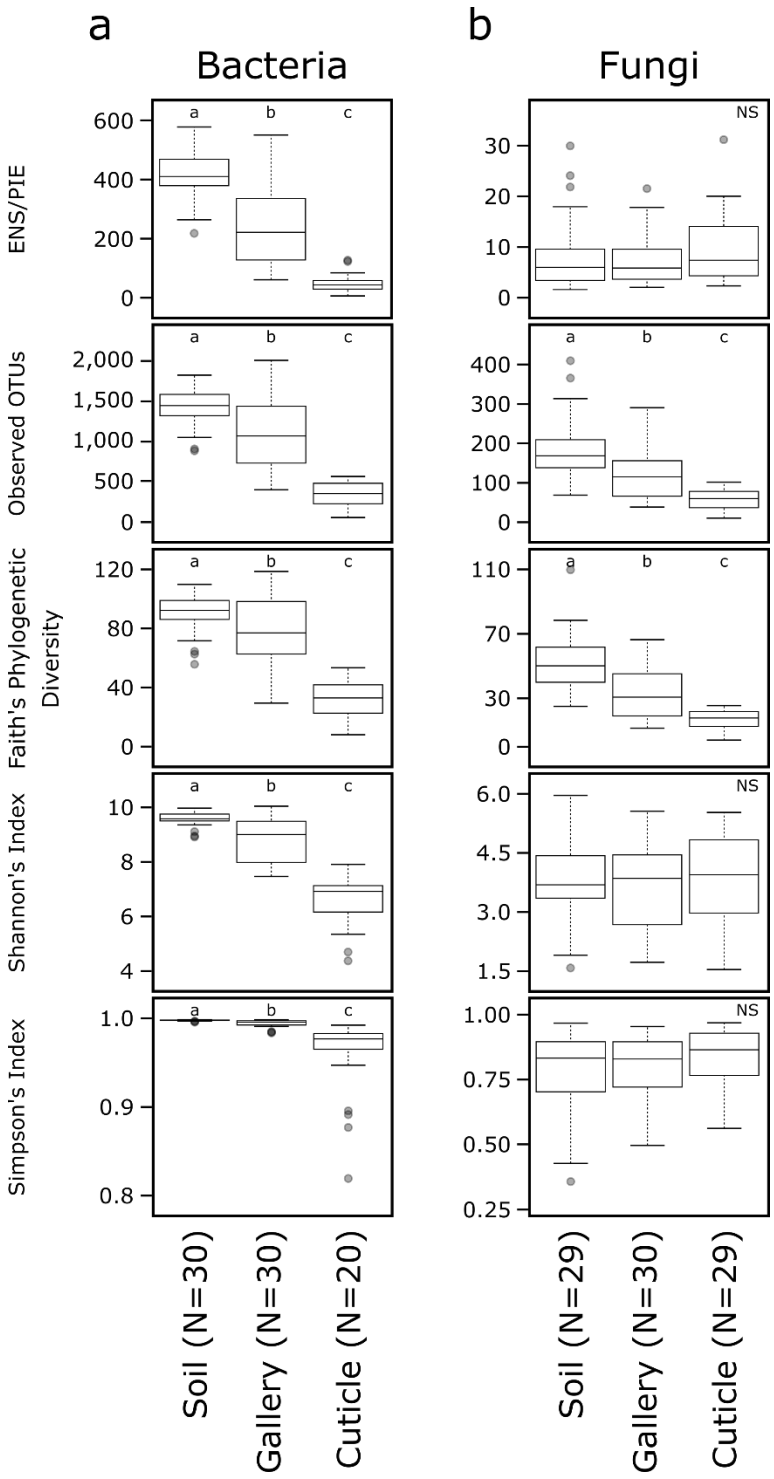

Figure IV-1 Alpha diversity metrics for bacterial and fungal communities in *R. flavipes* colonies

Boxplots show values for Effective Number of Species/Probability of Intra- or Interspecific Encounter (ENS/PIE), Observed OTUs, Faith's Phylogenetic Diversity, Shannon Diversity, and Simpson Diversity in bacterial (a) and fungal (b) communities, separated by substrate. Letters denote significance ($p<0.001$).

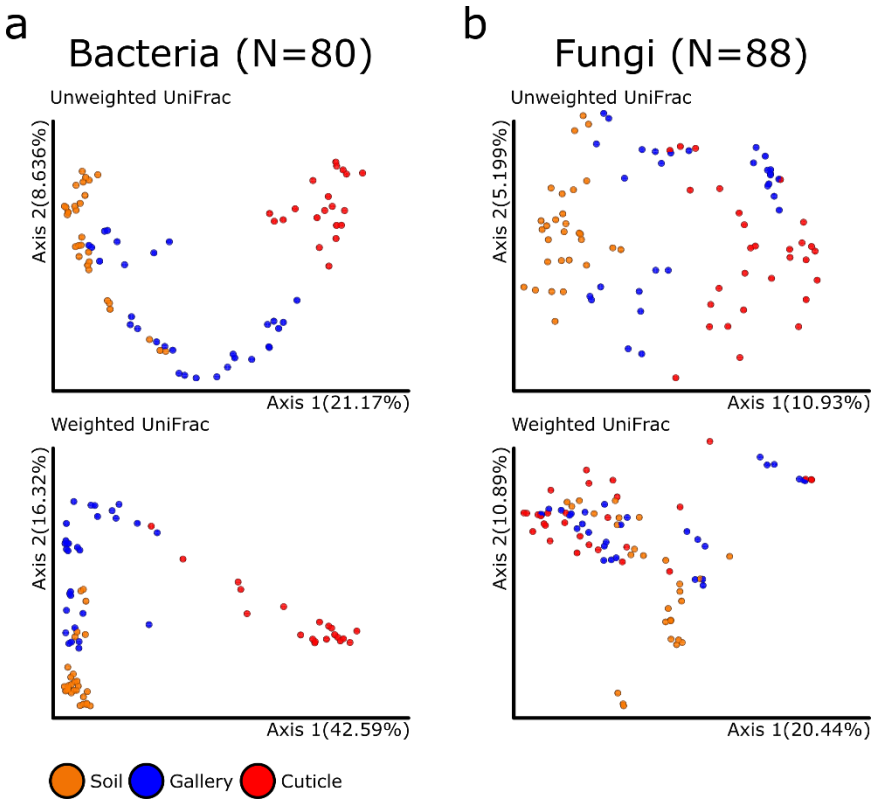

Figure IV-2 Bacterial and fungal community similarity in *R. flavipes* colonies

Principal Coordinates Analysis (PCoA) of UniFrac distances for bacterial (a) and fungal (b) communities. Each community is colored according to whether it was sampled from the soil, galleries, or cuticle.

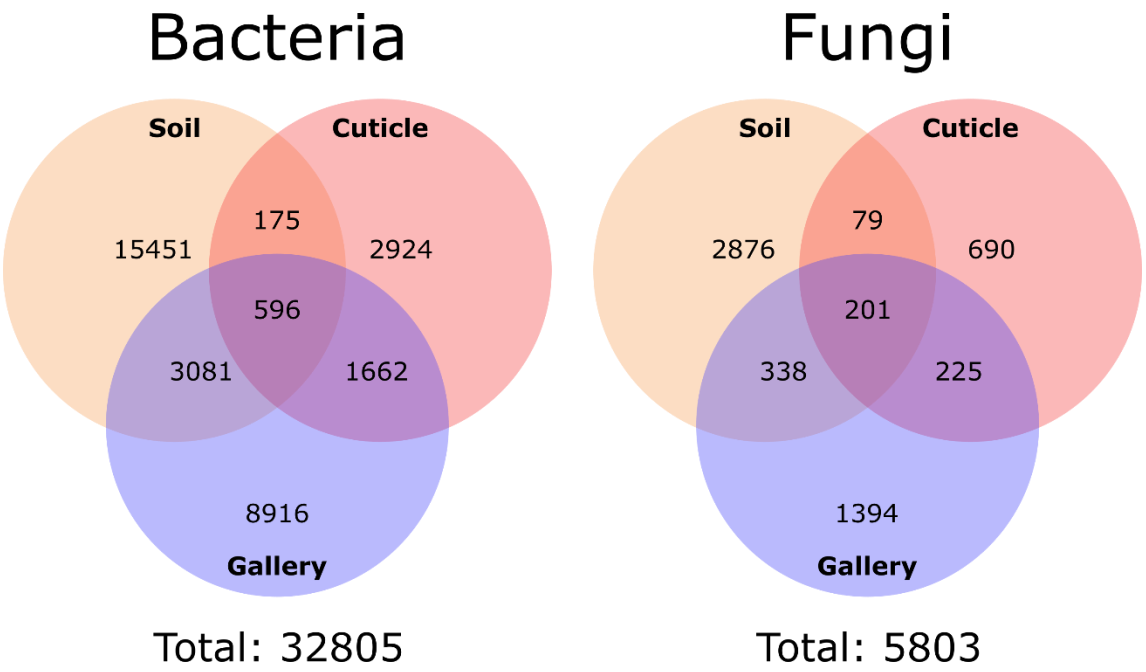

Figure IV-3 Venn diagram of classified bacterial and fungal ASVs in *R. flavipes* colonies

Venn diagram depicts the number of bacterial and fungal ASVs that were unique to a substrate (soil, gallery, or cuticle) or were shared between substrates.

