ABSTRACT

HALAWANI, OMAR. Lethal and Antimicrobial Responses to Bacterial Exposure across Ants. (Under the direction of Dr. Robert R. Dunn, Dr. Adrian Smith).

As the threat of microbial resistance rises, we try to increase our success in identifying novel antimicrobials by expanding our search beyond single source antibiotics. Observing other animal groups and their interactions with the bacteria in their environment may present new sources of antibiotic compounds and antimicrobial mechanisms. Ant societies (Hymenoptera: Formicidae) may have such interactions with their environmental micro-fauna, and investigation of these interactions could uncover new processes to control potentially pathogenic microbes. There are close interactions with ants and environmental bacteria, but more evidence is needed on how environmental bacteria impose evolutionary selection pressures on ant societies.

Whether general bacterial exposure is lethal to ants is largely unknown. As is whether ants' antimicrobial response is selective only to pathogens, if antibiotic use and production must have conditional requirements, or even if antimicrobial ability may vary between ant species. Conditional responses to the presence of a microbial threat would strengthen evidence for an evolutionary history of interactions among the ants and bacteria. Few species of ants have been studied extensively against non-entomopathogenic bacteria, with most studies selecting a particular ant species' ability to inhibit multiple bacteria. Further comparative studies promise potential insights to mechanisms in social immunity.

I tested if ants are susceptible to environmental bacteria by exposing four ant species to high concentrations of *Staphylococcus epidermidis* and *Escherichia coli* and measuring their mortality over 48hrs. I found that *E. coli* negatively affected

Dorymyrmex bureni and *Solenopsis invicta* ants but had non-significant effects on *Brachyponera chinensis* or *Aphaenogaster rudis.* Exposure to *S. epidermidis* did not produce significant mortality on any species. To address any potential conditional response in antimicrobial ability from bacterial exposure, a second experiment was conducted to test if surface extracts from bacteria exposed ants are more inhibitory from treatment exposures as compared to control exposure. *S. invicta* and *B. chinensis* showed no conditional response as tested. This experiment did not find evidence for conditional responses, but different conditions may be required.

Overall, the findings of this comparative study present evidence that some environmental bacteria may be affecting ants. While there was no evidence for conditional responses in antimicrobial ability in these experiments, the results correlated in that ants that displayed antimicrobial ability were in turn, negatively affected from exposure to the bacteria they could inhibit. If antimicrobial ability is paired with susceptibility, there may be interactions from bacteria producing pressure on certain ant species to use antimicrobial defenses.

Lethal and Antimicrobial Responses to Bacterial Exposure across Ants

by
Omar Halawani

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Biology

Biology

Raleigh, North Carolina
2020

APPROVED BY:

_____	_____
Dr. Robert R. Dunn	Dr. Adrian Smith
Committee Co-Chair	Committee Co-Chair

_____
Dr. Amy Grunden

BIOGRAPHY

This degree is an escalation of ideas that first began in a senior year undergraduate course with Dr. Dunn and Dr. Smith. We first explored social insect societies for ways to improve our public health, to now trying to discover new sources of antibiotics by learning about the public health needs of ant societies. While we have continued to reveal new discoveries that may be useful for humans, this project has always been about acknowledging and learning from ants.

TABLE OF CONTENTS

LIST OF TABLES v
LIST OF FIGURES vi

Chapter 1: Comprehensive review of ant antimicrobial ability and potential.....1

Antibiotic discovery 1
Ant antimicrobial ability 2
Ant behaviors inhibit entomopathogens 5
Ant antimicrobial compounds 6
Ant antimicrobial compounds inhibit non-entomopathogens in vitro 8
Conclusion 10

Chapter 2: Lethal and Antimicrobial Responses to Bacterial Exposure across Ants..... 11

Introduction.....11
Methods 17
Results 21
Discussion 29

References 34

Appendix 42
Appendix A: Supplemental information for chapter two 43

LIST OF TABLES

Table 1. Kruskal-Wallis test for water and isopropanol extract antimicrobial ability in *S. invicta* and *B. chinensis*. 43

LIST OF FIGURES

Figure 1. Timeline of antibiotic release and resistance acquisition. 2

Figure 2. Ant antimicrobial defenses.....5

Figure 3.1. Exposure mortality at 24 hrs 24

Figure 3.2. Exposure mortality at 48 hrs 24

Figure 4.1. *S. invicta* inhibition potential vs. *S. epidermidis* 27

Figure 4.2. *S. invicta* inhibition potential vs. *E. coli*.....27

Figure 5.1. *B. chinensis* inhibition potential vs. *S. epidermidis*. 28

Figure 5.2. *B. chinensis* inhibition potential vs. *E. coli*. 28

Figure 6. Measuring change in antimicrobial ability for *B. chinensis* and *S. invicta*.....29

Figure 7.1. H_2O *S. invicta* inhibition potential vs. *S. epidermidis* 44

Figure 7.2. H_2O *S. invicta* inhibition potential vs. *E. coli*.....44

Figure 8.1. H_2O *B. chinensis* inhibition potential vs. *S. epidermidis* 45

Figure 8.2. H_2O *B. chinensis* inhibition potential vs. *E. coli*.....45

Figure 9. H_2O Measuring change in antimicrobial ability for *B. chinensis* and *S. invicta*.....46

CHAPTER 1: Comprehensive Review of Ant Antimicrobial Ability and Potential ANTIBIOTIC DISCOVERY

Antibiotics have improved modern medicine's ability to treat and cure a wide range of ailments. These medicines inhibit microbial infections, usually by disrupting some essential function of the bacteria [1]. In microbial societies, antibiotics may be used by microbes to remove bacterial challenges in competition [2]. In human societies, antibiotics were discovered to inhibit bacterial pathogens and provided cures for previously debilitating or fatal infections. Antibiotic research peaked with a golden age of discovery in the mid-1900s, with many new classes of medicines that removed the threat of bacterial infections [2].

Antibiotics are characterized into classes based on their mode of action. The antibiotic classes we use in medicine tend to have modes of action that are generalized, to target a wide range of microbes. When antibiotics work, they can wipe out entire colonies of bacteria. Few colonies, though, may contain a mutation that allows them to survive an antibiotic [3]. This gene mutation leads to resistance. Colonies continue to acquire resistant traits as they survive antibiotic attacks, therefore unsuccessful antibiotic attacks promote resistant bacteria [3]. Resistance mechanisms can evolve to an individual antibiotic or against an entire antibiotic class [4]. The reliance on antibiotics with generalized mechanisms paired with widespread use have, over the past 60 years, produced a selective pressure on microbial pathogens to quickly acquire resistance [4]. The acquisition of resistance against entire classes of antibiotics has been increasing since the first use of penicillin [Fig. 1].

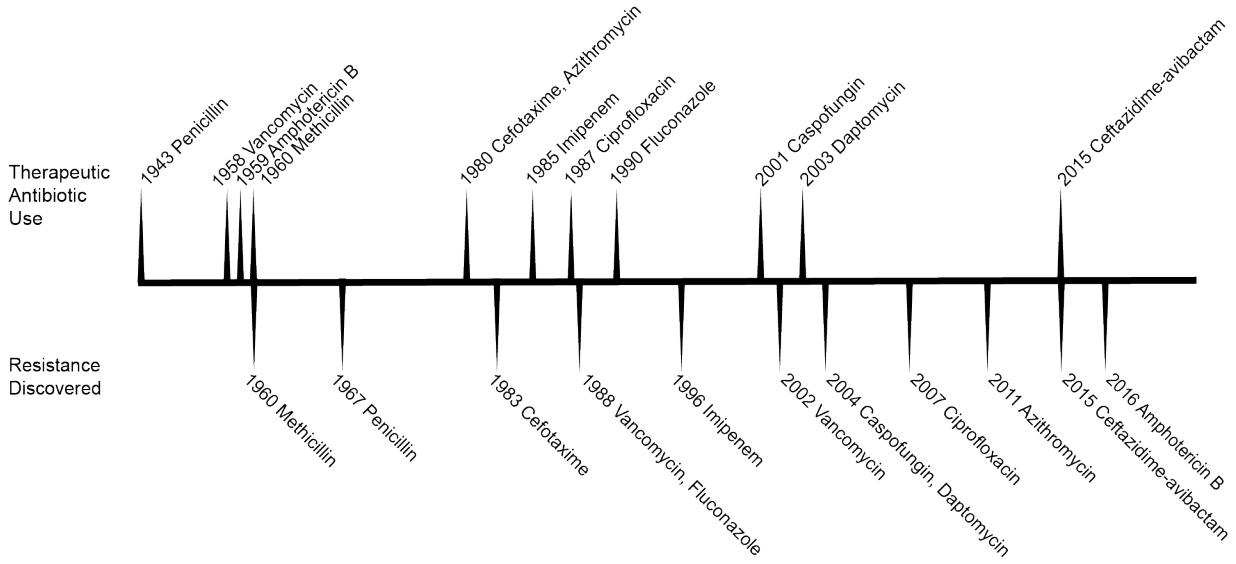

Figure 1. Timeline of therapeutic antibiotic use and resistance. Since the discovery of penicillin in 1943, classes of antibiotics have quickly been met with resistance after their widespread release. Data acquired from CDC.gov

To combat resistance, new antibiotic discovery spurred stronger clinical requirements that involve resistance trials [3]. Along with more rigorous testing, new effective compounds became increasingly difficult to find [5]. This slowed discovery nearly to a halt, but with the overwhelming threat of resistance, the need for new antibiotic discovery has been widely recognized as critically important for human health. In an urgent attempt to curb resistance, new antibiotic research has only produced modifications of current compounds [5]. This increases the immediate availability of medicine to patients but only slows the inevitable—multiple drug resistant bacteria.

Resistance to our current available pharmaceuticals threatens our ability to protect ourselves from pathogens. To combat resistance, pharmaceutical research has stressed that discovery of new effective medicine is an urgent matter for public health. Discovery of novel medicine is slow and challenging, but quick results have been seen by altering the mechanisms of currently available medication to circumvent resistant

microbes [6,7]. These newer mechanisms are still met with resistance, often quickly [6]. As the threat of microbial resistance rises, we try to increase our success by expanded our search from individual species that may produce antibiotics to searching for antimicrobial ability in other animal societies [8]. Observing other animal groups and their interactions with the bacteria in their environment may present new sources of antibiotic compounds and antimicrobial methods.

ANT ANTIMICROBIAL ABILITY

Ant societies (Hymenoptera: Formicidae) may have interactions with their environmental micro-fauna, and investigations of these interactions could uncover previously unknown methods to control potential microbial pathogens [1]. Ants are a large and diverse group, and literature already describes some components of ants' ability to resist microbial infection [11]. Ability to defend against microbes may be essential for ant societies, as defining traits of eusocial insects increase susceptibility to pathogens. Eusocial groups display multigenerational cooperation, where closely related workers manage brood care, foraging, resource sharing, and waste removal [12,13]. This creates high genetic similarity, which increases colony level risk from infectious pathogens, even in common behaviors such as in waste removal or leaving the nest to forage. Resource sharing and trophallaxis further increase interaction rates. These eusocial behaviors lead to interactions that are similarly mirrored in all social living groups, and by studying ant behaviors, there is a potential for novel insights regarding antimicrobial defense for other groups.

