THE ROLE OF QUORUM SENSING BACTERIA *PROTEUS MIRABILIS* ON THE BEHAVIORAL ECOLOGY OF THE RED IMPORTED FIRE ANT, *SOLENOPSIS INVICTA* BUREN (HYMENOPTERA: FORMICIDAE)

A Dissertation

by

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ABSTRACT

Interkingdom interactions describe dynamic and complex relationships between prokaryotes and eukaryotes sharing an environment. In particular, bacteria-animal interactions studies have hypothesized the intrinsic role bacteria have on animal behavior. In this study, foraging rate and nestmate recognition behaviors of the red imported fire ant (RIFA) *Solenopsis invicta* (Buren) (Hymenoptera: Formicidae) were used to determine whether quorum sensing bacteria *Proteus mirabilis* played a role in these ant behaviors. First, a regional and colony level bacterial survey of *S. invicta* was conducted to determine the bacterial communities found on the worker caste, reproductive caste, brood and midden using 16S rRNA gene v4 region amplicon sequencing. The bacterial survey revealed high bacterial diversity in adult castes versus conserved, low bacterial diversity in brood. Ecological regions were important in establishing the available bacterial community. *Proteus* bacteria was found in all ecoregions and colony member functional categories. Behavioral assays and quantification of bacteria concentrations on ants, established that *P. mirabilis* played no significant role in nestmate recognition of *S. invicta* colonies. Foraging responsiveness experiments revealed 10^5 CFU/mL *P. mirabilis* increased attraction to high carbohydrate honey water baits in different *S. invicta* colonies. The models and methodologies used in this study can be used to evaluate additional bacteria-ant interactions or develop the mechanisms underlying *S. invicta* increased attraction to high carbohydrate baits exposed to *P. mirabilis.*

DEDICATION

I dedicate my dissertation to my parents, Maria Esperanza Espinoza and Jose Aniceto Espinoza because I owe them my life, my faith, and the chance to pursue the opportunities this country has to offer. *Los amo con toda mi alma y les dedico todo mi trabajo*.

To my supportive and sweet mother, thank you for always listening to me and providing the love-filled wisdom only you know how to give. You are my best friend, my rock, my heart, none of this would have been possible without all of your hard work and dedication. Words cannot define your unending love and support, and they fall short in helping me express my gratitude to you. *Te amo*.

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CHAPTER I

INTRODUCTION

The microbial world was first revealed to human-kind through the eyes of Anton van Leeuwenhoek, the Father of Microbiology. In 1677, van Leeuwenhoek made some improvements on the microscope of the time, increasing magnification, and making possible the observation of "animalcules". Over the following centuries, the ubiquity of microorganisms and their role in disease were investigated, germ theory developed by Louis Pasteur from 1860-1864, and postulates identifying causative agents of disease established by Robert Koch in 1884. Until more recent decades, microbial research was limited to only the study of microbes that could be grown in culture via viability based methods. The "great plate count anomaly" limited the microbes that could be used in laboratory studies (Staley and Konopka, 1985). Culturable-biased study methods imposed a limit on the advancement of microbiology, detecting less than 1% of the total population of bacteria in the world. Innovations in technology and microbiology in the past decades have made the study of unculturable microbes possible, and further paved the way of sequencing technologies that amplified highly informative genetic material to reveal more about these unseen microbes (Lasken and McLean, 2014; Woese and Fox, 1977). In conjunction with other scientific applications, microbiologists continue to discover the incredible diversity and ubiquity of microbes on Earth, studying their role and function in many ecosystems (Caporaso *et al.*, 2011; Hay, 2009; Lozupone and Knight, 2007).

Communication between organism is facilitated when these organisms occupy the same ecosystem, sharing space and resources in their immediate environments. Interkingdom interactions describe the complex communication systems that exist transversely between prokaryotic and eukaryotic organisms, made possible by the fundamental molecular chemistry that drives life's cellular functions (Lowery *et al.*, 2008; Pacheco and Sperandio, 2009). Mediating molecules shared between the converging chemical pathways of prokaryotic and eukaryotic organism have driven the co-evolution of many microbe-eukaryotic host interactions, eliciting changes in microbial cellular functions and contributing to host adaptations. Yet, these interactions are not limited to only symbiotic relationships and many animal-microbe interactions have served as models to include pathogenic, and even facultative, opportunistic relationships. Beyond nutrient cycling and trophic dynamics, interkingdom interactions address direct exchanges of information relayed between the two interacting organisms, whether it be a symbiotic signal evolutionarily established to convey specific information from sender to receiver, or a facultative cue that has become associated with affiliated information (Smith and Harper, 2003). For example, the longstanding reciprocal dynamics with microbes have been used to better understand animal behavior (Archie and Theis, 2011; Ezenwa *et al.*, 2012; Ezenwa and Williams, 2014). Whether a single microbial strain or a consortium of multiple microbes found in the environment, or the microbiome found within the body of the host animal, we are beginning to discover the existence of underlying mechanisms influencing animal behavior, and ultimately affecting the evolutionary trajectory of many animal taxa.