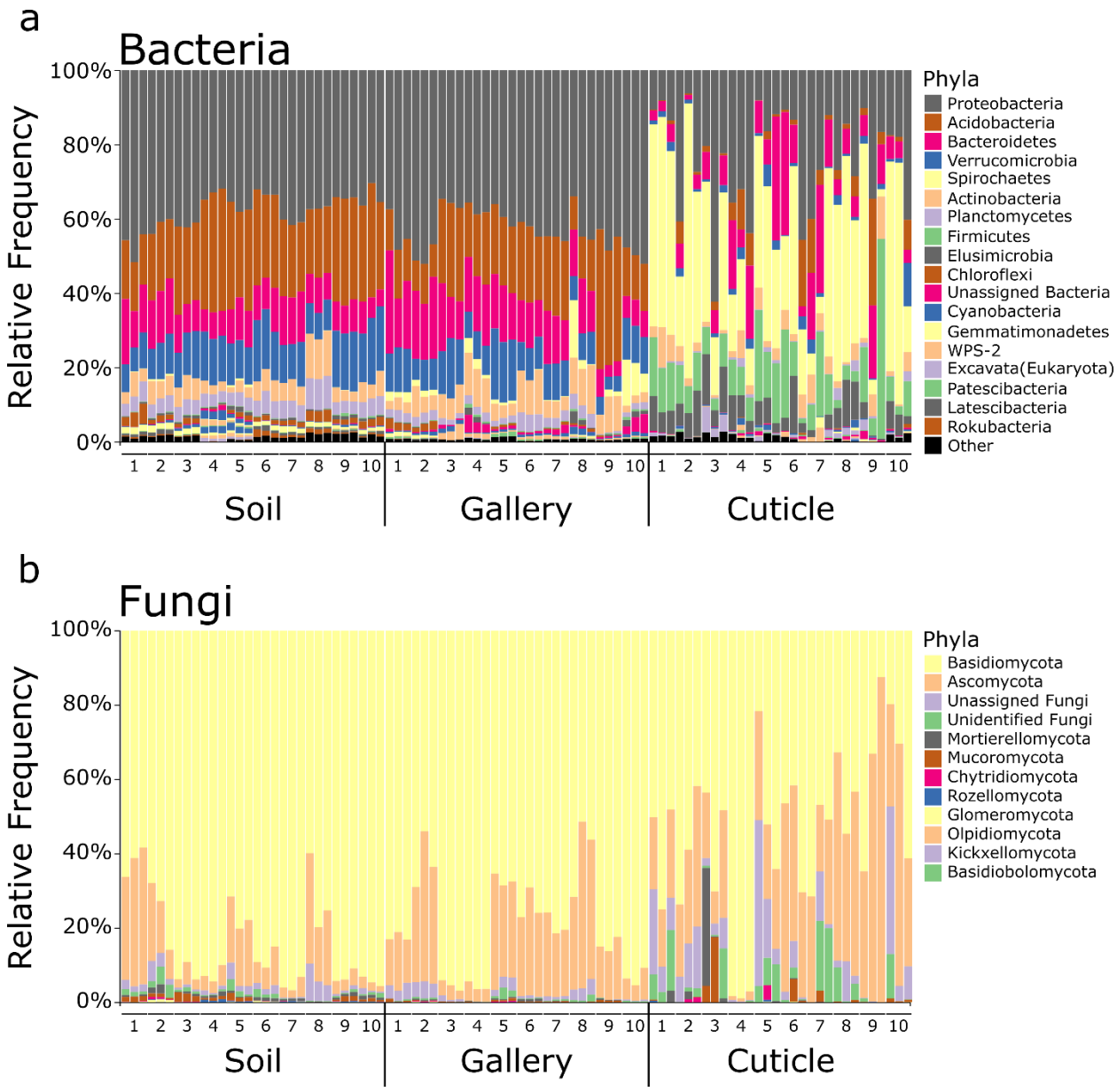

Figure IV-4 Relative frequency of bacterial and fungal phyla in *R. flavipes* colonies

Phylum-level taxonomic profile of bacterial (a) and fungal (b) communities in each sample. Phyla identified from kingdoms other than bacteria and fungi are noted as such.

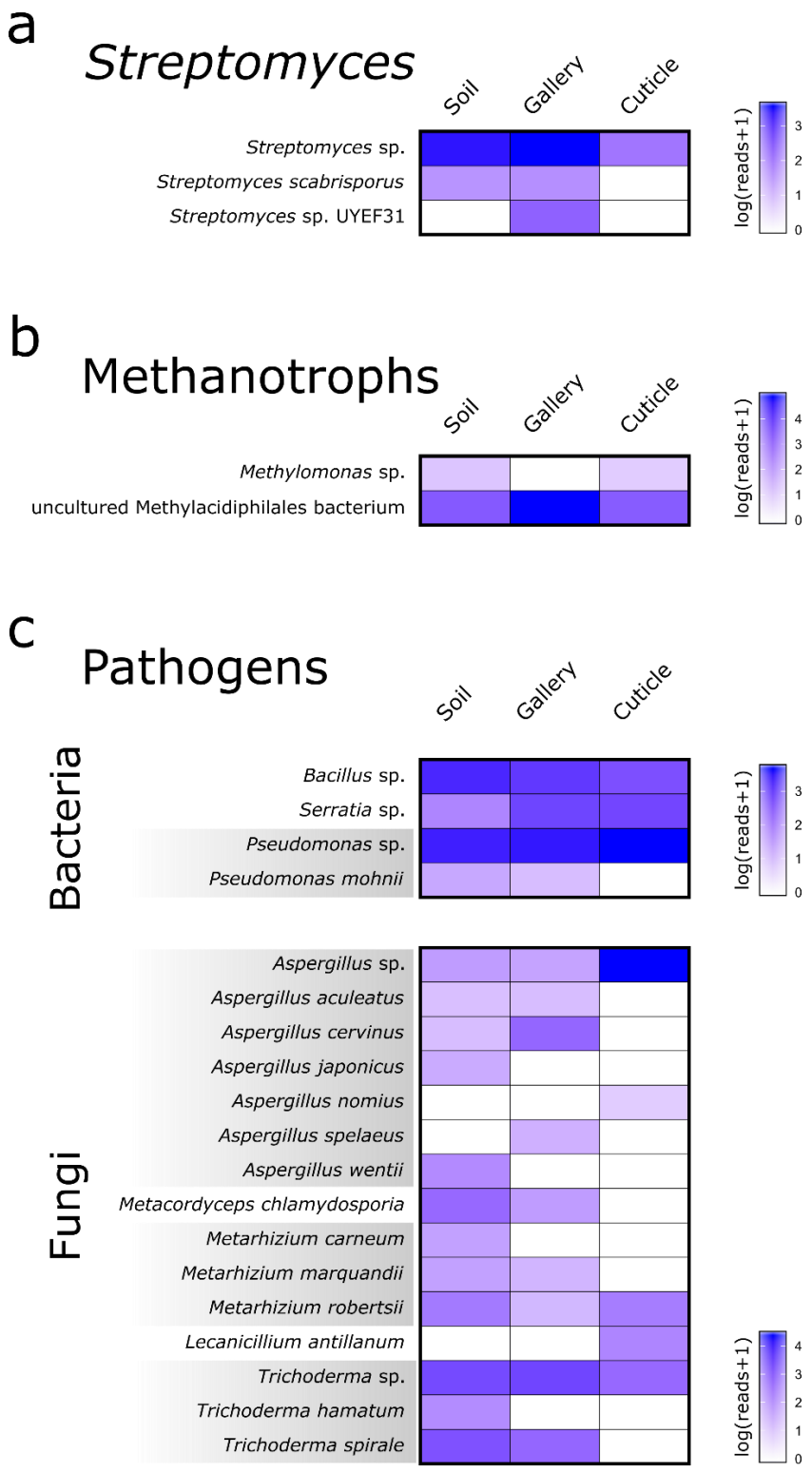

Figure IV-5 Relative abundance of bacterial and fungal species of interest

Heatmaps depict the relative abundance across substrates for *Streptomyces* species (a), methanotrophic bacteria (b), and pathogenic microbes (c). Heatmap scales are based on the log value of the number of reads attributed to each taxon. Pathogenic microbes from the same genus are grouped together in shaded areas. Taxa that have only been identified to genus-level could not be classified further using the SILVA or UNITE databases.

Discussion

We characterized bacterial and fungal communities associated with *R. flavipes* in the surrounding soil, the gallery substrate, and the surface of the insect cuticle. The highest diversity was found in the soil and diversity was reduced in the galleries and on the cuticle. While bacterial communities of these substrates are distinct from each other, fungal communities are not as clearly separated. Microbes associated with specialized nest structures in other termite species were also harbored in *R. flavipes* foraging galleries. Harmful microbes were inconsistent in their relative abundance. Some pathogens were absent in galleries and cuticle, while others were highly concentrated.

In this study we did not attempt to identify multicellular soilborne threats to termites, such as mites or nematodes, only the diversity of bacteria and fungi in the soil, which was quite high. Likely, the microbes identified are just a fraction of what subterranean termite colonies actually encounter. Thus, the already high diversity observed in the soil no doubt underestimates the actual microbial pressure experienced by termites. Despite this, a significant reduction in bacterial and fungal diversity in the galleries and on the cuticle of *R. flavipes* was observed. This reduction in microbial diversity may weed out many harmful microbes and suggests that termite colonies maintain selective communities. When examining the bacterial community, samples separated according to the substrate from which they were sampled. However, this was not the case for fungal communities, which suggests that *R. flavipes* colonies are less likely to have specific fungal associations in their nest and may have less control over the exclusion of harmful fungi.