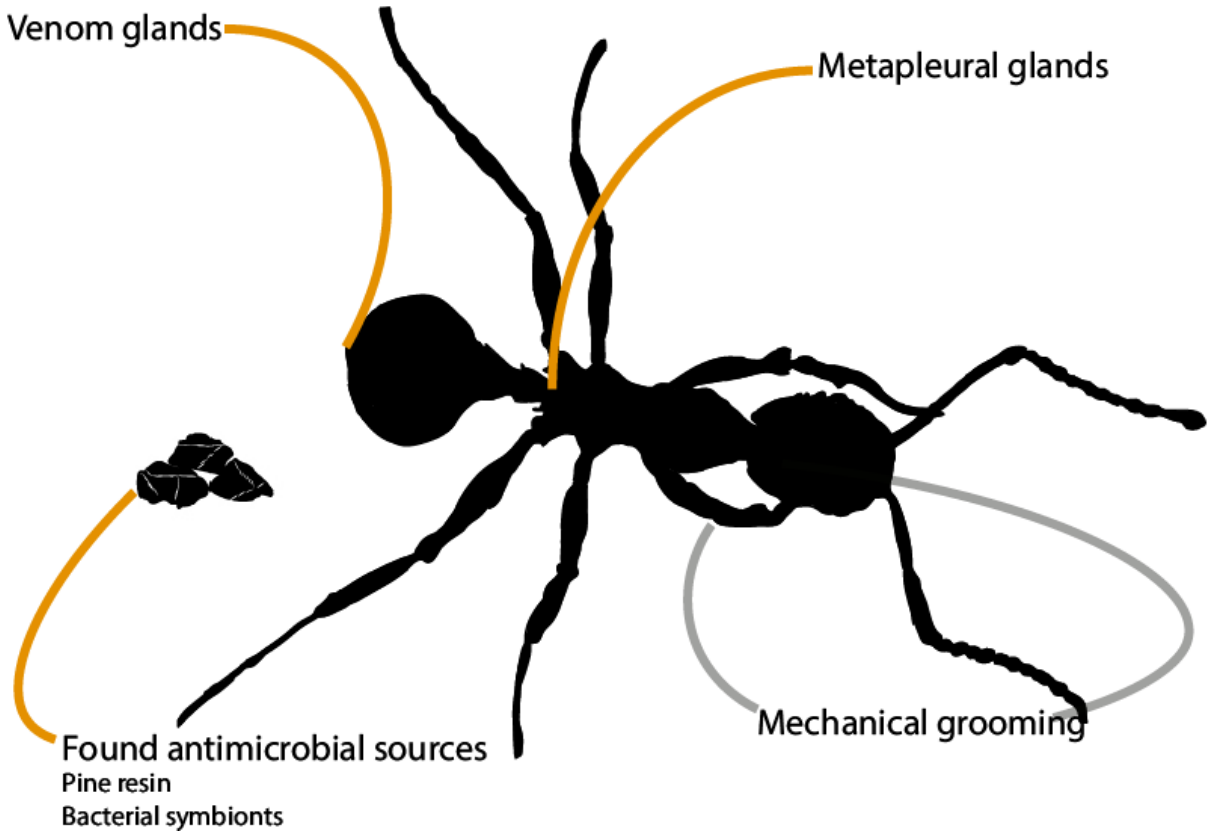

Figure 2: Ant antimicrobial defenses. Ants primary antimicrobial defense can be grouped into two categories. Mechanical defense via grooming removes foreign objects but also facilitates the use of antimicrobial products produced by their venom glands, metapleural glands, or from found objects.

Ants' antimicrobial defenses are mostly derived from mechanical and chemical defenses. [Fig. 2]. These defenses have been most studied in four ways. The first is ant grooming, a behavior found throughout social groups that mechanically removes foreign objects and facilitates the rest of ants' antimicrobial defenses [13]. The second is the use of antimicrobial products. Antimicrobial products can be self-produced or found in nature and are used in grooming, brood care, and nest care [11,14]. Antimicrobial compounds have been shown to inhibit entomopathogens, and evidence suggests that deploying antimicrobial compounds can be adjusted or conditional [11]. These compounds work in tandem with ant behaviors to inhibit entomopathogenic microbes [14]. Studied separately,

antimicrobial compounds are analyzed for their mode of action and how ants distribute them [15,16]. Lastly, the efficacy of ant antimicrobial products is tested against human pathogens in vitro, a promising angle of research for discovering new human medicines [17]. It is unknown if ants need to defend against human-associated bacterial pathogens, but the ability to inhibit resistant pathogens might indicate a new antibiotic source [11].

ANT BEHAVIORS INHIBIT ENTOMOPATHOGENS

Ants display two forms of grooming behavior: self-grooming or allogrooming, the grooming of nest-mates. The behavior is driven by a technique of using limbs and mouth parts to physically remove foreign objects, which are digested and inhibited in the gut [18]. Ants constantly groom themselves, particularly after foraging and exposure to contaminants. Allogrooming consists of ant workers grooming other worker ants, alates and the queen, and brood [13]. Ants use both behaviors to maintain a supposed hygienic standard, as workers have been seen to adjust behaviors to any entomopathogenic threats.

Grooming seems to be important for defense, *Acromyrmex echinatior* ants infected with *Metarhizium* had significantly lower pathogen transmission rates and higher survivability if they were groomed by their nest-mates [19]. Self and allogrooming behavior have been studied extensively against the fungal entomopathogen, *Metarhizium anisopliae* [20]. In a comparative study of *Polyrhachis dives, A. echinatior, Formica fusca, and Myrmica ruginodis,* grooming rates were shown to increase when workers were treated with *Metarhizium,* and higher grooming rates increased survivability [21]. *A. echinatior* allogrooming also doubled after introducing an infected worker to groups. Similar responses where antimicrobial behavior was modified to

exposure by a pathogen were seen in *Formica selysi* [20], *Oecophylla smaragdina* [22], and *Ectatomma ruidum* [23]. Recognizing infection can also help colony survivability, *Camponotus aethiops* ants reduced their interaction rates with nest-mates, including brood, when they were infected with *Metarhizium* [24]. Their cuticular hydrocarbon signal did not change to alert nest-mates, but as the infection progressed, sick ants would remove themselves from their nests.

In many cases, ants seem to have recognized infections and responded to entomopathogenic threats by increasing their rates of grooming and allogrooming. While grooming behavior is driven by mechanical removal, the behavior also facilitates the spread of antimicrobial compounds.

ANT ANTIMICROBIAL COMPOUNDS

Antimicrobial compounds are usually self-produced secretions sourced from ant metapleural glands [25] or venom glands [26]. Metapleural glands are paired exocrine glands found on the propodeum/petiole of ants in most subfamilies, that excrete strong antimicrobial compounds which inhibit known entomopathogens [25]. The glands secrete small amounts of compounds, mostly acids, that the ants spread in grooming and brood care [25]. Metapleural gland secretions from *Acromyrmex subterraneus* and *Polyrhachis dives* protected brood and nests from possible entomopathogenic infections [27]. Gland usage can be complex, too. Another leaf cutter ant, *Acromyrmex octospinosus*, displayed robust use of their metapleural gland [28]. Workers up-regulated gland production against more virulent entomopathogens and their secretions were more effective in vitro at inhibiting pathogens if workers had been challenged by the

entomopathogen before extracting glandular secretions. These experiments suggest antimicrobial ability can be induced under specific conditions.

The other major source of antimicrobials is ant venom. Primarily evolved for predation and defense, recent venom studies also indicate they may be an effective antimicrobial [29]. Venoms are a mixture of alkaloids, acids, amines, and peptides delivered via stinger or sprayed via acidopore, and have wide taxonomic diversity throughout ant species [26]. In some cases, venom use significantly contributed to the effectiveness of allogrooming when used in concert. This mechanism involves collecting venom in the mouths of workers to be spread on nest-mates, including brood [21,30]. Brood are regularly cleaned with venom in *Lasius neglectus,* both directly by spraying and indirectly by spreading venom with their mouth parts, increasing their survivability [30]. Venom was found to be an important component of defense in ants with or without a metapleural gland, supporting the hypothesis that all ants may have strong antimicrobial ability [21]. The wide diversity of metapleural gland compounds and venom gland compounds offers a large potential source of new antimicrobial compounds for antibiotic use against human pathogens.

Ants have been found to work with other organismal products for antimicrobial defense, such as using found natural products or compounds from symbiotic organisms. *Formica paralugubris* ants collect conifer resin which is found throughout their nests and shows antimicrobial activity against entomopathogens [31]. In fungus farming ants, a *Streptomyces* symbiont which defends fungus farms and the colonies from entomopathogenic fungi is found on adult worker cuticles [32]. Similarly, *Actinomycete* symbionts defend the fungus farm in *A. octospinosus* [33]. For many species, ant sanitary

behavior may be complex and more expansive than just using self-produced antimicrobial compounds.

ANT ANTIMICROBIALS INHIBIT NON-ENTOMOPATHOGENS IN VITRO

Human medicine has been met with bacterial resistance quickly [6], but ants have seemingly co-evolved with entomopathogens while maintaining the ability to produce effective antimicrobial compounds. Few species of ants have been studied extensively against non-entomopathogenic bacteria, with most studies selecting a particular ant species ability to inhibit multiple bacteria. These in vitro studies suggest ant venom has had a complex evolutionary history, from more general prey capture and mammalian defense to more evolved compounds that display antimicrobial ability.

Pachycondayla goeldii venom was synthesized and analyzed for antimicrobial activity against various environmental bacteria and found to have strong inhibitory effects on gram negative and gram positive bacteria [34]**.** In a comparative analysis of 20 ant species, surface extracts from workers were tested against *S. epidermidis* to identify antimicrobial ability [35]. Ability was suggested to be phylogenetically linked, *Solenopsis invicta*, *S. molesta*, and *Monormium minimum* showed the strongest ability, and some Formicines showed strong inhibition from surface extracts. *S. invicta* have antimicrobial alkaloids in their venom that were effective inhibitors of *S. epidermidis* and *E. coli,* a gram positive and gram negative bacterium, respectively, suggesting general antimicrobial ability [36]. Fire ant venom may have broad, strong antimicrobial ability; in other studies, the venom has been shown to inhibit complex *Pseudomonas fluorescens* biofilms [37] and disrupt quorum-sensing signaling [16], and synthetic venom derivatives have been used to inhibit specific human pathogens [38]. The venoms from ants in the

genus *Solenopsis* were determined to have similar composition and properties, and all shared potential antimicrobial ability [39]. There were similar properties found in closely related ants of the *Monomorium* genus [39], further suggesting antimicrobial ability towards non-entomopathogenic bacteria may be characteristic of certain groups.

General ability may not be in all ants, but antimicrobial ability has still been noted against specific bacteria. In a study of antimicrobial ability of the venom from three *Crematogaster* ants, only one species displayed general antimicrobial ability against gram positive and negative bacteria [40]. Ability may even be selective, *Formica rufa* venom proteins have some antimicrobial properties but could only inhibit *E. coli* [41]. Alternatively, ant venom can be more complex. Venom from *Dinopera quadriceps* was tested in a crystal violet bioassay, where tetrazolium salts are added to an incubated well plate to determine bacterial count [17]. The venom was an effective antimicrobial against *Staphylococcus aureus*, a human pathogen that has acquired antibiotic resistance. In the diversity of ant venoms, there may be variety in effectiveness of antimicrobial compounds but the possible linkage of antimicrobial ability to phylogeny emphasizes the need for comparative studies across ant societies.

Venoms, though, are not the only source of diverse antimicrobial ability. Similar ability can be found from metapleural gland secretions. *S. invicta* and *S. geminata* produce effective antimicrobial compounds from their metapleural glands, although venom is likely their primary method of disinfection [42]. *Atta sexdens rubroplisoa* metapleural gland secretions show general antimicrobial ability and were able to inhibit gram negative and gram positive bacteria, in vitro [43]. *Myrmecia gulosa* metapleural gland secretions show specific mechanisms against different bacteria [44], with the ability

to disrupt gram negative cell walls and the ability to disrupt the plasma membrane and cytoplasm against all tested microbes—including yeast. Hemolymph from *M. gulosa* contains an antimicrobial peptide, this could be used in both metapleural gland and venom gland production [45]. Antimicrobial ability might vary across the phylogeny, but certain species have demonstrated the ability to produce antimicrobial compounds capable of inhibiting non-entomopathogenic, environmental bacteria.

General antimicrobial ability isn't limited to self-produced compounds. As with entomopathogens, symbiotic fungi in fungus-farming ant colonies produce antimicrobial compounds capable of inhibiting multiple antibiotic resistant pathogens, even in resistance trials [46]. *Formica paralugubris* ants collect conifer resin to distribute throughout their nest. The resin has strong antimicrobial ability against multiple non-entomopathogens, including an opportunistic pathogen [47].

CONCLUSION

As the literature stands today, we understand antimicrobial ability in ants mostly through single species case studies. Identifying which bacteria can be inhibited is beneficial, but slow if the goal is to identify new antibiotic sources. While results are promising, discovery is slow and future applications are limited. Potentially, understanding the evolution of antimicrobial ability in ant societies could more easily identify antibiotics in ants. While we've begun to understand which ant antimicrobial compounds inhibit human associated bacteria, we do not understand why ants may use them in nature. A more practical approach to discovery might be identifying ant societies that interact with microbes that are similar to human pathogens. By identifying species that display a need to defend against human pathogens, we can target discovery at

species with similar requirements for antimicrobial defense. We may be able to identify novel compounds by studying the life history of ant societies and describing which species share our need to defend against pathogenic microbes. We may be able to recognize indicators for strong antimicrobial ability across ant species if we know the possible interactions between human associated, non-entomopathogenic bacteria and ant societies.

CHAPTER 2: Lethal and Antimicrobial Responses to Bacterial Exposure across Ants

INTRODUCTION

Living in societies can increases individuals' survivorship, brood-care, and foraging success [12]. However, social life can come with drawbacks, such as an increase in pathogen susceptibility from high levels of relatedness between group members [2, 19]. As a result, many social animal species have evolved collective defenses [48]. Some defenses include hygienic behaviors such as allogrooming—the grooming of nest-mates—or the removal of waste and dead individuals [11]. Other defenses include the production of prophylactic antimicrobial compounds [49]. In ants, particular prophylactic antimicrobial compounds are most well-documented in response to fungal entomopathogens [50]. However, the microbial-rich environments of ground-nesting ants facilitates interactions with many more microorganisms beyond fungi and known entomopathogenic species [51]. Whether these bacteria kill ants is largely unknown, as is whether ants' antimicrobial response is selective only to pathogens, if expression must have conditional requirements, or even if antimicrobial ability may vary between species. Further comparative studies promise potential insights to unseen mechanisms in social immunity.