Microbial communities are shaped by many abiotic and biotic factors, such as nutrient availability, pH, competition, metabolic cooperation, and temperature (Pepper *et al.*, 2015). How might the animal's environment concede to the establishment and development of an associated microbe? Is the animal itself the host environment where microbes are to be found? Specifically, the functions and mechanisms of microbes interacting with animals will be further defined by their relevance to the animal. Particular to bacteria, is the chemical communication termed quorum sensing (Fuqua *et al.*, 1994). Quorum sensing describes a system of communication where bacterial cells produce autoinducer molecules detected by the cells around them. Upon reaching a specific cell density and detecting these molecules, the bacterial cells engage in multicellular behavior and regulated gene expression. The activated gene expressions can regulate a multitude of possible phenotypic traits associated with pathogenicity, motility, or symbiosis (Miller and Bassler, 2001). In addition, the same quorum sensing molecules activating coordinated gene expression in bacterial cells can affect an interacting animal, as is the case of the symbiotic bioluminescent *Vibrio fisheri* and the Hawaiian bobtail squid *Euprymna scolopes* (Boettcher *et al.*, 1996). The *lux* gene system in *V. fisheri* produces acyl-homoserine-lactone (AHL) autoinducer synthase that activates increased coordinated transcription of luciferase, essentially prompting the bacterially-activated bioluminescence within the bobtail squid housing the bacteria. *V. fisheri* density thresholds for activation of the quorum sensing system and expression of luciferase are closely coordinated with the squid's circadian rhythm to protect it against detection by predators (Koch *et al.*, 2014). Using Tinbergen's four questions addressing the study of

ethology (Tinbergen, 1963), we can develop experiments that explore the effects quorum sensing bacteria have on the developmental biology, evolutionary history, functional adaptations, and mechanisms driving animal behavior. Several insect models have already been explored.

Quorum sensing bacteria implications on insect resource finding have provided further insight on interkingdom interactions. For example, increased blood-feeder attraction of adult yellow fever mosquitoes, *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) were found to be mediated by the presence of *arg* gene expression in quorum sensing *Staphylococcus epidermidis* (Zhang *et al.*, 2015). Arthropod vector *Ae. aegypti* was more attracted to blood-feeders containing wild type *arg* + *S. epidermidis*, the underlying means of this attraction is not known. However, it is likely that *S. epidermidis* quorum sensing molecules are used as cues by mosquitoes locating hosts to feed on, as this bacterium often dominates areas of the human skin microbiota. The attraction to oviposition sites of green blow flies *Lucilia sericata* (Diptera: Calliphoridae) have also been affected by the presence of quorum sensing bacteria, in this case, *Proteus mirabilis* (Ma *et al.*, 2012; Tomberlin *et al.*, 2012). *P. mirabilis* is a ubiquitous, Gram-negative bacterium belonging to the *Enterobacteriaceae* family, commonly associated with pathogenicity in human urinary tract infections. *P. mirabilis* bacterial strains have the *lux* gene system, activating autoinducer production and in turn activating biofilm formation and swarming motility of bacterial cells (Daniels *et al.*, 2004; Schneider *et al.*, 2002; Stankowska *et al.*, 2012). Using this model, *P. mirabilis* was extracted from the salivary glands of *L. sericata* maggots and used to identify putative cues used by *L. sericata* in the increased attraction to oviposition

sites where the swarming bacteria was present. Lactic acid, phenol, indole and ammonia were among the fly attractants identified to also stimulate swarming in *P. mirabilis.* Indole is as a widespread interkingdom signal and cue found in the environment, a secondary metabolite derived from the essential amino acid tryptophan. The production of indole by *P. mirabilis* is of great relevance to the study of interkingdom interactions because it's derivatives are widespread in both prokaryotic and eukaryotic systems (Lee *et al.*, 2015; Tomberlin *et al.*, 2017). The presence of *P. mirabilis* in the environment may play a role in the attraction of other arthropods exploiting resources similar to *L. sericata,* such as insects that scavenge and utilize decomposing ephemeral resources. *Solenopsis invicta* (Buren) (Hymenoptera: Formicidae), is an insect that could possibly partake in interkingdom interactions with *P. mirabilis* for three particular reasons: (1) *S. invicta* readily scavenges for food and has been seen located near decomposing ephemeral resources (Pechal *et al.*, 2015); (2) *P. mirabilis* has been previously isolated from *S. invicta* (Chadee and Le Maitre, 1990)*;* and, (3) tryptophan, from which indole is derived, is an essential amino acid regularly found in the nutritional resources of *S. invicta* (Lanza, 1991; Lanza *et al.*, 1993).