Termites are characterized by their many intimate interactions with microbes. This is best studied in the termite gut, where obligate symbioses allow them to feed on difficult to digest food sources (Brune 2014, Brune and Dietrich 2015). In some species of termites, these symbiotic digestive processes have been externalized such that colonies obtain their food from cultivated

fungal gardens. Fungal cultivation in termites not only requires them to obtain their cultivar from the environment, but to also maintain their garden combs by weeding out unwanted microbes (Rouland-Lefèvre 2000, Nobre et al. 2010). The fungal comb is constructed from termite feces and likely evolved from a structure similar to the carton nest in *C. formosanus*, which is not used to cultivate fungi (Bignell 2006, Eggleton 2010, Aanen and Eggleton 2017). This carton is a network of spongy material that forms the center of the nest and contributes to colony homeostasis (Wood 1988), but is also known to harbor beneficial Actinobacteria. This bacterial phylum is known for its antimicrobial properties (Waksman and Lechevalier 1962, Goodfellow and Williams 1983) and, specifically, the genus *Streptomyces* is harbored in *C. formosanus* carton, providing additional disease resistance against *Metarhizium* fungi (Chouvenc et al. 2013b, Chouvenc et al. 2018). Interestingly, there is growing evidence that beneficial associations with Actinobacteria may also exist in the combs of fungus-growing termites (Kaltenpoth 2009, Visser et al. 2012, Benndorf et al. 2018). Similarly, in fungus-growing ant species, beneficial associations with microbes contribute to the inhibition of garden parasites (Currie et al. 1999, Little and Currie 2007). *Streptomyces* bacteria were identified in this study in *R. flavipes*. In *C. formosanus*, *Streptomyces* was thought to have been inoculated into the carton from surrounding soil, rather than from the termites themselves (Chouvenc et al. 2018). Our results suggest that this could also be the case in *R. flavipes*, as the soil harbored a higher relative abundance of *Streptomyces* than the cuticle.

Termites can also passively alter the microbial communities in the soil around them, as a result of the organic material produced by a colony (Jouquet et al. 2006). Many termite species produce methane as a result of the digestive processes in their gut (Brune 2014, Brune and Dietrich 2015) and the high numbers of individuals in a colony once led researchers to believe that termites could be significant contributors to the global methane budget (Sanderson 1996, Kirschke et al.

2013, Brune 2018). However, this has been found to not be the case and it has been confirmed that in three termite species, methanotrophic archaea and bacteria oxidize the methane produced by the colony (Nauer et al. 2018). These methanotrophs are harbored in the large mound structure that houses the colonies of many termite species, but their taxonomic identity remains unknown (Nauer et al. 2018).The methanotrophic bacterial groups, Methylacidiphilales and *Methylmonas* were identified in this study in *R. flavipes*. There was a high concentration of the methanotrophic bacterial order, Methylacidiphilales, in *R. flavipes* gallery substrate, while other methanotrophic bacteria, including the *Methylomonas* identified in the soil and cuticle, were absent. Associations with *Streptomyces* and methanotrophic bacteria have previously been known only from specialized nest structures, but our study found that even the amorphous galleries of *R. flavipes* carry these bacteria. Considering the examples of *Streptomyces* in carton material and methanotrophs in termite mounds, it is clear that even the termite nest serves a pivotal role in microbial interactions. Further testing is needed to definitively show that these bacteria have the same function, but their presence in the galleries does strongly suggest that this is the case.

We compared the relative abundance of known termite pathogens across three substrates and found considerable variation between different groups. Notably, although these genera have been associated with termite health, they can actually be quite versatile in ecological function and may not be obligate entomopathogens (Vega et al. 2009). Although *Metarhizium* has received the most attention as a termite pathogen and has been repeatedly associated with termites, its inability to be successfully used as a control agent has fostered doubt as to its actual ecological relevance to termites (Chouvenc et al. 2011b, Loreto and Hughes 2016). All *Metarhizium* species we identified had lower relative abundance in the galleries, compared to the soil, suggesting that growth may be inhibited in the galleries. Similar reductions in relative abundance were also

observed in the genera *Bacillus* and *Trichoderma* and this could be due to the antimicrobial effects of termite feces and salivary gland secretions that are incorporated into the galleries (Rosengaus et al. 1998a, Hamilton et al. 2011). The presence of *Streptomyces* in the galleries may also help inhibit these pathogens. Interestingly, *Metarhizium robertsii*, *Lecanicillium antillanum*, and the unclassified *Pseudomonas* sp., *Serratia* sp., and *Aspergillus* sp. had the highest relative abundance on the cuticle, indicating that some pathogens are able to penetrate the nest.

Unfortunately, as the samples used in this study were collected from mature colonies in the field, we are unable to determine whether factors such as colony age affect the presence of pathogens in the nest and, therefore cannot definitively explain, for example, why *Metarhizium robertsii* has higher relative abundance on the cuticle compared to the galleries. One possible explanation is that although some pathogens are able to penetrate the nest, the routine maintenance of a healthy colony prevents them from developing to a propagative stage (Chouvenc et al. 2013a). Termites have been shown to maintain strong immune defenses due to their social behavior (Rosengaus et al. 1998b), but as termite colonies decline in health from stress or age, epidemics are more likely to occur (Chouvenc et al. 2012, Chouvenc et al. 2013a). Phoretic mites are also commonly found in termite colonies, but do not appear to be detrimental until the entire colony begins to decline (Myles 2002, Wang et al. 2002). A similar example is seen in *R. speratus* where a 'termite-ball' fungus is able to mimic termite eggs and trick workers into bringing it into the nest (Matsuura 2006, Yashiro and Matsuura 2007, Mitaka et al. 2018). However, this fungus cannot germinate until the termites' nest has been vacated (Matsuura et al. 2000). Termite colonies are resistant to disease, but pathogens may accumulate in the nest over time. Potentially, the pathogen load that builds up in colonies, in addition to any additional stressors from the environment, could reach a threshold where the colony is no longer able to prevent an epidemic.

Surprisingly little is known about what actually kills termite colonies in nature. By using metagenomics, we are beginning to understand the complex interactions between subterranean termites and soil microbiota. The life of a subterranean termite colony can span years and during this time they are subject to the seasonality of their environment. Additionally, as colonies age from foundation to maturity, and eventually senescence, there are drastic changes to the demographics of individuals within a colony. There is still much to learn about how termite colonies interact with microbes in the environment. Understanding how microbiota-termite interactions change throughout the lifespan of a colony may elucidate the factors that contribute to natural colony collapse and provide novel targets for future termite management strategies.

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CHAPTER V

BEHAVIORAL CHANGES IN PATHOGEN-INFECTED TERMITE WORKERS FACILITATE THEIR REINTRODUCTION TO THE COLONY

Introduction

The evolution of eusociality in animals has repeatedly led to ecological success. This has reached a paradigm in social insects, which have rapidly diversified throughout different ecological niches (Wilson 1990). However, there are still costs to living in large groups, especially those that are densely packed with close relatives. Disease can spread more easily, as frequent interactions increase the chance of transmission (Hamilton 1987, Schmid-Hempel 1998, Godfrey et al. 2006) and related individuals are more likely to suffer from the same disease agent (Anderson and May 1985, Shykoff and Schmid-Hempel 1991, Nunn et al. 2006). Yet, social insect colonies are well protected against epidemics, due to a variety of individual and group-level defenses, such as allogrooming (Peng et al. 1987, Drees et al. 1992, Oi and Pereira 1993, Rosengaus et al. 1998b, Hughes et al. 2002, Wilson-Rich et al. 2007, Yanagawa and Shimizu 2007, Chouvenc et al. 2009b, Liu et al. 2019a), the transfer of antimicrobial substances through trophallaxis (Hamilton et al. 2011b), and corpse disposal (Sun and Zhou 2013). These defenses, collectively referred to as social immunity, take advantage of an organized workforce in order to mitigate the costs of social living (Cremer et al. 2007, Cremer et al. 2018, Liu et al. 2019b).

Division of labor is the hallmark of social insect colonies, whereby individuals are efficiently allocated into different colony tasks. Within a colony, reproduction is the responsibility of one, or few, individuals, while a larger workforce tends to all other needs. This social organization allows the reproductive caste to be protected from external threats, as some tasks, such as foraging, defense or corpse disposal, increase the risk of pathogen exposure (Durrer and

Schmid-Hempel 1994, Sun and Zhou 2013). Typically, colony members responsible for these risky tasks have reduced contact with high-value members of the colony (*i.e.,* reproductives and brood), thus decreasing the chance of disease transmission to these valuable group members (Wang and Mofller 1970, Naug and Camazine 2002, Naug and Smith 2007, Stroeymeyt et al. 2018). Even among the worker castes, particularly dangerous tasks are handled by more expendable individuals. Many social insects exhibit age polyethism, such that younger individuals work inside the nest while older workers are responsible for more hazardous tasks (Seeley 1982, Schmid-Hempel and Schmid-Hempel 1984, Sun and Zhou 2013). This serves to prolong the life of workers, and thus maximizes their contributions to the colony. Thus, division of labor improves social immunity by constraining disease transmission throughout the colony.

While the organization of the colony workforce provides a measure of passive immune defense, individuals still actively respond to imminent disease threats. Selective pressures associated with disease are thought to have played a large role in the evolution of eusociality (Gadagkar 1992). Consequently, many social insects are acutely sensitive to pathogen cues (Schmid-Hempel 1998) and, in some cases, can even discern the degree of pathogen virulence (Yanagawa et al. 2012). This strong detection ability allows social insects to adjust their behavior to reduce disease transmission risk. In several species of ants and bees, infected and contagious individuals self-isolate themselves, either by reducing their contact with nestmates or by leaving the nest entirely (Walker and Hughes 2009, Chapuisat 2010, Heinze and Walter 2010, Bos et al. 2012, Stroeymeyt et al. 2018, Geffre et al. 2020).

In termites, the typical response to pathogen cues is avoidance or shaking alarm displays to warn nestmates (Rosengaus et al. 1999, Yanagawa et al. 2015, Bulmer et al. 2019). In addition, termites can exhibit different behaviors towards infected nestmates. Termites rely heavily on

allogrooming to remove pathogenic spores from other workers (Rosengaus et al. 1998b, Chouvenc et al. 2009b, Davis et al. 2018, Liu et al. 2019a, Aguero et al. 2020). Although workers are typically repelled by olfactory pathogen cues, they may be attracted if those odors are presented alongside nestmate cues (Yanagawa et al. 2015). In some cases, workers will prevent the infected individuals from returning to the colony by sealing them into a chamber (Epsky and Capinera 1988). In the subterranean termite, *Reticulitermes flavipes*, when workers have incubated a lethal infection, the typical grooming response is replaced by cannibalism (Davis et al. 2018). It is currently unclear how termites determine when nestmates have developed lethal infections and can no longer be saved by grooming. If the infected individual is responsible for communicating its infection status, it may be either from increased shaking alarms or due to reduced movement. A lack of movement, or moribundity, has frequently been observed in diseased termites, but it is unknown if this is a signal meant to communicate with nestmates or just a symptom of disease (Chouvenc et al. 2009b, Davis et al. 2018).