Ants' response to bacteria in their environment, whether pathogenic or not, has not been well documented. Soil can contain a high diversity and density of bacteria [18]. Soil from ant nests have exhibited higher microbial diversity, including entomopathogens [52,53]. Ants, particularly those that are ground-dwelling species, inevitably interact with these bacterial species [50]. Ants are likely to interact when

behaviors like resource foraging and food storage additionally provide sufficient conditions to promote bacterial diversity in nests and nearby soil [54]. Most recorded bacterial interactions are limited to *Pseudomonas* or *Wolbachii* [50]—both taxa that are commonly found in soil. *Pseudomonas* species have not been shown to infect ants [55] and *Wolbachii* entomopathogens mostly target reproductive fitness [56]. In addition, many other apparently non-entomopathogenic, environmental bacteria have been recorded to exist on ants. For example, in urban settings, environmental bacteria have been found on ant cuticles, even carrying pathogens in potential nosocomial infections [57]. Non-entomopathogenic bacteria have also been sampled for in other ants; a whole body analysis of *Polyrhachis* ants found evidence of vertical transfer of bacterial strains following phylogeny, suggesting that ant societies have evolved alongside their bacterial micro fauna [58]. There are close interactions with ants and environmental bacteria, but more evidence is needed to suggest that bacteria produce an evolutionary selection pressure on ant societies.

Ant societies display evolved behavior as an entomopathogenic defense. Hygienic behaviors have been developed to reduce pathogenic load and increase survivorship of individuals and colonies [11]. Certain hygienic behaviors focus on nest sanitation to reduce pathogenic threats and are characteristic of most ant species. Most ants reduce pathogenic load by confining immature brood and the queen to deeper chambers, spatially separating more vulnerable members from potentially infected foragers that are leaving and entering the nest [12, 58]. Nests of *Azteca* ants suggest that workers controlled brood chambers for pathogens and where able to reduce bacterial populations, as opposed to abandoned nest chambers that were overtaken by a fungal

pathogen [60]. Grooming is another hygienic behavior found in all ant species that effectively inhibits pathogens from the cuticles of individuals, brood, and other nest-mates [21,22,30]. The mechanical removal of infectious agents facilitates chemical inhibition; grooming applies venom and other antimicrobial compounds to the cuticle [30].

There is evidence that ants can change grooming, allogrooming, and antimicrobial delivery rates in response to entomopathogenic threats, suggesting ants can recognize and appropriately respond to pathogenic threats. Worker ants of *F. selysi* increased self-grooming rates when they were infected with pathogenic spores, and groups would allogroom returning foragers for a longer duration if the group had previously been exposed to a pathogen [20]. In *Lasius neglectus,* workers could identify infected brood from healthy brood and would display a behavior called 'destructive disinfection' to kill, then remove infected brood to alleviate pathogenic threat to the colony [61]. These behaviors suggest ant societies can detect and combat entomopathogenic populations in their colonies. Further research into how ant societies control bacterial populations could identify potential novel interactions.

Some studies have suggested antimicrobial ability could work similarly to immune systems. Social insects have demonstrated similar benefits to immunization from low-risk challenges of pathogens to develop group immunity. An experiment that demonstrated this in termites found that groups had better survivorship when challenged with *Metarhizium anisopliae* if the group contained immunized individuals, with the entire colony benefiting through a social transfer of immunity among nest-mates [49]. *Lasius neglectus* ants demonstrated conditional requirements when defending from

entomopathogenic threats [15]. The act of allogrooming was shown to infect nest-mates with a low-level infection which then increased resistance in a subsequent exposure of the same pathogen but decreases resistance if the secondary exposure was from a different pathogen—two heterologous infections were more destructive superinfections [15]. The conditional behavior was that ants would decrease allogrooming rates while increasing antimicrobial use when nest-mates were infected with a secondary pathogen, to reduce risk from superinfections by avoiding infections [15]. Another conditional response was seen in *Formica exsecta,* where researchers tested if starvation affected workers when they were orally exposed to *Serratia marcescens, E. coli,* or *Pseudomonas entomophila,* two opportunistic pathogen of animals and a known bacterial entomopathogen, respectively [62]. Exposure to *S. marcescens* always increased mortality but exposure to *E. coli* or *P. entomophila* did not increase mortality but decreased the effects of starvation on mortality. In non-starved, normal condition, antimicrobial gene expression of ants exposed to *P. entomophila* and *E. coli* was up-regulated possibly as a general immune response from a high bacterial load exposure or as the ant's prophylactic response. Under the same conditions, exposure to *S. marcescens* induced a strong down-regulation in antimicrobial gene expression, which may be from an inability to maintain defenses under pathogenic stress or evidence of a trade-off to reserve energy. *S. marcescens* may be an entomopathogen and exposure led ants to restrict metabolic activity to increase survivability. Conditional responses improving antimicrobial ability strengthen evidence for evolutionary interactions among the ants and a diverse range of microbes, and conditional tests replicated with bacteria would suggest potential evolutionary adaptations.

There is a potential of finding antimicrobial compounds in ants that can inhibit bacteria. Antimicrobial ability has been described for <.1% of all ant species from in vitro studies towards specific target bacterial species. For example, one study of antimicrobial peptides synthesized by *Myrmecia gulosa* showed no inhibition of all challenged gram positive bacteria but inhibition of the gram negative bacterium *E. coli* was demonstrated [45]. *M. gulosa* has also been shown to produce metapleural gland secretions that can disrupt gram negative (*E. coli*) cell walls as well as gram positive (*Bacillus cereus*) plasma membranes [44]. Other studies have identified antimicrobial ability directly from ant venom. The venom from *Solenopsis invicta* contains multiple alkaloids with different individual antimicrobial ability against gram negative or gram positive bacteria, with a generally higher ability inhibiting gram positive bacteria [36]. In another study, *S. invicta* venom alkaloids were isolated and tested against *Pseudomonas fluorescens* biofilms, the venom was found to be an effective antibiotic, even in small concentrations [37]. General antimicrobial ability was tested in a previous study, where surface extracts of 20 ant species from four subfamilies were tested for antimicrobial ability against *S. epidermidis.* Antimicrobial ability was detected in 40% of species, with a strong indication of taxonomic linkage for ability [35]. Closely related species displayed similar ability, with strong inhibition found in the Solenopsid tribe ants that were tested and moderate ability found in all tested Formicines. The study used surface extracts of a wide range of ant species in a microwell plate assays and only tested antimicrobial ability against one gram positive microbe. Ant antimicrobial ability has varied across phylogeny and constructing comparative studies to explore

phylogenetic differences offers a wider understanding of how ant societies interact with their environment.

Ant antimicrobial compounds have shown inhibitory ability to non-entomopathogenic bacteria. Antimicrobial ability against these bacteria could be evidence of previously unknown relationships between environmental bacteria and the health of ant societies. There is evidence of ants exhibiting effective antimicrobial ability to potentially defend themselves from some bacteria. To further understand this potential evolutionary relationship, comparative experiments surveying how ants respond to non-entomopathogenic bacteria are needed.

Here, I have tested if ants are susceptible to infection from environmental bacterial using a gram positive bacterium (*Staphylococcus epidermidis)* and a gram negative bacterium (*Escherichia coli)* by exposing ants to possible bacterial challenges and measuring their mortality. To address any potential conditional response in antimicrobial ability, a second experiment tested if surface extracts from bacteria exposed ants are more inhibitory as compared to a control. An initial exposure to a bacterial challenge resulting in an increase of antimicrobial ability from ant surface extracts would indicate a conditional response for expressing antimicrobial compounds.

METHODS

Bacteria were sourced from Carolina Biological. *Staphylococcus epidermidis* and *Eschercia coli* strain K12 were kept in glycerol stocks at -80 C, then spread onto BD Difco LB Miller agar [Fisher Scientific] plates. Plates were incubated for 24hrs at 35°C. Bacteria were maintained as plates for experiment 1. For experiment 2, bacteria from

agar plates was also cultured in liquid BD Difco LB Miller media. Liquid incubated for 24 h at 35°C before use in assays.

Experiment 1: Ant mortality in response to exposure to non-entomopathogenic bacteria

I measured worker mortality across four ant species in response to exposure to two non-entomopathogenic bacteria. I collected *Solenopsis invicta* between June-December of 2018 in Raleigh, NC by collecting the tops of mounds with a shovel. *Aphaenogaster rudis* group ants were collected in Durham, NC and Raleigh, NC from July to August of 2018 by aspirating workers from within their nests. *Brachyponera chinensis* were collected in Raleigh, NC in December 2018 to May 2019 by opening dead logs to expose workers for collection via aspiration. *Dorymyrmex bureni* colonies were collected in Hoffman, NC in June 2019 by collecting mounds with a shovel. These species were chosen from the 20 species sampled in Penick et al. [35] study which they showed varying antimicrobial ability. Ants from *S. invicta* and *D. bureni* showed strong ability, whereas *A. rudis* and *B. chinensis* ants showed weak ability [35]. All colonies were removed from their nesting material and kept as lab colonies for <24 h in containers with Fluon around their edge, they were fed sugar water to avoid weakening possible antimicrobial ability, as reduced carbohydrate levels have been shown to lower antimicrobial expression [23].

200 *S. invicta* workers and 50 *B. chinensis*, *D. bureni*, and *A. rudis* workers were used for each exposure treatment. The difference in number of workers used for *S. invicta* and the other species was based on what could be consistently captured from colonies. Experiments with each species were replicated with 15 colonies, where each

colony was subject to three treatments. Treatments were exposure to *E. coli*, exposure to *S. epidermidis*, or exposure to sterile agar as control. Exposure experiments were performed in deep petri dishes (25 mm) with Fluon around their edges. Ants were exposed to a small piece of agar (10 mm x 9.7 cm^2) that had a bacterial lawn of *S. epidermidis*, *E. coli*, or no bacterial growth as a control. Bacterial lawns were grown from liquid culture grown overnight at 35 °C in a shaking incubator adjusted to a 0.5 MacFarland standard. In the experimental arena, agar pieces with and without bacteria were hydrated with 100 µl of MilliQ H_2O, this prevented agar from drying out and encouraged ant interaction with agar. Ant workers were introduced to exposure treatment after water was absorbed by agar by directly placing ants on agar to ensure at least one point of direct exposure. Mortality was measured by counting dead ants at 24 h and a continuous count at 48 h. Observations were recorded when ants tunneled into agar, indicating direct interactions.

I tested the effect of exposure of bacteria on the mortality of ants with 15 colonies from 4 species to test if contact of ants with the two bacteria lead to significant mortality when compared to the control. Data at 24 h and 48 h were graphed and analyzed with a Friedman's rank sum test with Finner post hoc pairwise comparison in R. Friedman's test is similar to a one-way ANOVA for an unreplicated complete blocked design of nonparametric data [63]. The Finner post hoc comparison procedure was used for indicating significance of treatment effects. Finner's post hoc uses a step-down p-value adjustment value, it rejects test statistics when $p_i > 1-(1-a)^{(k-1)/i}$ [62]. Friedman's statistical tests used at a significance level of $\alpha=0.05$ and Finner post hoc used an adjusted

threshold level of significance of α_1= 0.0975, α_2= 0.05, or a_3=0.0336. Analysis was carried out in R version 3.5.2 [64], using the packages scmamp [65] and devtools [64].

Experiment 2: Does exposure to bacteria lead to an increased presence of surface antimicrobials?

I compared the antimicrobial ability of extracted surface compounds from bacteria-exposed and non-exposed *S. invicta* and *B. chinensis* worker ants. In a previous study, these two species showed different levels of antimicrobial ability with *S. invicta* producing a strong response and *B. chinensis* lacking antimicrobial ability [35]. I collected *B. chinensis* ants by opening wood logs with their nests and aspirating workers. The ants were collected between January and May 2019. To collect *S. invicta* ants, the tops of nest mounds were removed, and workers were aspirated from removed soil. Ants were collected between June and December 2018. Both species were collected in Raleigh, NC.

The experiment consisted of an exposure treatment followed by extracting surface compounds of workers to be challenged by bacteria in a liquid culture well plate assay. 200 *S. invicta* workers and 50 *B. chinensis* workers were used for each exposure treatment. I used 15 colonies per species for treatments, in an incomplete block design. Treatments were exposure to *S. epidermidis*, *E. coli*, or sterile agar for control, following the protocol used for experiment 1 above. Bacterial lawns were grown from liquid bacterial cultures of *E. coli* and *S. epidermidis* adjusted to a 0.5 MacFarland standard after growing overnight at 35 °C in a shaking incubator. 100 µl of MilliQ H2O was pipetted under the agar to rehydrate media and encourage ants to interact with the agar. Exposure treatments ran for 6 h. Then, I removed any dead individuals from exposure

before freeze-killing the remaining living workers for extraction. Exposure times were determined from earlier trials, I selected a sub-lethal level of exposure as workers began to die after 6 h.

Surface compounds of 40 workers from each exposure treatment were extracted in either 360 µl isopropanol or 360 µl sterile MilliQ H2O for 15 min after an initial vortex spin for 15 s. Previous studies have shown effectiveness of using nonpolar compounds to extract antimicrobial properties, I wanted to capture a wider range of active compounds [35,66,67]. Extracts were filtered through 0.2 micron SEP filters [66]. Isopropanol extracts were evaporated in a vacuum centrifuge then resuspended in 360 µl LB. Water extracts were used directly after filtering.

I adjusted a 96 well plate assay protocol for testing antimicrobial ability against bacterial liquid culture [66]. I tested 10 worker equivalents of ant body-surface extracts (90 µl) against 100 CFU's of either *S. epidermidis* or *E. coli*. Bacterial controls of 100 CFU's bacteria (*S. epidermidis* or *E. coli*) with LB or H_2O were plated as maximum growth controls. Ant extracts with LB or H_2O were plated as minimum growth controls. Media controls were also plated to verify there was no contamination. Plates were incubated at 35 °C for 18 h before pipetting 10 µl of WST-8 [PromoKine, Heidelberg, Germany] followed by another hour of incubation to allow the salt to bind to available live cells. WST-8 salt binds to cellular membranes of organisms that are undergoing active transport, and therefore, provides a colorimetric method to analyze live microbial count in culture [17]. After incubation, optical density was measured at OD_{600} with 5 s shaking before reading using a plate reader [SpectraMax M5, Molecular Devices, USA].

OD_{600} readings of the well plate were comparable values across the plate because of WST-8 binding equally to available live cells [PromoKine, Heidelberg, Germany; 8]. Readings of exposure treatment workers were measured as percent inhibition adjusted relative to the control treatment workers. Antimicrobial ability was determined by comparing adjusted percent inhibition to plated maximum and minimum growth controls. Final results were reported as change in percentage of inhibition of microbes between treatment and control groups. Results provided either a positive or negative inhibition value based on the treatment inhibiting more or less, respectfully, of the microbial challenge.

I tested if antimicrobial ability showed a conditional response with a Kruskal Wallis rank sum test. The R. A Kruskal Wallis analysis tests non-parametric data for multiple groups when assumptions for ANOVA are not met—in our case, our data was not a complete design. Some colonies were comprised of two sampled nests to complete all three treatments. In these instances, a control and single treatment group were sampled from colonies twice—once for *E. coli* and once for *S. epidermidis*. All statistical tests used a threshold level of significance of $\alpha = 0.05$. Analysis was carried out in R version 3.5.2 [64], using the packages dplyr [68] and devtools [64].

RESULTS

Experiment 1: Ant mortality in response to exposure to non-entomopathogenic bacteria

The *E. coli* exposure treatments for *S. invicta* and *D. bureni* produced significant mortality after 48 h. No significant effects were observed against *S. epidermidis* or in other ant species. The effects of exposure for all ants to *S. epidermidis* and *E. coli* at 24

h and 48 h are shown in Fig. 3. Results for the exposures were transformed across species as proportions of dead ants to the size of the group at the beginning of the experiments. Control group responses were subtracted from treatment group responses to directly compare treatment effects. Exposure at 24 h seemed to affect ants similarly, but variance was higher than data collected at 48 h (Fig.3). This was because control colonies had more consistent mortality at 48 h.

S. invicta showed significant mortality to exposure (mortality at 48 h, Friedman's chi-squared = 8.933, p=0.01149 Fig. 3). There was significance from exposure to *E. coli* (Finner p= 0.02074, adjusted p-value threshold <0.0975) but not from *S. epidermidis* (Finner p= 0.08723, adjusted p-value threshold <0.05) or between treatment groups (Finner p= 0.4161, adjusted p-value threshold <0.0336). *D. bureni* also displayed significant mortality only to *E. coli* exposure (mortality at 48 h, Friedman's chi squared = 23.333, p = 8.575e-06, Finner p = 5.214e-04, adjusted p-value threshold <0.0975, Fig. 3). *A. rudis* exhibited significance differences in mortality (mortality at 48 h, Friedman's chi squared = 10.03, p = 0.006627, Fig. 3), but the only interaction that was significant was between exposure treatments (Finner p=0.0114, adjusted p-value threshold <0.0975). *E. coli* exposure was not significant (Finner p = 0.07024849) nor *S. epidermidis* (Finner p= 0.36525). *B. chinensis* showed no significant mortality to either treatment (mortality at 48 h, Friedman's chi-squared = 2.233, p = 0.3274, Fig. 3). All Friedman's tests used a significance level of α=0.05.

Post hoc analysis was also assessed with a Wilcoxon signed rank test and Holm-Bonferonni p-value adjustment for the 48 h data set. Wilcoxon post hoc test was less conservative and suggested significant mortality more often than the Finner multiple

comparison post hoc test. For *S. invicta,* both exposure treatments showed significant mortality *E. coli* p=0.0003052, *S. epidermidis* p=0.01058). *D. bureni* analysis was similar as before, with exposure only to *E. coli* displaying significant mortality (*E. coli* p=6.1043-05, *S. epidermidis* p=0.2131). *A. rudis* results differed, as *E. coli* exposure suggested significant mortality (*E. coli* p=0.009439, *S. epidermidis* p=0.06397).

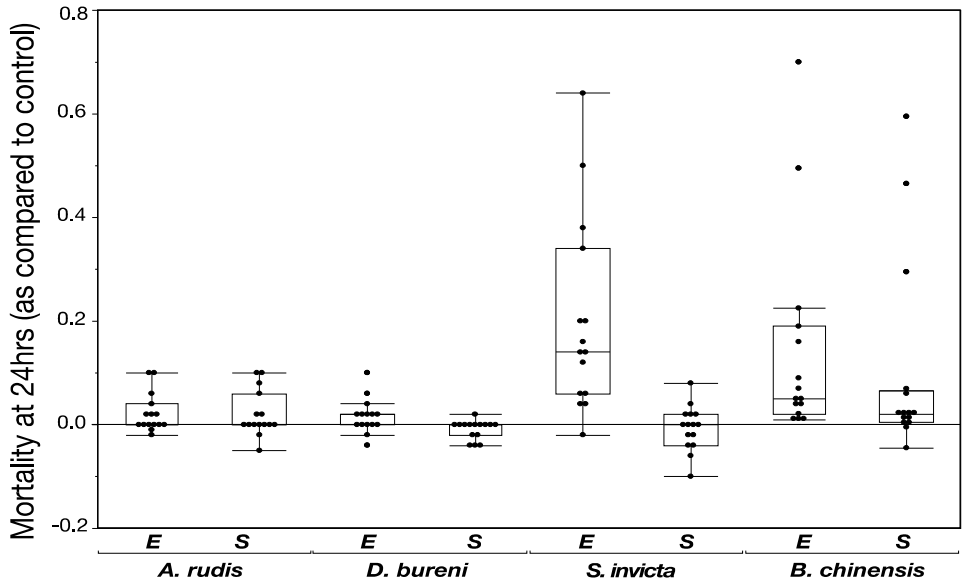

Figure 3.1 Exposure mortality at 24 h.

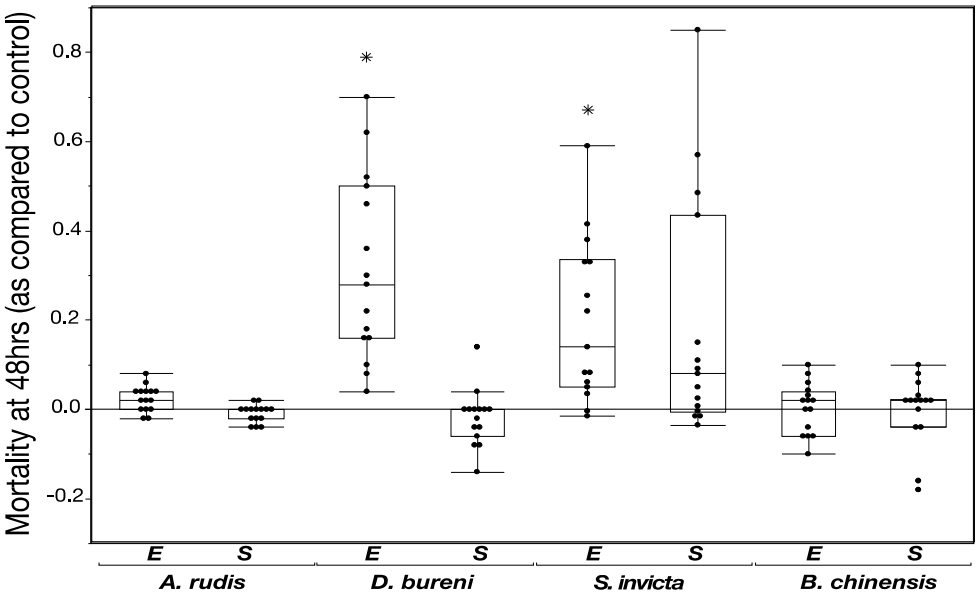

Figure 3.2 Exposure mortality at 48 h.

Figure 3. Mortality is shown for *S. epidermidis* (S) or *E. coli* (E) after adjusting treatment values to control value. 15 colonies were tested for each species, *A. rudis, B. chinensis, D. bureni,* and *S. invicta*, with each colony being exposed to a control and two experimental treatments. For *S. invicta*, 200 workers were in each exposure group. For *A. rudis, D. bureni,* and *B. chinensis,* 50 workers were in each exposure group. Exposure to *E. coli* significantly affected *S. invicta* and *D. bureni*. Box plot line represents median values, whiskers represent 1st and 3rd quartile, data points represent ant colonies, outliers are data outside of 1.5 IQR.

Experiment 2: Does exposure to bacteria lead to an increased presence of surface antimicrobials?

Exposure seemed to induce no conditional effect on antimicrobial production for both *S. invicta* and *B. chinensis.* Colonies of *S. invicta* and *B. chinensis* which were exposed to a bacterium were sampled for their surface extracts which were then subsequently tested against the same bacteria in a 96 well plate liquid assay to test if the strength of antimicrobial compounds on the surface of an ant's body increased. Antimicrobial ability was calculated from OD_{600} readings after WST-8 was added to wells and plates were incubated for one hour. OD_{600} readings were adjusted for media and normalized across plates by transforming results into percent inhibition compared to bacterial control growth. The raw inhibition results of the assay for *S. invicta* are shown in Fig. 4 and for *B. chinensis* in Fig. 5. I will primarily discuss surface extraction with isopropanol, as water extraction had similar results with higher variance [SI].

Figure 4 shows *S. invicta* has antimicrobial ability to inhibit *E. coli* or *S. epidermidis* with or without treatment exposures. Figure 5 shows *B. chinensis* lacks ability to completely inhibit *E. coli* but has antimicrobial ability to inhibit *S. epidermidis.* Water extracts for *S. invicta* reported similar ability, but *B. chinensis* antimicrobial ability could only be detected when extracted with isopropanol [SI].

To analyze a conditional response, treatment group results were adjusted to control groups to directly compare treatments. The results of the assay are shown in Fig. 6. In all species, previous exposure to these bacteria did not increase antimicrobial ability. In these figures some of the outlygin data points are percent inhibition measures beyond 100% and below 0%. Each data point is a measure of inhibition in wells where

half of the ant extract is added to an experimental well with live bacteria, however we report this measure relative to two other wells. One being only bacteria and media (a maximum bacterial growth control) and one being only media and the other half of the ant extract (a minimum growth control). For the outlying points beyond 100%, the half of the ant extract used as minimum growth control had a higher absorbency reading than the other half of the ant extract used in the experimental well. For the outlying points below 0%, the experimental well absorbency was higher. This resulted in extreme differences in antimicrobial ability but does not display a conditional response. *B. chinensis* water extracts likely contained cellular tissue that further exasperated extreme differences in ability [SI].

S. invicta showed no increased ability when extracted in isopropanol against *E. col*i (KW chi-squared = 0.36172, p = 0.5476, Fig. 6) or *S. epidermidis* (KW chi-squared = 1.3011, p = 0.254, Fig. 6). *B. chinensis* also showed no significant change in antimicrobial ability when surface extracts were extracted in isopropanol (*S. epidermidis* KW chi-squared = 0.010753, p = 0.9174, *E. coli* KW chi-squared = 2.4194, p = 0.1198, Fig. 6). Water extracts for *S. invicta* showed no increased ability against *E. coli* (KW chi-squared= 0.31355, p = 0.5755, SI) or *S. epidermidis* (KW chi-squared = 0.034839, p = 0.8519, SI). *B. chinensis* showed no significant change in ability in water extracts when challenged with *E. coli* (KW chi-squared = 2.0477, p = 0.1524, SI) or *S. epidermidis* (KW chi-squared = 0.36172, p = 0.5476, SI).