We set out to determine if pathogen-exposed workers of *R. flavipes* alter their behavior in the presence of nestmates. We examine immediate behavioral changes by measuring locomotion and shaking displays before and after reintroduction to a small group of nestmates. Then, we investigated gradual changes in behavior when infected workers return to a larger group within a nest. We use a fungal entomopathogen to infect termites and test if different incubation times affect these behavioral changes. Overall, our results assess how individual immune behaviors relate to social immunity.

Methods

Termite and pathogen preparation

In November 2019, groups of termites were collected from eight *R. flavipes* colonies in College Station, TX. Collection points were located at least 15 m apart from each other to ensure that each group of termites came from a different colony (Vargo 2003, DeHeer and Vargo 2004, DeHeer et al. 2005). For each colony, some termites were dyed blue, so that they could be identified among undyed nestmates. Termites were fed cellulose material containing Nile blue, which is a fat-soluble stain that is used to mark termites, for one week (Su 1991, Davis et al. 2018, Aguero et al. 2020). Pathogenic treatments were prepared from the conidia of the fungal pathogen, *Metarhizium robertsii*, suspended in a 0.1% TWEEN®80 (Sigma-Aldrich Chemie N.V, The Netherlands) solution at a concentration of $1x10^7$ conidia/mL. The 0.1% TWEEN®80 solution by itself was used as a control treatment. Individual termites were treated by 30 seconds of immersion in 0.5 mL in either a pathogen or a control solution. Treated individuals were individually moved to 60 mm diameter petri dishes lined with moistened filter paper and allowed to incubate for 15 minutes, 24 hours, or 48 hours. All experiments were started within one week from the time that termites were sampled.

Immediate changes in immune behaviors

After incubation, a five-minute video of the Petri dish was recorded. Locomotion (*i.e.*, time spent moving) and the number of shaking displays of each individual termite were counted. After 15 minutes, four undyed workers from the same colony were added to each Petri dish. In order to reduce the effects of this disturbance, an additional 15 minutes were allowed to pass before

recording another five-minute video. Locomotion and number of shaking events of the focal termites were counted. One to two replicates were tested for every treatment and incubation combination from four colonies. Additionally, although previous studies that have used Nile blue to mark termites have found that it does not affect mortality, it is unknown if the dye has any effect on behavior (Su 1991). To test for potential effects of Nile blue on termite behavior, 15 of these assays were prepared so that treated termites were undyed and the group of nestmates was dyed. Then, locomotion and shaking were compared between dyed and undyed termites.

Gradual changes to immune behaviors

Groups of 500 workers and five soldiers from each of four colonies were introduced into 25 cm x 25 cm planar arenas filled with moistened sand (Figure V-1). The planar arenas were constructed so that there was a single entrance that connected to an accessible container. The side of the arena opposite to the entrance was lined with discs of filter paper, serving as a food source. Each plate was divided into a 4x4 grid, so that observers could identify individuals in different areas of the arena. Starting from the entrance and leading to the bottom of the arena, four levels were identified as A-D (Figure V-1). Termites were introduced through the entrance and allowed to tunnel through the plate for one month, which was enough time for the colony to settle and form stable tunnels and chambers. The entrance was continuously supplied with filter paper, so that foraging workers could continually be collected and returned.

After one month, five workers were removed from the entrance of each plate. These workers were dyed blue and treated with either a pathogen or control solution. Treated workers were left to incubate for either 24 or 48 hours before being reintroduced to the entrance of the

arenas. In order to assess potential self-isolation, the number of dyed termites (*i.e.*, infected or control treated) was counted in each grid-square for 15 minutes, one day, and seven days after their reintroduction. In order to assess changes in social organization, the number of nestmates in each grid-square was also counted 15 minutes before the reintroduction of treated individuals, as well as 15 minutes, one day, and seven days after their reintroduction. Each treatment was replicated by using 2-3 different colonies.

Statistical analysis

Immediate changes in locomotion and shaking displays were determined with an analysis of variance (ANOVA) testing for individual and interaction effects between treatment (24-hour control, 48-hour control, 24-hour pathogen, and 48-hour pathogen) and observation time (15 minutes before and after being grouped with nestmates). A Tukey's honestly significant difference (HSD) test was used for pairwise comparisons between all treatments and observation times. The effects of dye on locomotion and shaking were determined using a Welch two-sample t-test. In the analysis of gradual changes in behavior, separate ANOVAs were used within all four treatment groups to test for significant differences between the four nest levels in the abundance of workers. Then, a Tukey's HSD was used to make pairwise comparisons between all grid levels and observation times within each treatment. Within each treatment, a Pearson's χ^2- test was used to determine if the number of dyed individuals differed between grid levels (A-D). All analyses were performed in the statistical software R 3.5.0 (R Core Team 2018).

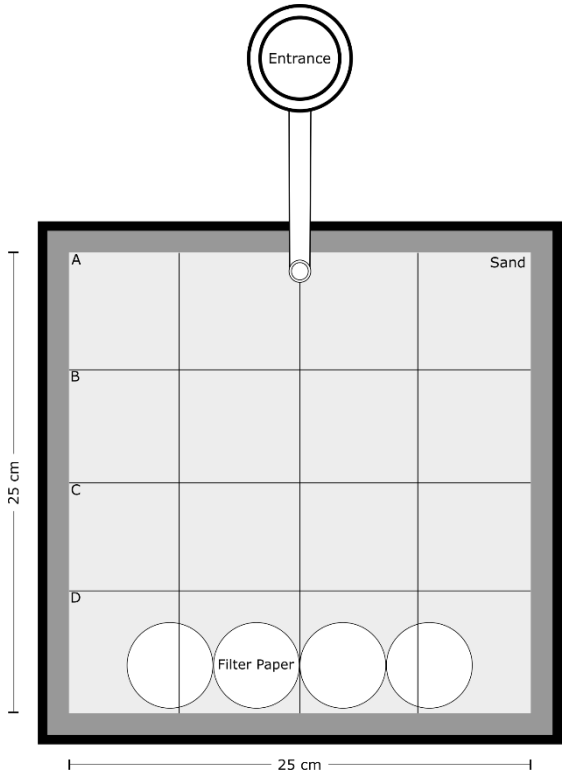

Figure V-1 Diagram of planar arenas

500 workers and five soldiers from each colony were placed into the arena entrance, which leads into a 25 cm x 25 cm planar arena filled with moistened sand. Each arena was divided into four levels (A-D). Level D contains filter paper discs that serve as a food source for the colony.

Results

Pathogen-exposed termites that had incubated for 24 hours showed lower levels of locomotion than all other treatments, except for the 24-hour control group ($p < 0.05$; Figure V-2a). However, within all treatments, there was no change in locomotion before or after the addition of nestmates. No differences in shaking alarm displays were observed between any treatments or observation times (Figure V-2b). Notably, shaking displays were only observed in a small fraction of the recorded videos (14%). Interestingly, the dye used to mark termites slightly increased locomotion, but did not significantly influence shaking displays (Appendix D-1).

In the experiments testing for gradual changes, colony density concentrated away from the entrance of the plates, as workers were significantly more abundant in level D ($p < 0.001$; Figure V-3). The abundance of workers did not change after the reintroduction of treated individuals, in any treatment (Figure V-3). Termites treated with a control solution (both at 24 and 48 hours) did not differ in abundance in any part of the plate (Figure V-3a-b). In contrast, termites in the 24-hour pathogen group were significantly more abundant in Level D, where colony density was concentrated ($p < 0.05$; Figure V-3c). However, termites that had been infected with a pathogen for 48 hours did not show this variation in abundance (Figure V-3d). This did not change over time, suggesting that infected termites do not self-isolate.

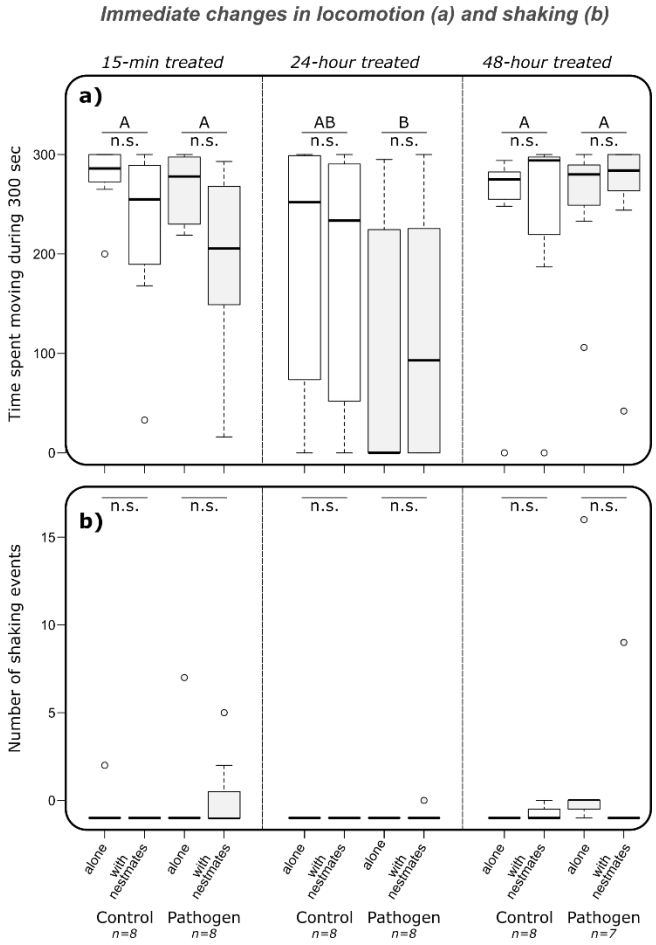

Figure V-2 Immediate changes in immune behaviors

(a) Termites that had incubated for 24 hours after pathogen exposure showed lower levels of locomotion than all other treatments (n = 4 colonies), except for the 24 hour control group ($p < 0.05$). Within all treatments, there was no change in locomotion before or after their reintroduction to nestmates. (b) No differences in shaking alarm displays were observed between any treatments (n = 4 colonies) or observation times (n = 4 colonies).