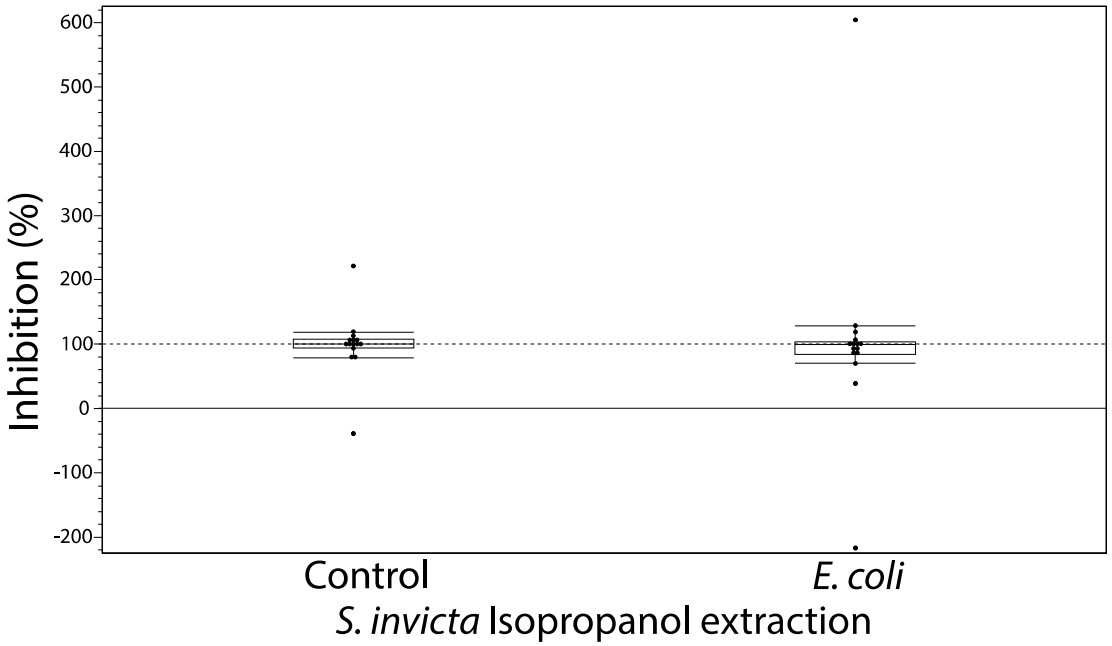

Figure 4.1 *S. invicta* inhibition potential against *E. coli.*

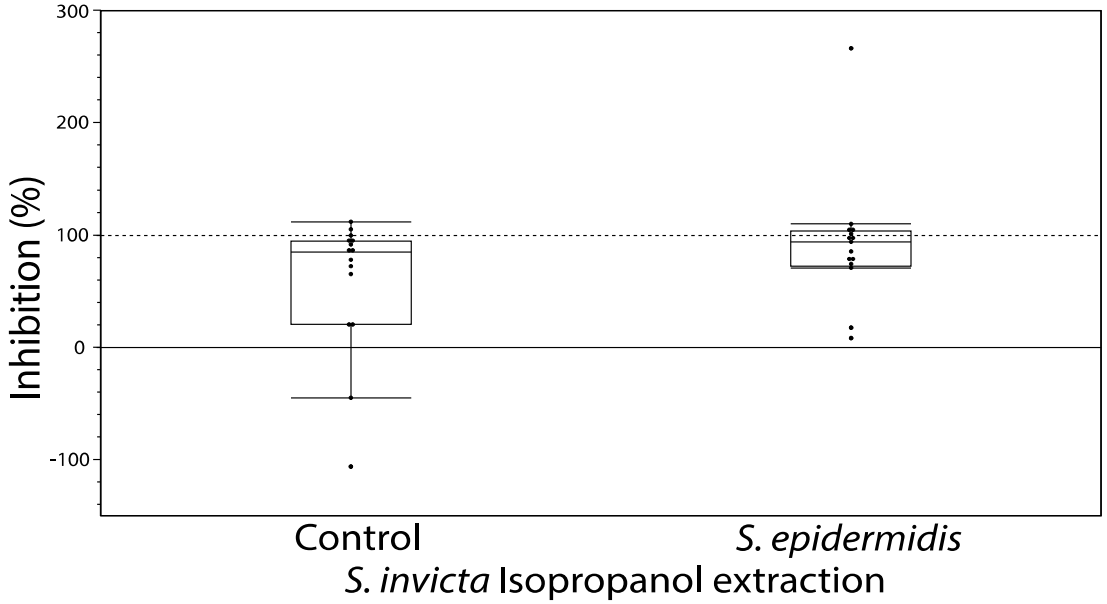

Figure 4.2 S. invicta inhibition potential against S. epidermidis.

Figure 4. *S. invicta* inhibition potential for control and treatment exposures for isopropanol extracts. Values are percent inhibition of extracts compared to growth controls. Maximum and minimum growth controls visualized at 100% and 0%, respectfully. *S. invicta* effectively inhibited bacterial challenge similarly with or without treatment exposures. Box plot line represents median values, whiskers represent 1st and 3rd quartile, data points represent ant colonies, outliers are data outside of 1.5 IOR.

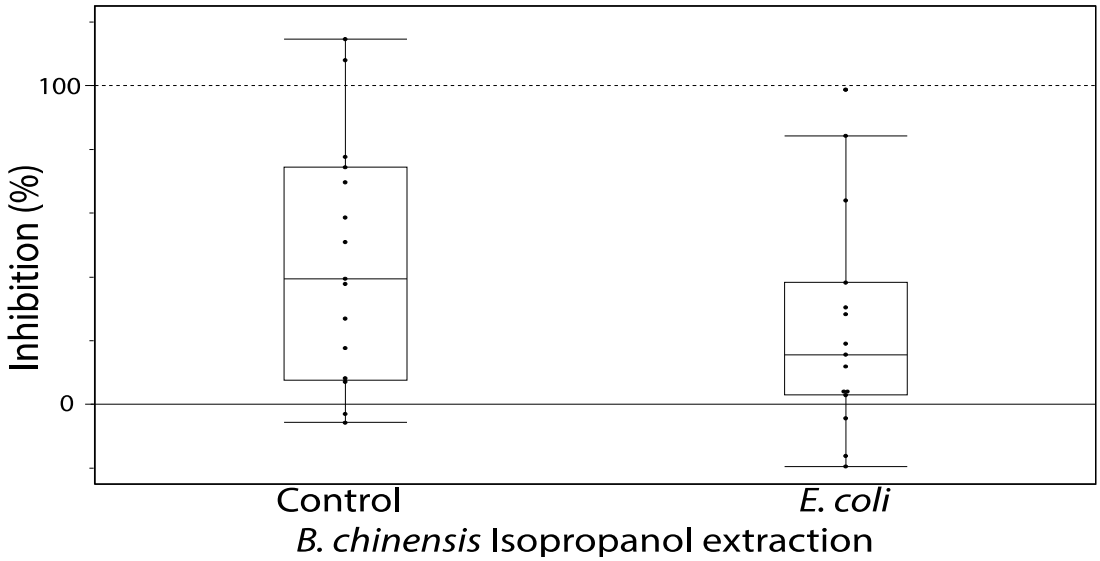

Figure 5.1 *B. chinensis* inhibition potential against *E. coli*.

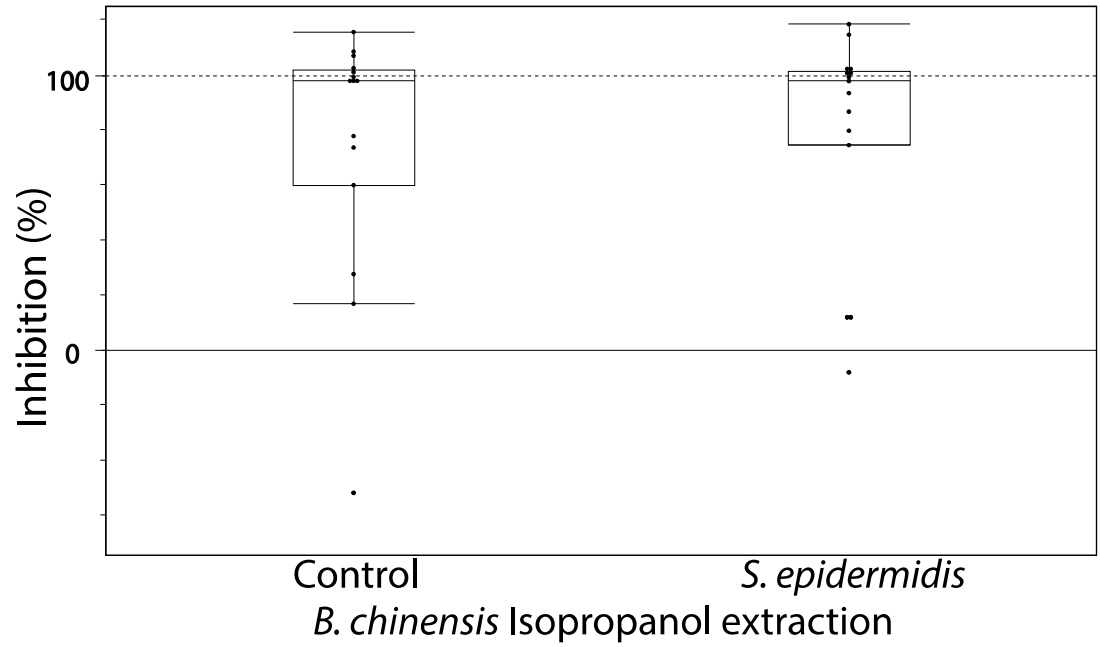

Figure 5.2 *B. chinensis* inhibition potential against *S. epidermidis*.

Figure 5. *B. chinensis* inhibition potential for control and treatment exposures for isopropanol extracts. Values are percent inhibition of extracts compared to growth controls. Maximum and minimum growth controls visualized at 100% and 0%, respectfully. *S. invicta* effectively inhibited bacterial challenge similarly with or without treatment exposures. Box plot line represents median values, whiskers represent 1st and 3rd quartile, data points represent ant colonies, outliers are data outside of 1.5 IOR.

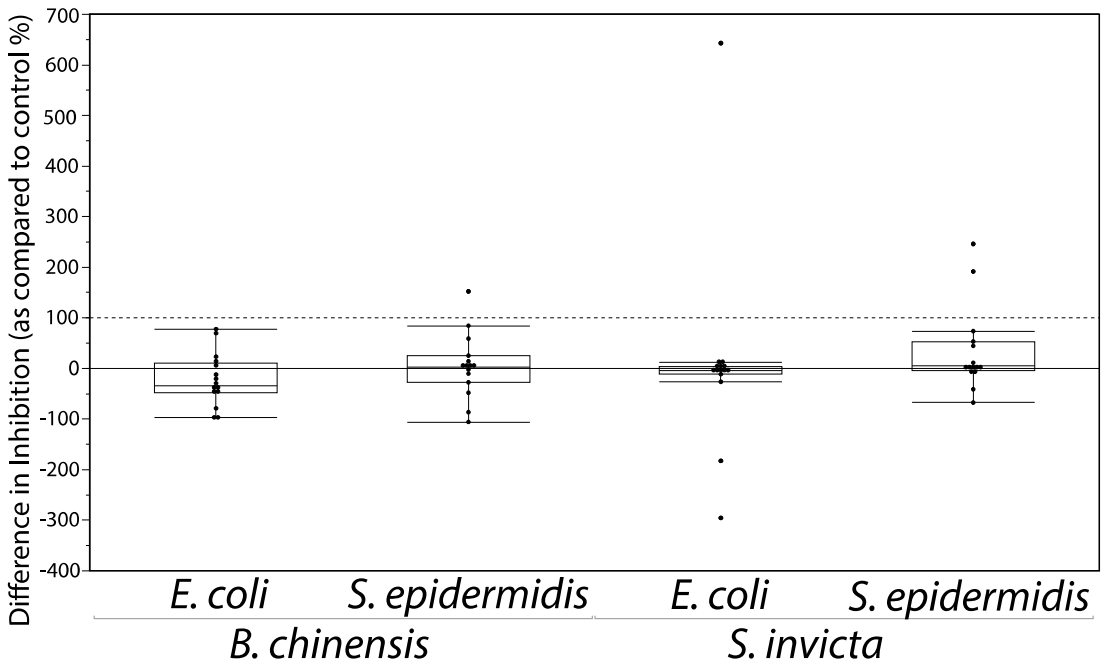

Figure 6. Measuring change in antimicrobial ability for *B. chinensis* and *S. invicta*. Measuring change in antimicrobial ability of isopropanol surface extracts of *B. chinensis* and *S. invicta* in a liquid assay against *S. epidermidis* and *E. coli* when previously challenged by those bacteria. Antimicrobial ability was tested against *S. epidermidis* and *E. coli* for *B. chinensis* and *S. invicta* after colonies were previously exposed to bacteria to test if there is a conditional response for antimicrobial ability. 15 colonies were tested for each species against each bacterium. OD_{600} measurements were recorded after WST-8 was added to each well. Results are a percentage of inhibition compared to bacterial control growth and adjusted to ant control treatment groups. Box plot line represents median values, whiskers represent 1st and 3rd quartile, data points represent ant colonies, outliers are data outside of 1.5 IOR.

DISCUSSION

Here, I showed that an exposure to a common bacterium—*Escherichia coli*—could kill two ant species, *Solenopsis invicta* and *Dorymyrmex bureni*. Exposure to *Staphylococcus epidermidis* had no significant effect on all tested ants. Also, *Solenopsis invicta* and *Brachymyrmex chinensis* were subject to a nonlethal exposure followed by an antimicrobial assay of their surface extracts to test for a conditional response in antimicrobial ability. As tested, I found no evidence of a change in antimicrobial ability akin to a conditional response.

My first experiment aimed to address the unknown issue of whether ant species can be negatively affected from exposure to environmental bacteria, the findings provide evidence that certain species can be affected from non-entomopathogenic bacteria (Fig. 4). I tested if environmental bacteria affect ants in species that had demonstrated ability or lacked ability in Penick et al. comparative analysis [35]. The bacterial challenge from *E. coli* negatively affected *S. invicta* and D*. bureni*—two species that displayed antimicrobial ability previously [35]. *B. chinensis* and *A. rudis* group ants did not show ability nor did they die from exposure. This could indicate a linkage between susceptibility and antimicrobial ability. This linkage may suggest certain ants evolved antimicrobial ability against environmental bacteria that may have detrimental effects.

Other potentially entomopathogenic bacteria have been found interacting with ant species. Previously, a *Pseudomonas* species was found on dead ant bodies. Upon exposure to the species, 50% of live ants were killed ants [55]. *E. coli* and *Staphylococcus aureus* were found in nests of *Camponotus compressus*. This mandibular secretions of *C. compressus* showed antimicrobial ability against *E. coli* and *S. aureus* in vitro [70]. Previously, environmental bacteria were not considered to affect ants, but collectively these findings suggest that some species of ants can be killed by environmental microbes. Undoubtedly such effects are contingent on the densities of microbes, the health of colonies and many other factors and yet they can occur.

In *S. invicta* exposure trials, there was high mortality when ants were exposed to *S. epidermidis*. Eleven of the 15 colonies in *S. invicta* showed negative effects from exposure to *S. epidermidis*. *A. rudis* was also killed by *S. epidermidis* but the effect was

modest. Because the variation in mortality among replicates and treatments at 24 hours was great, I chose to focus on comparisons after 48 hours [SI]. Since 48 h data counts were cumulative for the entire experiment they represent a more biologically relevant response and count of mortality across species, as they account for initial variation in worker condition in the first 24 h.

The second experiment aimed to induce a conditional response in antimicrobial ability, in an attempt to uncover the potential for ants to produce more antimicrobials when exposed to more environmental microbes. Neither ant species tested showed any evidence of the ability to increase antimicrobial abilities in response to a microbial challenge. It is of note that during these tests that *S. invicta* demonstrated similar antimicrobial abilities to those observed in Penick et al. [35]. *B. chinensis*, however, displayed antimicrobial activity in our assay that had not been previously observed. Surface extracts were found to inhibit *S. epidermidis* but only when extracted via isopropanol. When we used ethanol extracts, as in Penick et al., they showed no antimicrobial ability. *B. chinensis* may have non-polar antimicrobial compounds that neither ethanol nor water could capture (Figs. 4-6).

In the work here, we have only considered some of the potential antimicrobial responses. We can't rule out the possibility that ants response to exposures to bacterial pathogens via mechanisms other than those on which we focused. For example, ants can change their grooming rates when exposed to pathogens [20] and, in previous studies, have shown quick changes in venom deployment when threatened by pathogens [21]. In addition, antimicrobial behaviors and compounds produced by ants may be selective. This selectively could have two forms. Ants could selectively increase

behaviors or upregulate antimicrobial production contingent on the bacteria species to which they are exposed (and its abundance). In addition, the behavior or antimicrobial may be selective with regard to its action. For example, previously *L. neglectus* showed specific upregulation in antimicrobial gene expression only when exposed to a specific entomopathogenic fungus, as the gene that regulated anti-fungal expression increased. Antibacterial and antiviral genes had no change [71]. Even with some evidence for immune priming, *F. selysi* ants exposed to a certain entomopathogenic fungi showed no evidence of conditional responses for antimicrobial expression [72], and antimicrobial expression may be specific against pathogens or even limited to certain ant societies.

For antimicrobial assays with both species, water extracts showed similar results but allowed for more contamination from the extraction process. Extracellular tissue may have remained in surface extracts after filtration. This would introduce more matter into wells, altering optical density comparison readings, as well as feeding bacteria more than experimental protocols. This difference was exacerbated when comparing experimental and control treatments. Statistical analysis for water extractions also showed no significant change in antimicrobial ability [SI]. While the results presented did not suggest there were conditional responses, the methods described provide an assay that can be used to evaluate more of the ant phylogeny for ability and hidden mechanisms involved. Further studies may still present conditional responses, as even with fungal entomopathogens, these mechanisms are not ubiquitous across ant species [72].

The ant species tested I considered here are diverse with regard to their taxonomic position and life history. They differed in diet, colony size, and nesting

location. In this light, it is perhaps unsurprising that these species also differed in their response to environmental microbes. As more species are studied it is likely that responses will prove to be even more varied. I tested 4 ant species out of the perhaps twenty thousand extant species globally.

In the future, it will be both interesting and useful to better understand what factors drive differences among ant species in their responses to environmental microbes. It is generally understood that larger colony sizes increase transmission rates and therefore increases risk from pathogens [11]. Fungal entomopathogens typically produce spores that increase the transmission rates and virulence [73] but these are traits that don't exist in all bacteria—certainly not the bacteria in this experiment. It is unknown how bacteria are spread among ant colonies and possible that transmission of bacteria may have different dynamics than fungi. If bacteria are present but transmission is slow, it is possible that smaller colonies, where individual ants are more valuable and all individuals tend to be exposed to the same environment, may actually be at greater risk from environmental microbes. In this context, smaller colonies might be expected to develop stronger resistance to bacteria since allogrooming may not be as effective as compared to larger colonies.

Nesting locations may also be an important factor in influencing susceptibility to environmental microbes. Some ant species build ground nests, moving soil into mounds and creating chambers which can be sanitized with antimicrobial compounds from the ants. This behavior may tend to create "cleaner" nests and colonies would then maintain a hygienic standard. Fire ants, for example, create soil mounds that they spray with venom to maintain microbial populations [73]. Other ants find nest sites in their

environment, such as under rocks or inside dead wood and do not modify those nest sites heavily. Living in dead wood may increase ant species interactions with microbes as dead wood also harbors fungi and bacteria [74]. *A. rudis* and *B. chinensis*, two species used in my experiment, live in dead wood, and displayed no effect from exposure in my experiments. This may be because the two species have developed immune responses or lack susceptibility from being commonly exposed to bacteria.

A first line of defense for insects against microbes is the cuticle. The cuticle prevents pathogens from infecting the hemocoel of insects, but if a pathogen infects past the cuticle the ants humoral defenses protect against infection in ways similar to other animals [9]. Pathogens can have functions specific to breaching the thick waxy protective layer; fungi can excrete enzymes to break through the cuticle to infect insects [10]. Since infection can be curbed by preventing this breach, cuticle and external defenses may be an important investment for insects. Some ants, such as *B. chinensis* and *A. rudis*, show even greater investment in cuticle sculpturing, including raised and punctate cuticles that may be thicker than ants with a smooth cuticle. Cuticle sculpturing could have possible antimicrobial properties. Sculpturing may prevent infection through physically limiting microbes from penetrating and infecting the ants. Additionally, more sculptured cuticles may also contain nanostructures which have been identified in other insect groups to create an antimicrobial surface [75], wherein these structures create a higher stress surface that can lyse bacterial cell walls. *B. chinensis* and *A. rudis* share similar life histories and were unaffected from microbial exposure. This may be an indication of underlying mechanisms that certain ant species would have evolved from closely interacting with bacteria in their environment.

Another life history change may be with possible symbiotic relationships ants have evolved. Different ant phyla have been sampled for their microflora and show many similarities, but actual bacterial species have not been identified. Further experiments conducted to understand the microbial populations found on ants may show antimicrobial producing microbes. Experiments can also attempt to induce a conditional response of these symbionts by measuring population changes before and after a challenge, or by measuring the difference in secondary metabolites before and after a challenge.

My experiment used two bacterial species and four ant species, and I found a wide range of effects from possible interactions ants may have with bacteria in their environment. There are thousands more of ant species and millions more of bacterial species that will continue to provide new findings for possible interactions between the two groups. Targeting some more infectious bacteria might show novel insights with how ants defend against sporulating pathogens such as *Bacillus spp*. It is still not understood if the mechanisms of infection for bacteria exist in ants as they do in other animals. Understanding these mechanisms of infection in ants might also point at ant defenses against bacteria. Ants, like humans, have faced bacteria as a possible threat for hundreds of millions of years. As possible pathogens and with an evolutionary relationship of hundreds of millions of years, bacteria have likely produced selective pressures on ants and their antimicrobial defenses.

References

1. Clardy, J., Fischbach, M. A. & Currie, C. R. The natural history of antibiotics. *Curr. Biol.* **19**, 1–8 (2009).
2. Sengupta, S., Chattopadhyay, M. K. & Grossart, H. P. The multifaceted roles of antibiotics and antibiotic resistance in nature. *Front. Microbiol.* **4**, 1–13 (2013).
3. Zaman, S. Bin *et al.* A review on antibiotic resistance: alarm bells are ringing. *Cureus* **9**, (2017).
4. Jørgensen, P. S. *et al.* Use antimicrobials wisely. *Nature* **537**, 159–161 (2016).
5. Silver, L. L. Challenges of antibacterial discovery. *Clin. Microbiol. Rev.* **24**, 71–109 (2011).
6. World Health Organization. Antibacterial Agents. (2017).
7. Theuretzbacher, U. Antibiotic innovation for future public health needs. *Clin. Microbiol. Infect.* **23**, 713–717 (2017).
8. Littmann, J., Zorzet, A. & Cars, O. Antimicrobial Resistance - A threat to the world's sustainable development - Dag Hammarskjöld Foundation. *Ups. J. Med. Sci.* **121**, 159–164 (2016).
9. Sheehan, G., Garvey, A., Croke, M. & Kavanagh, K. Innate humoral immune defences in mammals and insects: The same, with differences? *Virulence* **9**, 1625–1639 (2018).
10. Hillyer, J. F. Insect immunology and hematopoiesis. *Dev. Comp. Immunol.* **58**, 102–118 (2015).
11. Cremer, S., Armitage, S. A. O. & Schmid-Hempel, P. Social Immunity. *Curr. Biol.* **17**, 693–702 (2007).
12. Schmid-Hempel, P. *Parasites in social insects.* (Princeton, N.J. : Princeton University Press, [1998], 1998).
13. Hölldobler 1936-, B. & Wilson, E. O. *The ants*. (Cambridge, Mass. : Belknap Press of Harvard University Press, 1990., 1990).
14. Baracchi, D. & Tragust, S. Venom as a component of external immune defense in Hymenoptera. *Evolution of Venomous Animals and Their Toxins* 1–17 (Springer Netherlands, 2015). doi:10.1007/978-94-007-6727-0_3-1.
15. Konrad, M. *et al.* Ants avoid superinfections by performing risk-adjusted sanitary care. *Proc. Natl. Acad. Sci.* **115**, 2782–2787 (2018).
16. Park, J., Kaufmann, G. F., Bowen, J. P., Arbiser, J. L. & Janda, K. D. Solenopsin A, a venom alkaloid from the Fire Ant *Solenopsis invicta*, inhibits quorum-sensing signaling in *Pseudomonas aeruginosa* . *J. Infect. Dis.* **198**, 1198–1201 (2008).
17. Lima, D. B. *et al.* Antimicrobial effect of *Dinoponera quadriceps* (Hymenoptera: Formicidae) venom against *Staphylococcus aureus* strains. *J. Appl. Microbiol.* **117**, 390–396 (2014).
18. Kaltenpoth, M. & Engl, T. Defensive microbial symbionts in Hymenoptera. *Funct. Ecol.* **28**, 315–327 (2014).
19. Hughes, W. O. H., Eilenberg, J. & Boomsma, J. J. Trade-offs in group living: transmission and disease resistance in leaf-cutting ants. *Proc. R. Soc. B Biol. Sci.* **269**, 1811–1819 (2002).
20. Reber, A., Purcell, J., Buechel, S. D., Buri, P. & Chapuisat, M. The expression and impact of antifungal grooming in ants. *J. Evol. Biol.* **24**, 954–964 (2011).