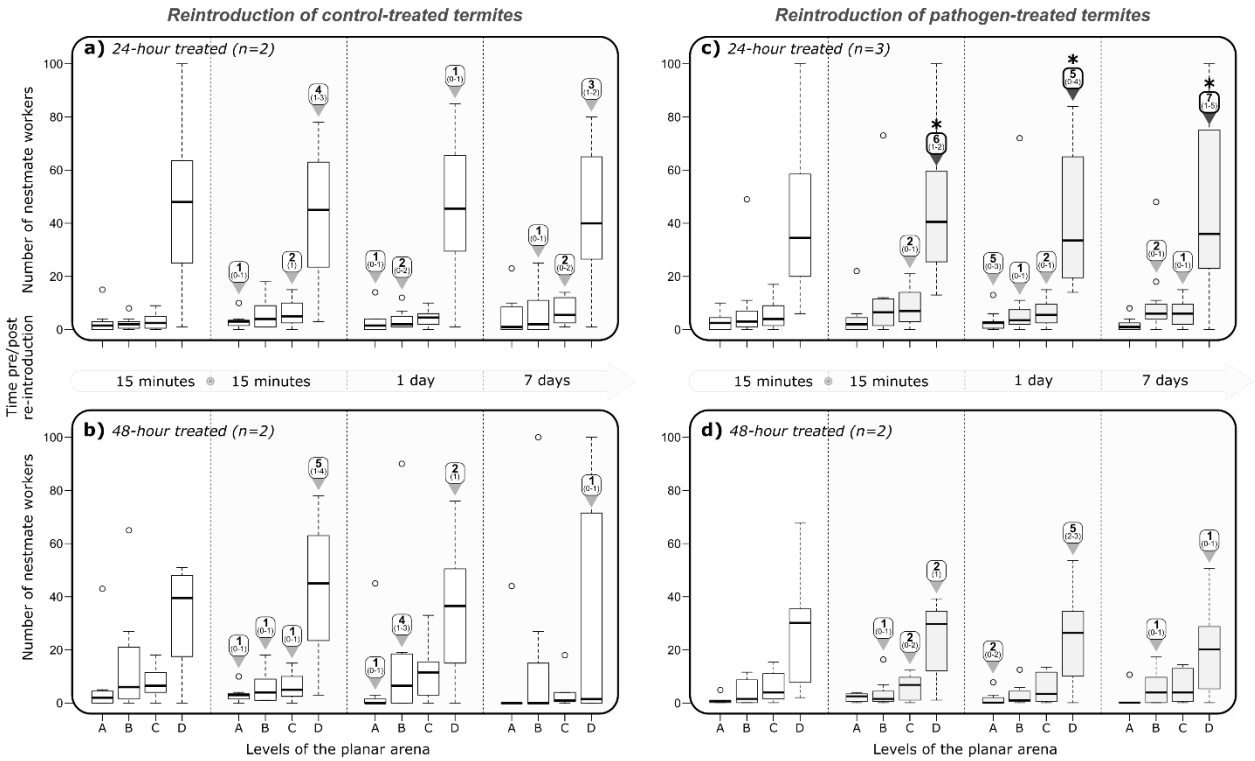

Figure V-3 Gradual changes in immune behaviors

Boxplots depict the number of undyed workers found in all plate levels over time. N in each plot indicates the number of different colonies tested with each treatment. For all four treatments, worker density was consistently concentrated in level D, furthest from the entrance ($p < 0.05$). The values above each box indicate the number of dyed individuals found in each level (the total sum in bold and the variation across replicates beneath it). (a, b, d) In almost all treatments, dyed individuals were not significantly more abundant in any level. (c) The 24-hour treated pathogen group is the one exception, as dyed individuals were significantly more abundant in level D ($p < 0.05$).

Discussion

We found that individual workers did not alter their locomotion or shaking behaviors in the presence of nestmates, regardless of their treatment. Although workers that had incubated a pathogen for 24 hours after exposure showed less locomotive activity than most other treatments, the presence of nestmates did not increase or reduce their movement. This coincides with the results found in the planar arenas. No dyed individuals were excluded from any part of the colony. In fact, within 15 minutes after reintroduction, termites that had been exposed to a pathogen for 24 hours were significantly more abundant in the densest parts of the colony. Thus, infected workers do not self-isolate and may be drawn to larger groups, despite the increased risk of spreading disease.

Although moving into dense areas of the colony may promote the spread of disease, it also increases the chance of being groomed by nestmates. Termites can self-groom, but it is much more efficient to be groomed by others (Rosengaus et al. 1998b, Yanagawa and Shimizu 2007, Chouvenc et al. 2009b, Liu et al. 2019a). Thus, the cost for a colony to risk spreading disease by grooming, appears to be outweighed by the benefit of rescuing workers before they develop a lethal infection (Davis et al. 2018). Additionally, termite nest material exhibits strong antimicrobial activity from feces (Rosengaus et al. 1998a), defensive salivary secretions (Bulmer et al. 2010, Hamilton et al. 2011a), and beneficial bacteria (Chouvenc et al. 2018). By traveling to more active parts of the nest, infected individuals may also be seeking areas with the strongest antimicrobial activity and may sanitize themselves in the process.

Even when a termite has developed a lethal infection and can no longer be saved, they do not become infective until the fungus has killed them and sporulates from their body. By seeking out nestmates, workers could be inviting cannibalistic responses for the safe disposal of their

bodies before they can become infective. The termite gut serves an important role in termite immunity, as harmful spores are inhibited in the alimentary tract (Chouvenc et al. 2009b). Indeed, when an infected worker may no longer be saved from infection, the response of nestmates switches from grooming to cannibalism (Davis et al. 2018). Reduced movement has been suggested as the signal for nestmates to begin cannibalization (Chouvenc et al. 2009a, Davis et al. 2018). Additionally, shaking displays are used to signal pathogen presence and may also play a role in communicating infection status (Rosengaus et al. 1999, Yanagawa et al. 2015, Bulmer et al. 2019). We found no changes in locomotion or shaking displays when infected termites were grouped with nestmates, which suggests that these behaviors are not used by workers to selectively signal infections to nestmates. Potentially, if reduced locomotion does not rely on the presence of nestmates, then it may be a symptom of deteriorating health. This difference may not be possible to discern from behavioral studies and may require a more thorough analysis of physiological or transcriptional changes in infected individuals.

Shaking displays appear to be unrelated to the communication of infection status, as only few shaking displays were observed. Locomotion, however, was significantly lower in pathogen-exposed workers that had incubated for 24 hours, as opposed to 15 minutes. Notably, though, termites that had incubated for 48 hours after exposure did not show any reduced movement. It is possible that, as an infection progresses, behavior could continue to change and termites may resume moving. In addition, there was no significant difference in locomotion between 24-hour pathogen treated termites and 24-hour control treated termites. Both of these treatments were tested during the same day, so there is the potential that unknown environmental factors, such as changes in temperature, may have an effect.

Behavioral changes after infection are not always the result of social immune defenses. Several pathogens can manipulate their host's behavior to benefit their own transmission. More complex examples of manipulation appear to require a degree of host specialization (Lafferty and Shaw 2013). Carpenter ants infected with *Ophiocordyceps* fungi descend from arboreal nests to find optimal conditions for fungal growth (Hughes et al. 2011). Similarly, in honeybees, workers infected with Israeli acute paralysis virus show decreased aggression towards other colonies, increasing the chance of transmission to new hosts (Geffre et al. 2020). A reduction in movement is one of the most common examples of host manipulation, as it increases the chance of the current host being predated and further transmitting the disease (Lafferty and Shaw 2013). If this is the case, then cannibalism and the inhibitory strength of the termite gut may have evolved in response. Potentially, the dye we used to mark termites may also have had some influence on behavior. Nile blue was originally used to monitor termite colonies in the field and reportedly does not affect mortality (Su 1991). However, the effect of this dye on termite behavior has not been investigated. We have identified a slight change in the locomotion of dyed termites, which may warrant further investigation into overlooked effects that this dye has on termites.

We show that *R. flavipes* workers that are infected with a fungal pathogen do not self-isolate from the colony in the planar arenas. However, subterranean termite colonies can be much more complex in nature. Foraging ranges can extend hundreds of square meters and mature colonies typically contain much more than the 500 individuals we used in our arenas (Vargo and Husseneder 2009). While infected termites are uninhibited from dense pockets of workers, it is unknown if the same is true for reproductive chambers. Additionally, the concentration of pathogenic spores used in this study is much higher than what termites are expected to encounter naturally (Chouvenc et al. 2011, Loreto and Hughes 2016). Lower pathogen titers are typically not

used in studies of social immunity, as differences in colony survival are less likely to be seen. Potentially, termite workers may behave differently when exposed to less lethal pathogen concentrations. Expanding on these results by testing the influence of additional castes and altering pathogen titers will increase our understanding of how individual behaviors translate to overall colony organization.

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CHAPTER VI

SUMMARY OF RESULTS

In my first objective, I sought to test if genetic diversity influenced individual immunity in *Reticulitermes flavipes*. To do this, I sampled colonies throughout the US (Texas, North Carolina, Maryland, and Massachusetts) and determined the level of inbreeding of each colony. I challenged individual termite workers from each of these colonies with two strains of a pathogenic fungus, one 'local' strain present in the soil surrounding sampled colonies and another 'naïve' strain, collected outside the range of this species. I found natural variation in the level of inbreeding between colonies, but this variation did not explain differences in susceptibility to either pathogen. Colonies, however, had either relatively high or low susceptibility to both strains. This bimodal distribution suggests that additional factors, such as the influence of a few selective loci or immune priming, may influence survival.