21. Graystock, P. & Hughes, W. O. H. Disease resistance in a weaver ant, *Polyrhachis dives*, and the role of antibiotic-producing glands. *Behav. Ecol. Sociobiol.* **65**, 2319–2327 (2011).
22. Tranter, C. & Hughes, W. O. H. Acid, silk and grooming: alternative strategies in social immunity in ants? *Behav. Ecol. Sociobiol.* **69**, 1687–1699 (2015).
23. Kay, A. D. *et al.* A carbohydrate-rich diet increases social immunity in ants. *Proc. R. Soc. B Biol. Sci.* **281**, 20132374–20132374 (2014).
24. Bos, N., Lefèvre, T., Jensen, A. B. & D'Ettorre, P. Sick ants become unsociable. *J. Evol. Biol.* **25**, 342–351 (2012).
25. Yek, S. H. & Mueller, U. G. The metapleural gland of ants. *Biol. Rev.* **86**, 774–791 (2011).
26. Touchard, A. *et al.* The biochemical toxin arsenal from ant venoms. *Toxins (Basel).* **8**, 1–28 (2016).
27. Tranter, C., Graystock, P., Shaw, C., Lopes, J. F. S. & Hughes, W. O. H. Sanitizing the fortress: Protection of ant brood and nest material by worker antibiotics. *Behav. Ecol. Sociobiol.* **68**, 499–507 (2014).
28. Yek, S. H., Nash, D. R., Jensen, A. B. & Boomsma, J. J. Regulation and specificity of antifungal metapleural gland secretion in leaf-cutting ants. *Proc. R. Soc. B Biol. Sci.* **279**, 4215–4222 (2012).
29. Schmidt, J. O. Biochemistry of insect venoms. *Annu. Rev. Entomol.* **27**, 339–368 (2003).
30. Tragust, S. *et al.* Ants disinfect fungus-exposed brood by oral uptake and spread of their poison. *Curr. Biol.* **23**, 76–82 (2013).
31. Chapuisat, M., Oppliger, A., Magliano, P. & Christe, P. Wood ants use resin to protect themselves against pathogens. *Proc. R. Soc. B Biol. Sci.* **274**, 2013–2017 (2007).
32. Currie, C. R., Scott, J. a., Summerbell, R. C. & Malloch, D. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* **398**, 701–704 (1999).
33. Poulsen, M., Bot, A. N. M., Nielsen, M. G. & Boomsma, J. J. Experimental evidence for the costs and hygienic significance of the antibiotic metapleural gland secretion in leaf-cutting ants. *Behav. Ecol. Sociobiol.* **52**, 151–157 (2002).
34. Orivel, J. *et al.* Ponericins, New antibacterial and insecticidal peptides from the venom of the ant *Pachycondyla goeldii*. *J. Biol. Chem.* **276**, 17823–17829 (2001).
35. Penick, C. A. *et al.* External immunity in ant societies: sociality and colony size do not predict investment in antimicrobials. *R. Soc. Open Sci.* **5**, 171332 (2018).
36. Jouvenaz, D. P., Blum, M. S. & MacConnell, J. G. Antibacterial activity of venom alkaloids from the imported fire ant, *Solenopsis invicta* Buren. *Antimicrob. Agents Chemother.* **2**, 291–293 (1972).
37. Carvalho, D. B. de *et al.* Fire Ant venom alkaloids inhibit biofilm formation. *Toxins (Basel).* **11**, 420 (2019).
38. Sullivan, D. C., Flowers, H., Rockhold, R., Herath, H. & Nanayakkara, N. P. Antibacterial activity of synthetic Fire Ant venom: The Solenopsins and Isosolenopsins. *Am. J. Med. Sci.* **338**, 287–291 (2009).
39. Jones, T. H., Blum, M. S. & Fales, H. M. Ant venom alkaloids from *Solenopsis* and *Monorium* species. Recent developments. *Tetrahedron* **38**, 1949–1958 (1982).

40. Quinet, Y. *et al.* Antibacterial properties of contact defensive secretions in neotropical *Crematogaster* ants. *J. Venom. Anim. Toxins incl. Trop. Dis.* **18(4)**, 441–445 (2012).
41. Taguchi, S., Bulet, P. & Hoffmann, J. A. A novel insect defensin from the ant *Formica rufa*. *Biochimie* **80**, 343–346 (1998).
42. Cabrera, A., Williams, D., Hernández, J. V., Caetano, F. H. & Jaffe, K. Metapleural- and postpharyngeal-gland secretions from workers of the ants *Solenopsis invicta* and *S. geminata. Chem. Biodivers.* **1**, 303–311 (2004).
43. Do Nascimento, R. R., Schoeters, E., Morgan, E. D., Billen, J. & Stradling, D. J. Chemistry of metapleural gland secretions of three attine ants, *Atta sexdens rubropilosa, Atta cephalotes,* and *Acromyrmex octospinosus* (Hymenoptera: Formicidae). *J. Chem. Ecol.* **22**, 987–1000 (1996).
44. Mackintosh, J. A. *et al.* Antimicrobial mode of action of secretions from the metapleural gland of *Mytmecia gulosa* (Australian bull ant). *Can. J. Microbiol.* **41**, 136–144 (1995).
45. Mackintosh, J. A., Veal, D. A., Beattie, A. J. & Gooley, A. A. Isolation from an ant *Myrmecia gulosa* of two inducible O-glycosylated proline-rich antibacterial peptides. *J. Biol. Chem.* **273**, 6139–6143 (1998).
46. Qin, Z. *et al.* Formicamycins, antibacterial polyketides produced by *Streptomyces formicae* isolated from African *Tetraponera* plant-ants. *Chem. Sci.* **8**, 3218–3227 (2017).
47. Christe, P., Oppliger, A., Bancalà, F., Castella, G. & Chapuisat, M. Evidence for collective medication in ants. *Ecol. Lett.* **6**, 19–22 (2003).
48. Meunier, J. Social immunity and the evolution of group living in insects. *Philos. Trans. R. Soc. B Biol. Sci.* **370**, 19–21 (2015).
49. Traniello, J. F. A., Rosengaus, R. B. & Savoie, K. The development of immunity in a social insect: Evidence for the group facilitation of disease resistance. *Proc. Natl. Acad. Sci.* **99**, 6838–6842 (2002).
50. Schlüns, H. & Crozier, R. H. Molecular and chemical immune defenses in ants (Hymenoptera: Formicidae). *Myrmecological News* **12**, 237–249 (2009).
51. Hoggard, S. J., Wilson, P. D., Beattie, A. J. & Stow, A. J. The effectiveness of antimicrobial defenses declines with increasing group size and genetic similarity. *Ann. Entomol. Soc. Am.* **106**, 53–58 (2013).
52. Baird, R., Woolfolk, S. & Watson, C. E. Survey of bacterial and fungal associates of Black/Hybrid Imported Fire Ants from mounds in Mississippi. *Southeast. Nat.* **6**, 615–632 (2007).
53. Dauber, J., Schroeter, D. & Wolters, V. Species specific effects of ants on microbial activity and N-availability in the soil of an old-field. *Eur. J. Soil Biol.* **37**, 259–261 (2001).
54. Delgado-Baquerizo, M., Eldridge, D. J., Hamonts, K. & Singh, B. K. Ant colonies promote the diversity of soil microbial communities. *ISME J.* **13**, 1114–1118 (2019).
55. Lofgren, C. S., Banks, W. A. & Glancey, B. M. Biology and control of Imported Fire Ants. *Annu. Rev. Entomol.* **20**, 1–30 (1975).
56. Wenseleers, T. *et al.* Widespread occurrence of the micro-organism *Wolbachia* in ants. *Proc. R. Soc. B Biol. Sci.* **265**, 1447–1452 (1998).

57. Antônio Pesquero, M., Carla Carneiro, L. & Pires, De Jesus, D. Insect/Bacteria association and nosocomial infection. in *Salmonella - A Diversified Superbug* (InTech, 2012). doi:10.5772/intechopen.69704.
58. Ramalho, M. O., Bueno, O. C. & Moreau, C. S. Microbial composition of spiny ants (Hymenoptera: Formicidae: Polyrhachis) across their geographic range. *BMC Evol. Biol.* **17**, 1–16 (2017).
59. Pie, M. R., Rosengaus, R. B. & Traniello, J. F. A. Nest architecture, activity pattern, worker density and the dynamics of disease transmission in social insects. *J. Theor. Biol.* **226**, 45–51 (2004).
60. Lucas, J. M. *et al. Azteca* ants maintain unique microbiomes across functionally distinct nest chambers. *Proc. R. Soc. B Biol. Sci.* **286**, 20191026 (2019).
61. Pull, C. D. *et al.* Destructive disinfection of infected brood prevents systemic disease spread in ant colonies. *Elife* **7**, 1–30 (2018).
62. Stucki, D., Freitak, D., Bos, N. & Sundström, L. Stress responses upon starvation and exposure to bacteria in the ant *Formica exsecta*. *PeerJ* **2019**, 1–17 (2019).
63. García, S., Fernández, A., Luengo, J. & Herrera, F. Advanced nonparametric tests for multiple comparisons in the design of experiments in computational intelligence and data mining: Experimental analysis of power. *Inf. Sci. (Ny).* **180**, 2044–2064 (2010).
64. R Core Team. R: A Language and Environment for Statistical Computing. **R Foundati**, URL https://www.R-project.org/ (2017).
65. Calvo, B. & Santafe, G. scmamp: Statistical comparison of multiple algorithms in multiple problems. *R J.* **Accepted f**, (2015).
66. Stow, A. *et al.* Antimicrobial defenses increase with sociality in bees. *Biol. Lett.* **3**, 422–424 (2007).
67. Turnbull, C. *et al.* Antimicrobial strength increases with group size: implications for social evolution. *Biol. Lett.* **7**, 249–252 (2011).
68. Wickham, H., Francois, R., Henry, L. & Müller, K. dplyr: A Grammar of data manipulation. **R package**, (2018).
70. Mohana, P., Gunasekaran, C. & Selvarasu, P. Assemblage of nest soil bacteria and evaluate the antibacterial activity of carpenter ant (*Camponotus compressus*) mandibular extracts against selective gram staining bacteria. **3**, 269–274 (2018).
71. Konrad, M. *et al.* Social transfer of pathogenic fungus promotes active immunization in ant colonies. *PLoS Biol.* **10**, (2012).
72. Reber, A. & Chapuisat, M. No evidence for immune priming in ants exposed to a fungal pathogen. *PLoS One* **7**, (2012).
73. Obin, M. S. & Vander Meer, R. K. Gaster flagging by fire ants. *J. Chem. Ecol.* **11**, 1757–1768 (1985).
74. Bani, A. *et al.* The role of microbial community in the decomposition of leaf litter and deadwood. *Appl. Soil Ecol.* **126**, 75–84 (2018).
75. Sun, M. *et al.* Differences in nanostructure and hydrophobicity of cicada (*Cryptotympana atrata*) forewing surface with the distribution of precipitation. *Appl. Bionics Biomech.* **2018**, (2018).

APPENDIX

Appendix A: Supplemental Information

Species	Extraction	Challenge	Kruskal-Wallis results
S. invicta	H_2O	*E. coli*	chi-squared = 0.31355, df = 1, p-value = 0.5755
S. invicta	H_2O	*S. epidermidis*	chi-squared = 0.034839, df = 1, p-value = 0.8519
B. chinensis	H_2O	*E. coli*	chi-squared = 1.0327, df = 1, p-value = 0.3095
B. chinensis	H_2O	*S. epidermidis*	chi-squared = 3.4069, df = 1, p-value = 0.06493
S. invicta	Isopropanol	*E. coli*	chi-squared = 0.36172, df = 1, p-value = 0.5476
S. invicta	Isopropanol	*S. epidermidis*	chi-squared = 1.3011, df = 1, p-value = 0.254
B. chinensis	Isopropanol	*E. coli*	chi-squared = 1.4972, df = 1, p-value = 0.2211
B. chinensis	Isopropanol	*S. epidermidis*	chi-squared = 0.15527, df = 1, p-value = 0.6936

Table 1. Kruskal-Wallis test for water and isopropanol extract antimicrobial ability in *S. invicta* and *B. chinensis*. Ants were challenged with exposure prior to surface extracts being collected to be tested against bacteria in a well plate assay. Treatment ants were exposed to the challenging bacteria and controls were exposed to sterile agar. Inhibition values were recorded as percent inhibition then controls were used to compare treatment groups. Kruskal-Wallis test for non-parametric, incomplete block design, showed no significant increase in antimicrobial ability if ants were previously exposed to bacteria. There is no evidence for conditional response as tested.