The effects of genetic diversity were further examined by testing its influence on group immunity. I artificially increased genetic diversity by merging together groups of termites from different colonies and, then, exposed those groups to a fungal pathogen. I confirmed previous findings that colonies of *R. flavipes* will readily merge and showed that workers will equally groom both nestmates and non-nestmates after merging. Despite this, the survival of these merged colonies was not improved after exposure to a fungal pathogen, but instead leveled to that of the more susceptible or the more resistant colony. Thus, it appears that increased genetic diversity does not improve immunity in this species.

Next, I characterized the bacterial and fungal communities in foraging galleries of *R. flavipes* in order to determine if the galleries cultivate a more favorable microbial community than

the surrounding soil. I compared microbial communities in soil near termite colonies, termite foraging tunnels, and on the cuticle of termites. Bacterial and fungal diversity was highest in the soil, less in the galleries, and lowest on the cuticle. Bacterial communities clustered together according to the substrate from which they were sampled, but this clustering was less clear in fungal communities. Most of the identified bacterial and fungal taxa were unique to one substrate, but soil and gallery communities have very similar phylum-level taxonomic profiles. When examining specific microbes, I found that the foraging galleries of *R. flavipes* harbored beneficial *Streptomyces* and the methanotrophic Methylacidiphilales. These two bacterial groups have previously been associated with a few other termite species on specialized nest structures (Chouvenc et al. 2018, Nauer et al. 2018). Their presence in the amorphous foraging galleries of *R. flavipes* suggests that these microbial associations are more widespread than what has been demonstrated so far. When I identified pathogenic bacteria and fungi in the samples, I found several groups that were relatively abundant on the cuticle. Subterranean termites have strong immune defenses, so it is surprising to find pathogens on the termite cuticle. Likely, pathogens accumulate within termite nests over time, but healthy colonies may be able to inhibit their propagation.

In my final objective, I examined how individual *R. flavipes* workers, which have been infected with a fungal pathogen, alter their behavior in the presence of nestmates. It has been suggested that termites may signal their infection status to others by becoming moribund. I found that, although workers may reduce their movement as their infection progresses, there were no immediate changes before or after rejoining their nestmates. Termites will display shaking alarms when exposed to pathogens, however this was also uninfluenced by the presence of others. Therefore, changes in infected individuals' behavior do not appear to be triggered by the presence

of nestmates. Next, I tracked the movement of infected workers after returning to a colony and found that they did not self-isolate. Rather, they traveled to the areas in the nest that had the most workers. Potentially, the response of other colony members, such as grooming or cannibalism, may outweigh the increased risk of spreading disease.

Much of what we know about social immunity comes from studies examining Hymenoptera, but the results presented here demonstrate the diversity of mechanisms that can bring about the same level of colony-wide pathogen resistance. Genetic diversity has a strong influence on pathogen susceptibility in several Hymenopteran species (Liersch and Schmid-Hempel 1998, Baer and Schmid-Hempel 1999, Baer and Schmid-Hempel 2001, Palmer and Oldroyd 2003, Tarpy 2003, Hughes and Boomsma 2004, Seeley and Tarpy 2006, Tarpy and Seeley 2006, Reber et al. 2008, Bourgeois et al. 2012, Mattila et al. 2012, Evison et al. 2013, Lee et al. 2013), yet it does not appear to influence immunity in *R. flavipes*. Additionally, whereas metapleural glands allow ants to control harmful microbes in their nests (Brown Jr 1968, Hölldobler and Engel-Siegel 1984, Beattie et al. 1986, Ortius-Lechner et al. 2000), termites have accomplished the same feat through alternative means. Furthermore, although there are several examples in the Hymenoptera that infected workers will self-isolate from the colony (Walker and Hughes 2009, Chapuisat 2010, Heinze and Walter 2010, Bos et al. 2012, Stroeymeyt et al. 2018, Geffre et al. 2020), diseased termites appear to rely on the responses of other colony members. As more studies are conducted on the variations of immune defenses across taxa, we will more fully understand the ways in which groups protect themselves from disease.

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APPENDIX A

A-1 Location of sampling sites

Code	Location	Total	Simple	Extended	Mixed
TX1	Lick Creek Park, College Station, TX	7	5	2	0
TX2	Lake Bryan, Bryan, TX	7	4	3	0
NC1	Schenk Forest, Raleigh, NC	9	6	3	0
NC2	Schenk Forest, Raleigh, NC	6	5	1	0
MD1	Gunpowder Falls State Park, Baltimore County, MD	7	5	2	0
MD2	Towson University Field Station, Monkton, MD	10	9	1	0
MA1	Cutler Park, Needham, MA	12	6	6	0
MA2	Middlesex Fells, Medford, MA	10	8	1	1

The location of all sampling sites, with the number of colonies (and their family type) collected from each site.

A-2 DNA extraction and PCR protocols

DNA Extraction:

1. Individual termite workers were placed in a 1.5mL tube.
2. 100 µL of cell lysis solution and 1 µL of proteinase K were added to each tube.
3. Samples were ground in solution using a pestle.
4. Samples were placed in a water bath at 55°C for 3 hours.
5. Samples were removed from the water bath and kept in a freezer at -20°C for 30 minutes.
6. 35 µL of 8M ammonium acetate was then added to thawed samples.
7. Samples were centrifuged for 7 minutes at 10K rpm.
8. The supernatant was pipetted into new tubes containing 100 µL cold isopropanol.
9. Samples were then centrifuged for 5 minutes at 10K rpm.
10. Isopropanol was poured out of tubes, leaving behind pelleted DNA.
11. 400 µL 100% Ethanol was added to each sample.
12. Samples were centrifuged for 5 minutes at 10K rpm.
13. Ethanol was poured out, and samples were placed in a vacuum for 15 minutes to remove any excess alcohol.
14. Dry DNA pellets were resuspended in 100 µL of 1X TE buffer overnight.

PCR Protocols

Multiplex 1

Reagent	volume (µl)/sample
5X Buffer	2.5
RS13 forward	0.42
RS13 reverse	0.42
RS33 forward	0.3
RS33 reverse	0.3
RS62 forward	0.33
RS62 reverse	0.33
Rf15-2 forward	0.22
Rf15-2 reverse	0.22
RS43 forward	0.17
RS43 reverse	0.17
RS16 forward	0.14
RS16 reverse	0.14
Rf6-1 forward	0.06
Rf6-1 reverse	0.06
RS10 forward	0.06
RS10 reverse	0.06
ddH_2O	4.5
Taq	0.1
Extracted DNA	2
Total	12.5

Multiplex 2

Reagent	volume (µl)/sample
5X Buffer	2.1
Rf 24-2 forward	0.25
Rf 24-2 reverse	0.25
ddH_2O	8.8
Taq	0.1
Extracted DNA	1
Total	12.5

Primer sequences and thermocycler programs

Multiplex 1

94°C for 3 min; 7 cycles of 94°C for 30 sec, 62°C for 30 sec (-1°C each cycle), 72°C for 30 sec; then 31 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec; final extension 72°C for 5 min.

Multiplex 2

94°C for 3 min; 35 cycles of 94°C for 30 sec, 60°C for 60 sec, 72°C for 120 sec; final extension 72°C for 10 min.

A-3 Generalized linear model results

```
glm formula = Local_hazard_ratio ~ Fic * Site * Family_type


Deviance Residuals:
    Min      1Q  Median      3Q     Max
 -4.773  -1.541       0   1.108   5.855

Coefficients: (3 not defined because of singularities)
                          Estimate  Std. Error t value Pr(>|t|)
(Intercept)                7.3682      1.3311   5.536 2.47E-06 ***
fstat                     -6.1036     11.136   -0.548   0.5868
siteMA2                    8.4049      7.6021   1.106   0.2759
siteMD1                    8.0505      8.6883   0.927     0.36
siteMD2                   -4.0102      3.8365   -1.045   0.3025
siteNC1                   -2.9906      3.9809   -0.751   0.4571
siteNC2                   -6.9457      3.8036   -1.826   0.0757 .
siteTX1                   -3.6784      2.7841   -1.321   0.1943
siteTX2                   -5.4371      4.5978   -1.183   0.2443
familyS                   -6.0262      4.2927   -1.404   0.1685
fstat:siteMA2             -3.4129     15.0229   -0.227   0.8215
fstat:siteMD1            213.5651    176.4163   1.211    0.2335
fstat:siteMD2             11.8971     17.0494   0.698    0.4895
fstat:siteNC1             17.9535     30.6328   0.586    0.5613
fstat:siteNC2             -1.8306     17.8034   -0.103   0.9186
fstat:siteTX1            -19.0564     38.2771   -0.498   0.6215
fstat:siteTX2            -12.2364     30.0059   -0.408   0.6857
fstat:familyS              0.2759     16.9341   0.016    0.9871
siteMA2:familyS           -6.0164     11.8337   -0.508   0.6141
siteMD1:familyS           -4.6944     11.525    -0.407   0.6861
siteMD2:familyS            7.8277      5.9283   1.32     0.1946
siteNC1:familyS            5.002      7.9852   0.626    0.5348
siteNC2:familyS            5.784      5.9934   0.965    0.3406
siteTX1:familyS            8.9918     6.1611   1.459    0.1527
siteTX2:familyS            14.246     6.7855   2.099    0.0425 *
fstat:siteMA2:familyS      NA         NA       NA       NA
fstat:siteMD1:familyS    -208.2715   177.6152  -1.173   0.2483
fstat:siteMD2:familyS      NA         NA       NA       NA
fstat:siteNC1:familyS     -12.092     36.786   -0.329   0.7442
fstat:siteNC2:familyS      NA         NA       NA       NA
fstat:siteTX1:familyS      34.4747    44.3596   0.777    0.4419
fstat:siteTX2:familyS      44.2944    35.8091   1.237    0.2237
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for gaussian family taken to be 10.47756)

Null deviance: 755.27  on 66  degrees of freedom
Residual deviance:  54453  on 38  degrees of freedom
AIC: 369.54
```

```
glm formula = Naive_hazard_ratio ~ Fic * Site * Family_type


Deviance Residuals:
    Min      1Q  Median      3Q     Max
-56.068 -18.145  -1.348   5.539 107.375

Coefficients: (3 not defined because of singularities)
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)                 54.85      15.57   3.523  0.00113 **
fstat                       40.35     130.23   0.310  0.75837
siteMA2                     99.73      88.90   1.122  0.26898
siteMD1                    106.60     101.61   1.049  0.30076
siteMD2                    -34.03      44.87  -0.758  0.45287
siteNC1                    -52.06      46.56  -1.118  0.27053
siteNC2                    -45.89      44.48  -1.032  0.30879
siteTX1                    -48.62      32.56  -1.493  0.14365
siteTX2                    -57.90      53.77  -1.077  0.28835
familyS                    -58.70      50.20  -1.169  0.24957
fstat:siteMA2             -110.11     175.69  -0.627  0.53460
fstat:siteMD1             2650.07    2063.13   1.284  0.20675
fstat:siteMD2              116.77     199.39   0.586  0.56156
fstat:siteNC1              -58.65     358.24  -0.164  0.87083
fstat:siteNC2               18.15     208.21   0.087  0.93098
fstat:siteTX1              -99.55     447.64  -0.222  0.82520
fstat:siteTX2             -249.84     350.91  -0.712  0.48083
fstat:familyS             -104.54     198.04  -0.528  0.60064
siteMA2:familyS           -103.31     138.39  -0.747  0.45995
siteMD1:familyS           -137.29     134.78  -1.019  0.31483
siteMD2:familyS             69.08      69.33   0.996  0.32539
siteNC1:familyS             83.37      93.38   0.893  0.37760
siteNC2:familyS             42.81      70.09   0.611  0.54503
siteTX1:familyS             92.63      72.05   1.286  0.20638
siteTX2:familyS            141.77      79.35   1.787  0.08200 .
fstat:siteMA2:familyS          NA         NA      NA       NA
fstat:siteMD1:familyS    -2781.80    2077.15  -1.339  0.18845
fstat:siteMD2:familyS          NA         NA      NA       NA
fstat:siteNC1:familyS      124.80     430.20   0.290  0.77333
fstat:siteNC2:familyS          NA         NA      NA       NA
fstat:siteTX1:familyS      200.15     518.77   0.386  0.70178
fstat:siteTX2:familyS      588.56     418.78   1.405  0.16802
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for gaussian family taken to be 1432.967)

    Null deviance: 101903  on 66  degrees of freedom
Residual deviance:  54453  on 38  degrees of freedom
AIC: 699.06
```

Results from a generalized linear model was used to determine whether all of the recorded variables (FIC, family type, and sampling site) individually influenced the hazard ratios or if there were any interaction effects.

APPENDIX B

B-1 DNA Extraction and PCR Protocols

DNA Extraction:

1. Either an individual termite worker or fungal conidia were placed in a 1.5mL tube.
2. 100 µL of cell lysis solution and 1 µL of proteinase K were added to each tube.
3. Samples were ground in solution using a pestle.
4. Samples were placed in a water bath at 55°C for 3 hours.
5. Samples were removed from the water bath and kept in a freezer at -20°C for 30 minutes.
6. 35 µL of 8M ammonium acetate was then added to thawed samples.
7. Samples were centrifuged for 7 minutes at 10K rpm.
8. The supernatant was pipetted into new tubes containing 100 µL cold isopropanol.
9. Samples were then centrifuged for 5 minutes at 10K rpm.
10. Isopropanol was poured out of tubes, leaving behind pelleted DNA.
11. 400 µL 100% Ethanol was added to each sample.
12. Samples were centrifuged for 5 minutes at 10K rpm.
13. Ethanol was poured out, and samples were placed in a vacuum for 15 minutes to remove any excess alcohol.
14. Dry DNA pellets were resuspended in 100 µL of 1X TE buffer overnight.

PCR:

Reaction Template

Reagent	**Per Sample**
DNA template	2.0 µL
Forward Primer	0.2 µL
Reverse Primer	0.2 µL
5x PCR Buffer	5 µL
Taq Polymerase	0.06 µL
Water	18.54 µL

Primer sequences and thermocycler programs

Termite - 16S

LR-J-13007 (5'–TTACGCTGTTATCCCTAA-3')

LR-N-13398 (5'-CGCCTGTTTATCAAAAACAT-3')

94°C for 2 min; 41 cycles of 94°C for 45 sec, 50°C for 45 seconds, 72°C for 1 min; final extension 72°C for 5 min.

Fungus – IGS

Ma-IGSspF (5'-CTACCYGGGAGCCCAGGCAAG-3')

Ma-IGSspR (5'-AAGCAGCCTACCCTAAAGC-3')

94°C for 3 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min; final extension 72°C for 5 min.

Sequencing:

Following PCR, amplified DNA was purified using the EXOSAP-it PCR purification kit (Affymetrix), then sequenced using the ABI BigDye Terminator v.3.1 Cycle Sequencing Kit on an ABI 3500 Genetic Analyzer (Applied Biosystems). Base calling and sequence alignment was performed using the software Geneious v.9.1. Aligned sequences were BLAST searched on Genbank for species ID.

B-2 Pairwise comparisons of merged colony survival

Figure 3a	Control S	Pathogen S	Control S/U	Pathogen S/U	Control U
Pathogen S	2.90E-06	-	-	-	-
Control S/U	0.21272	3.50E-05	-	-	-
Pathogen S/U	1.80E-06	0.55632	1.50E-05	-	-
Control U	0.36613	7.80E-06	0.55632	2.90E-06	-
Pathogen U	4.07E-02	0.00078	0.32749	0.00029	0.14666

Figure 3b	Control P	Pathogen P	Control P/R	Pathogen P/R	Control R
Pathogen P	3.90E-12	-	-	-	-
Control P/R	0.5603	1.10E-13	-	-	-
Pathogen P/R	7.00E-12	0.746	2.40E-13	-	-
Control R	0.1602	2.00E-15	0.344	4.40E-15	-
Pathogen R	1.20E-05	0.0021	1.20E-06	0.0031	6.50E-08

Figure 3c	Control T	Pathogen T	Control T/U	Pathogen T/U	Control U
Pathogen T	2.35E-03	-	-	-	-
Control T/U	0.17454	5.30E-04	-	-	-
Pathogen T/U	2.35E-03	0.75188	5.30E-04	-	-
Control U	0.38616	4.60E-04	0.4414	4.60E-04	-
Pathogen U	5.09E-01	0.01411	8.23E-02	0.01413	1.47E-01

Figure 3d	Control Q	Pathogen Q	Control Q/R	Pathogen Q/R	Control R
Pathogen Q	1.50E-02	-	-	-	-
Control Q/R	0.111	3.16E-01	-	-	-
Pathogen Q/R	1.50E-06	0.013	6.20E-05	-	-
Control R	1	1.10E-02	0.095	5.10E-07	-
Pathogen R	9.70E-07	0.011	3.70E-05	0.983	4.50E-07

Figure 3e	Control S	Pathogen S	Control S/T	Pathogen S/T	Control T
Pathogen S	6.50E-12	-	-	-	-
Control S/T	0.99	6.50E-12	-	-	-
Pathogen S/T	2.74E-01	1.70E-07	2.74E-01	-	-
Control T	0.212	1.00E-13	0.212	4.00E-02	-
Pathogen T	2.12E-01	9.50E-07	2.12E-01	8.86E-01	2.40E-02

Figure 3f	Control P	Pathogen P	Control P/Q	Pathogen P/Q	Control Q
Pathogen P	1.40E-06	-	-	-	-
Control P/Q	1	1.40E-06	-	-	-
Pathogen P/Q	9.20E-09	1.27E-01	9.20E-09	-	-
Control Q	0.1017	7.20E-05	0.1017	5.90E-07	-
Pathogen Q	2.50E-05	6.26E-01	2.50E-05	8.92E-02	1.50E-03

Pairwise comparisons of survival distributions using a Log-Rank test. Each table corresponds to one of the plots of survival curves in Figure III-3. All analyses were performed in the statistical software R 3.5.0 (https://www.r-project.org/).

APPENDIX C

C-1

Termite DNA Extraction:

1. Individual termite workers were placed in a 1.5mL tube.
2. 100 µL of cell lysis solution and 1 µL of proteinase K were added to each tube.
3. Samples were ground in solution using a pestle.
4. Samples were placed in a water bath at 55°C for 3 hours.
5. Samples were removed from the water bath and kept in a freezer at -20°C for 30 minutes.
6. 35 µL of 8M ammonium acetate was then added to thawed samples.
7. Samples were centrifuged for 7 minutes at 10K rpm.
8. The supernatant was pipetted into new tubes containing 100 µL cold isopropanol.
9. Samples were then centrifuged for 5 minutes at 10K rpm.
10. Isopropanol was poured out of tubes, leaving behind pelleted DNA.
11. 400 µL 100% Ethanol was added to each sample.
12. Samples were centrifuged for 5 minutes at 10K rpm.
13. Ethanol was poured out, and samples were placed in a vacuum for 15 minutes to remove any excess alcohol.
14. Dry DNA pellets were resuspended in 100 µL of 1X TE buffer overnight.

Termite 16S PCR:

Reaction Template

Reagent	**Per Sample**
DNA template	2.0 µL
Forward Primer	0.2 µL
Reverse Primer	0.2 µL
5x PCR Buffer	5 µL
Taq Polymerase	0.06 µL
Water	18.54 µL

Primer sequences and thermocycler programs

Termite - 16S

LR-J-13007 (5'–TTACGCTGTTATCCCTAA-3')

LR-N-13398 (5'-CGCCTGTTTATCAAAAACAT-3')

94°C for 2 min; 41 cycles of 94°C for 45 sec, 50°C for 45 seconds, 72°C for 1 min; final extension 72°C for 5 min.