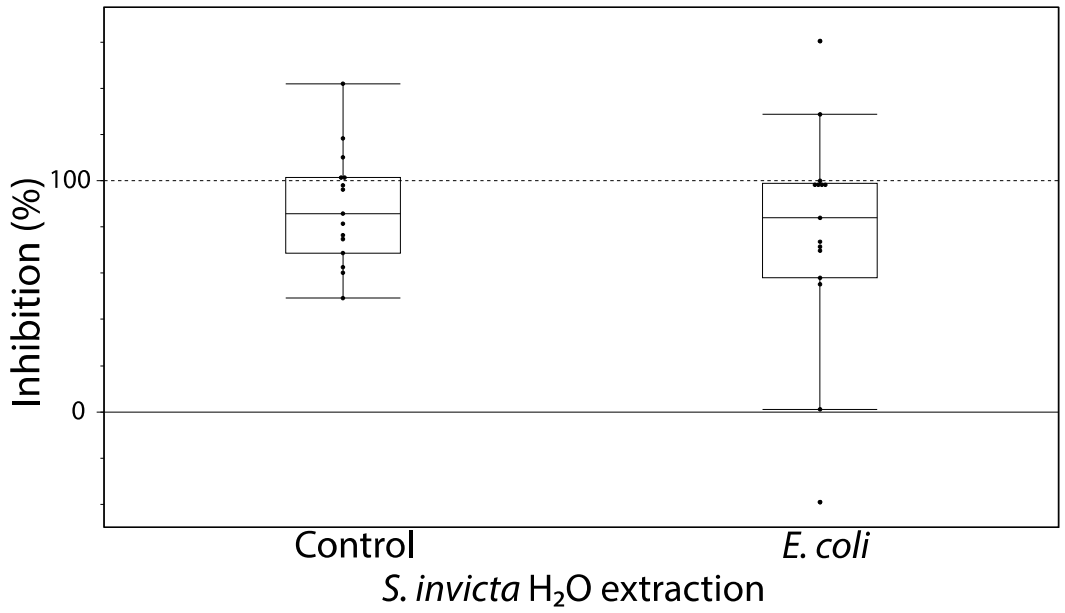

Figure 7.1. H_2O *S. invicta* inhibition potential against *E. coli.*

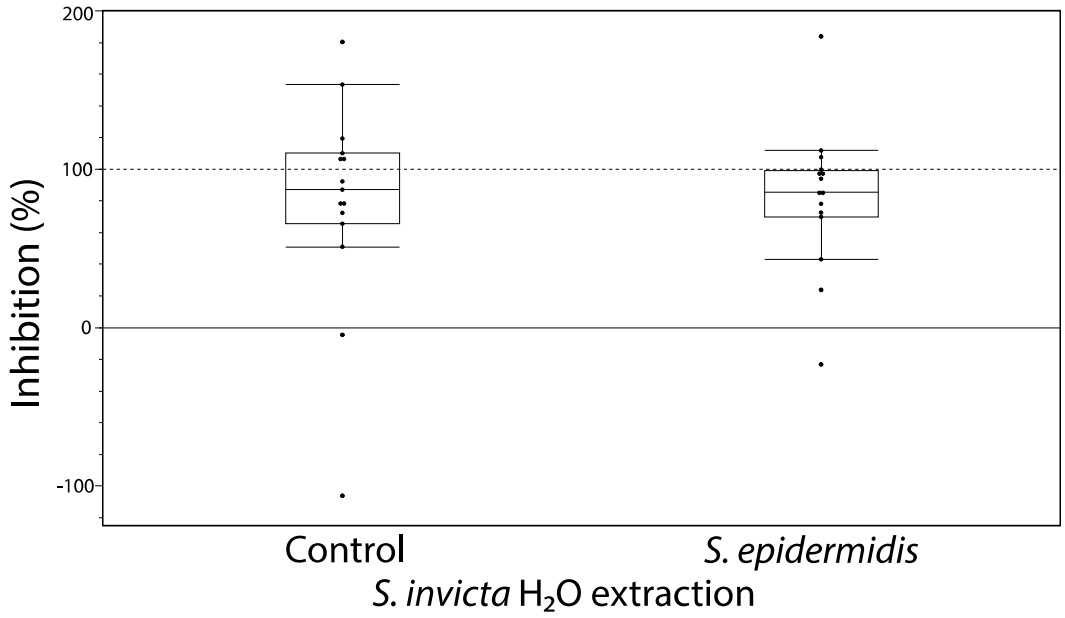

Figure 7.2 H_2O *S. invicta* inhibition potential against *S. epidermidis.*

Figure 7. *S. invicta* inhibition potential for control and treatment exposures for water extracts against S epidermidis. Values are percent inhibition of extracts compared to growth controls. Maximum and minimum growth controls visualized at 100% and 0%, respectfully. *S. invicta* effectively inhibited bacterial challenge similarly with or without treatment exposures. Box plot line represents median values, whiskers represent 1st and 3rd quartile, data points represent ant colonies, outliers are data outside of 1.5 IOR.

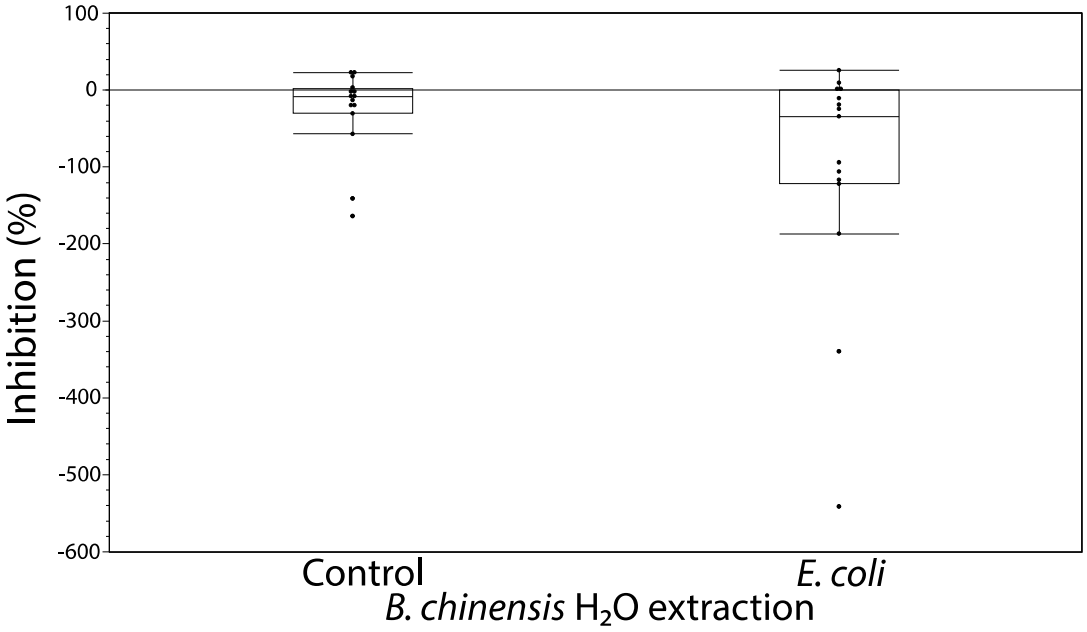

Figure 8.1. H_2O *B. chinensis* inhibition potential against *E. coli.*

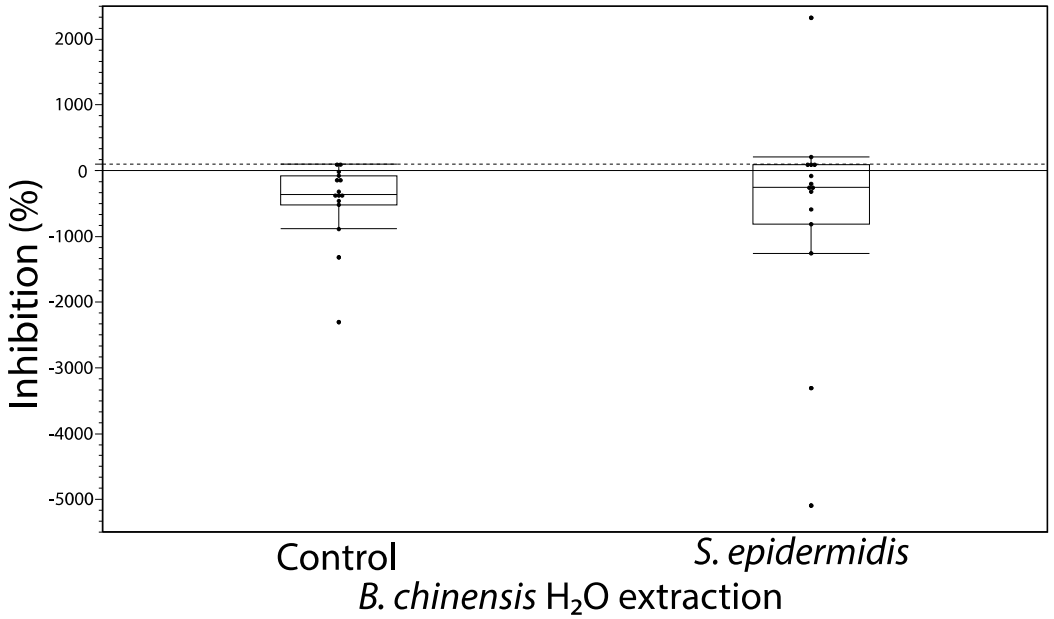

Figure 8.2 . H_2O *B. chinensis* inhibition potential against *S. epidermidis.*

Figure 8. *B. chinensis* inhibition potential for control and treatment exposures for water extracts against S epidermidis. Values are percent inhibition of extracts compared to growth controls. Maximum and minimum growth controls visualized at 100% and 0%, respectfully. *S. invicta* effectively inhibited bacterial challenge similarly with or without treatment exposures. Box plot line represents median values, whiskers represent 1st and 3rd quartile, data points represent ant colonies, outliers are data outside of 1.5 IOR.

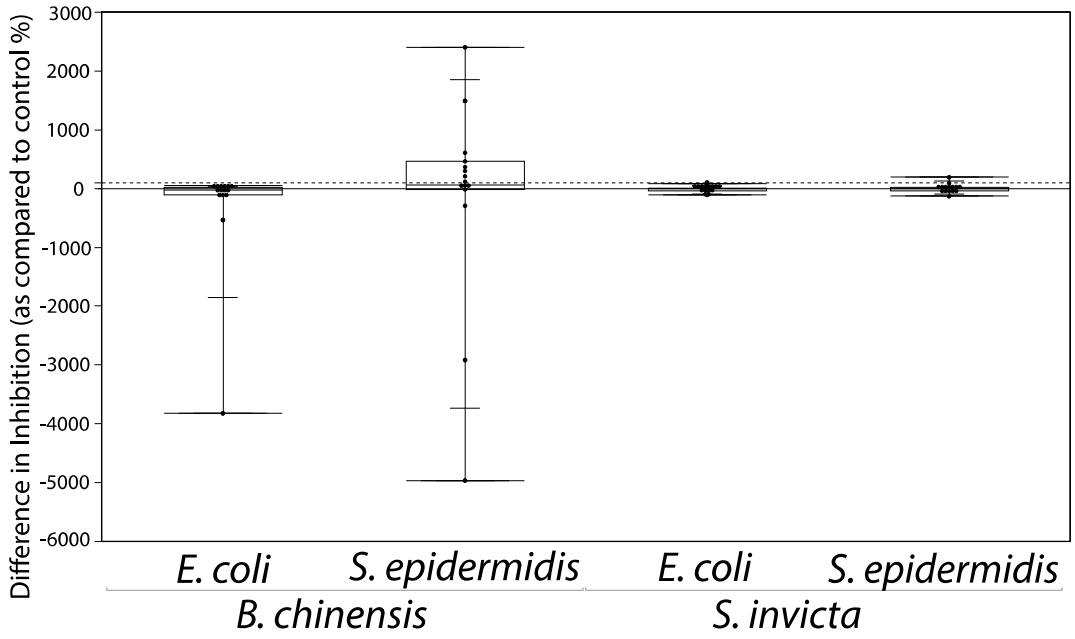

Figure 9. H_2O Measuring change in antimicrobial ability for *B. chinensis* and *S. invicta.* Measuring change in antimicrobial ability of water surface extracts of *B. chinensis* and *S. invicta* in a liquid assay against *S. epidermidis* and *E. coli* when previously challenged by bacteria. Antimicrobial ability was tested against *S. epidermidis* and *E. coli* for *B. chinensis* and *S. invicta* after colonies were previously exposed to bacteria to test if there is a conditional response for antimicrobial ability. 15 colonies were tested for each species against each bacterium. OD600 measurements were recorded after WST-8 was added to each well. Results are a percentage of inhibition compared to bacterial control growth and adjusted to ant control treatment groups. Box plot whiskers represent 97.5th quartile, 90th quartile, 10th quartile, and 2.5th quartile, from top to bottom.