Phenol:Chloroform extraction protocol for termite cuticle extraction

1. Pipette 250µL Cell Lysis Buffer and 3µL Proteinase K into each wash sample. Flick sample tubes to mix.
2. Incubate samples at 37°C overnight.
3. Pipette 3µL of Proteinase K into each sample. Flick sample tubes to mix.
4. Place samples in 65°C water bath for 1 hour.
5. Repeat steps 3-4.
6. Pipette 250µL of Phenol:Chloroform into each sample. Shake samples under fume hood for 5 minutes.
7. Centrifuge at 12,000 x g for 4 minutes.
8. Pipette supernatant into a new Eppendorf tube.
9. Repeat steps 6-8.
10. Pipette 250µL Chloroform into each sample. Shake samples under fume hood for 5 minutes.
11. Centrifuge at 12,000 x g for 4 minutes.
12. Pipette supernatant into a new Eppendorf tube.
13. Pipette 65µL of Ammonium Acetate and 500µL of ice cold 95% Ethanol into each sample. Flick samples to mix.
14. Place samples in freezer overnight.
15. Centrifuge for 10 minutes at 12,000 x g.
16. Pour off excess ethanol.
17. Pipette 100µL of 70% ethanol into each sample.
18. Repeat steps 15-16.
19. Let samples air dry to remove excess ethanol
20. Pipette 50µL ddH_20 into each sample and incubate at room temperature overnight.
21. Store samples in freezer.

PCR protocols for bacteria and fungi

Reaction Template

Reagent	**Per Sample**
DNA template	1.0 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
2x Master mix	5 µL
Water	13.0 µL
Total volume	25.0 µL

Primer sequences and thermocycler programs

Bacteria - 16S v4

515F (5'– GTGCCAGCMGCCGCGGTAA-3')

BAC806R (5'- GGACTACHVGGGTWTCTAAT-3')

94°C for 3 min; 35 cycles of 94°C for 45 sec, 50°C for 60 sec, 72°C for 90 sec; final extension 72°C for 10 min.

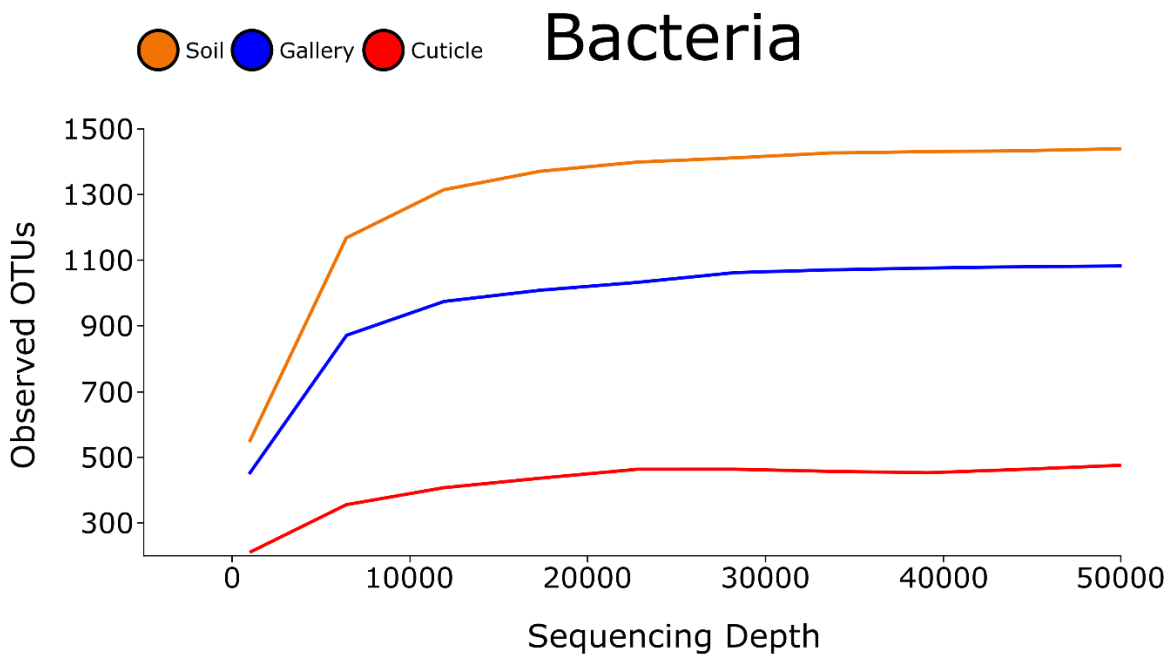
Fungi - ITS2 gene region

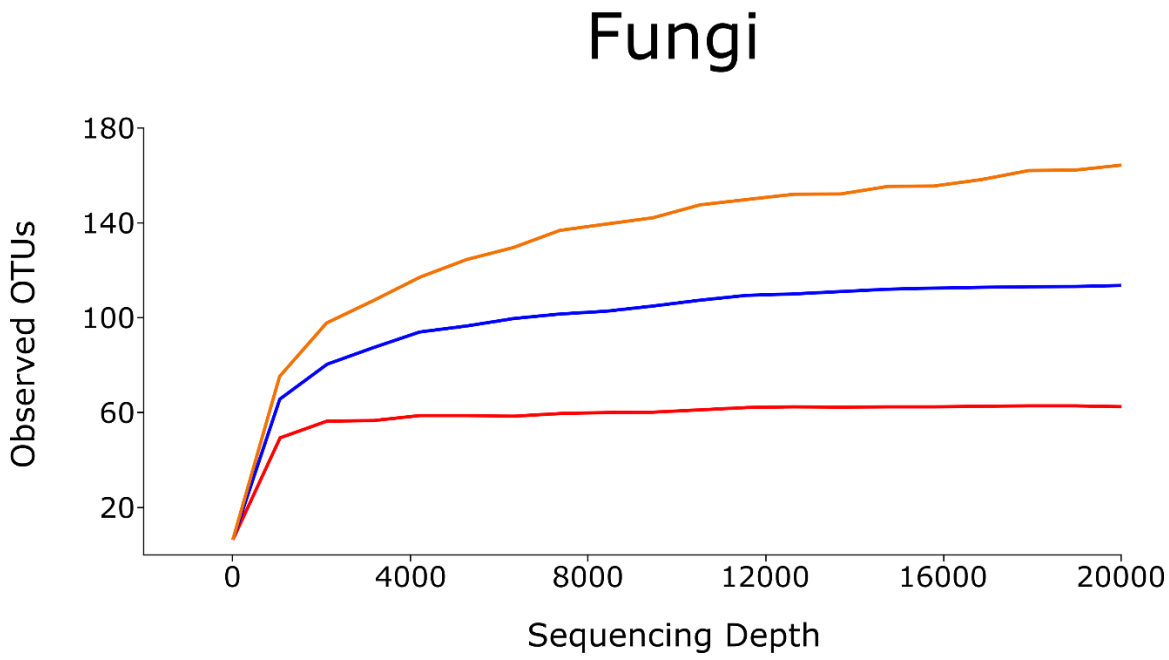
ITS3 (5'- GCATCGATGAAGAACGCAGC -3')

ITS4 (5'- TCCTCCGCTTATTGATATGC -3')

95°C for 2 min; 40 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min; final extension 72°C for 10 min.

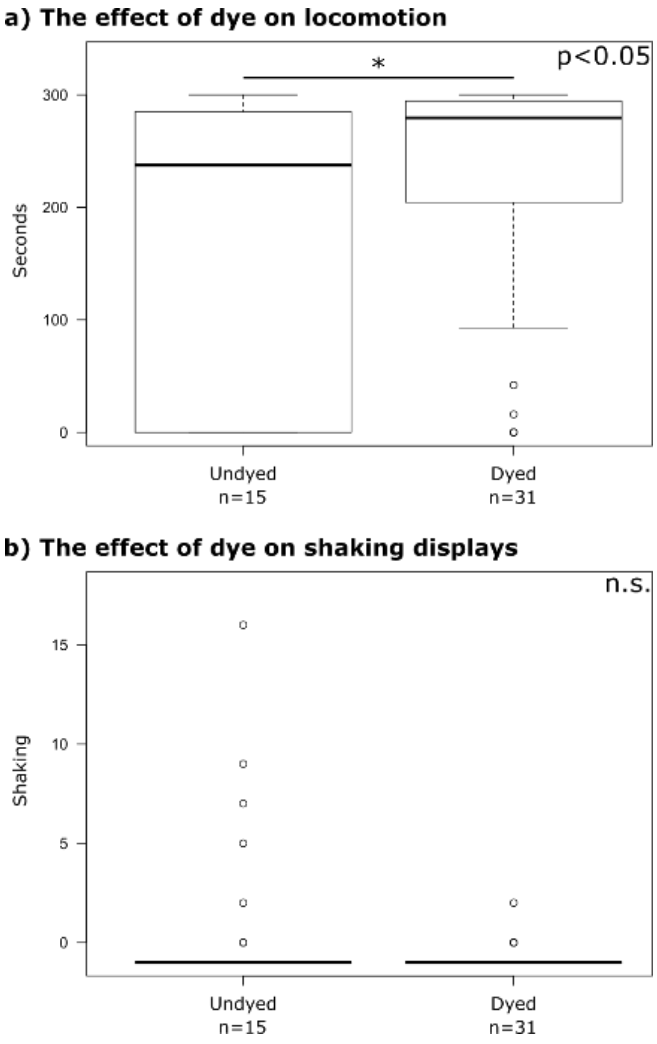
C-2 Rarefaction curves of observed OTUs for bacterial and fungal communities





APPENDIX D

D-1 Dye effect on immune behaviors



(a) Termites that were dyed (n = 31) had significantly higher locomotion than undyed termites (n = 15; $p < 0.05$). (b) There was no significant difference in shaking displays between dyed and undyed termites.