S. invicta, the red imported fire ant, belongs to the subfamily Myrmicinae. *S. invicta* is an active predator and scavenger, foraging by searching, recruiting worker nestmates, and transporting food resources back to the nest colony. *S. invicta* is native to the floodplains of the Mato Grosso Plateau in Brazil, and other neighboring regions in South America. In regions beyond its native range, *S. invicta* has become a notorious pest in urban and agricultural systems (Ascunce *et al.*, 2011) and commonly identified as the

red imported fire ant (RIFA). In the United States alone, *S. invicta* has been introduced multiple times, first in Alabama and then again in Florida, and continually expanded throughout the southeastern region, costing millions of dollars to control every year (Drees *et al.*, 2013; Drees and Gold, 2003; Lard *et al.*, 2002). Much of *S. invicta* success as an invasive species, like other invasive species, has to do with the absence of natural enemies and competitors in new environment, but also its resilience and adaptability to disturbed environments (King and Tschinkel, 2013; LeBrun, 2005; LeBrun *et al.*, 2012; Stuble *et al.*, 2013; Tschinkel and King, 2013). Native ants have proven to be less adept at dealing with drastic seasonal changes in the environment, where *S. invicta* evolved strategies to deal with the habitat disturbances and temporal fluctuations in their native environment. In Texas, the polygynous *S. invicta* social form (Greenberg *et al.*, 1985; Porter *et al.*, 1991) has become the one more commonly encountered, sharing reproductive tasks among multiple queens and causing the successful range expansion and establishment of fast growing populations (Morrison *et al.*, 2004). *S. invicta* is also an invasive species in India, Australia, and southeastern China, with the likely potential of being introduced to additional countries via transport made possible by globalization. For decades, research on *S. invicta* biology has translated to many insights for applications to help control expanding populations, nevertheless, *S. invicta* populations have persisted (Drees *et al.*, 2013). Studies involving bacteria and *S. invicta* interactions could generate innovative solutions and offer new strategies for how to mitigate invasive populations.

Like all eusocial organism, *S. invicta* is part of a colony with cooperative group care, overlapping generations, and a caste system. There are two different castes in the

colony, the worker caste, responsible for locating food, feeding and caring for the brood (larvae and eggs) and the queen, and protecting the colony. In addition, workers are constantly removing debris and dead ants from the colony, forming refuse piles outside the colony (Howard and Tschinkel, 1976). Tasks can be further divided by age and size of the worker (Cassill and Tschinkel, 1996, 1999b). Adult *S. invicta* can only consume a liquid diet, due to the constricted petiole which does not allow the passage of particles greater than 0.88 μg (Vinson, 1986). All solid food collected by worker ants is distributed to the fourth instar larvae in the colony. Larvae then digest the solid food, to be redistributed via trophallaxis once it has been digested to liquid form (Cassill and Tschinkel, 1995; Sorensen *et al.*, 1985). The second caste, the reproductive caste or queen, lays hundreds of eggs per day. Via a haplodiploidy sex determination system, the queen bears female sterile workers or reproductive females from fertilized eggs or reproductive males from unfertilized eggs. Communication between colony members is mediated by a diverse repertoire of pheromones produced by multiple glands in the ants' body, controlling behaviors like trail following and queen grooming (Vander Meer, 1983; Vander Meer and Alonso, 2002; Vander Meer *et al.*, 1988). Chemical communication is essential to the survival and success of *S. invicta* because these ants are required to produce and decipher these semiochemicals to function as unified colony. This well-established and evolutionarily adapted system of communication in ants may also be adapted to respond to environmental cues they encounter in the environment, such as those released by quorum sensing bacteria *P. mirabilis*. Possible *S. invicta* behaviors to be affected by the presence of quorum sensing bacteria are those where colony members are actively

searching for information to better assess their environment via eavesdropping on environmental cues.

The research objectives described in this dissertation aimed to identify the presence of bacteria on *S. invicta* ant colonies across varying ecoregions and investigate possible effects of a quorum sensing bacteria *P. mirabilis* on the behavioral ecology of *S. invicta.* Using *S. invicta* and *P. mirabilis* as an animal-bacteria model, this research explores the possible implications interkingdom interactions and communication play in the success of an invasive eusocial species.

CHAPTER II

BACTERIAL COMMUNITY DIVERSITY OF *SOLENOPSIS INVICTA* BUREN BASED ON COLONY, ECOREGION, AND FUNCTIONAL CATERGORY

Introduction

Social interactions, population structure, and the environment of animals regulate associated bacterial communities. Determining the structure of insect bacterial communities could lead to further ecological integration and knowledge of the biotic factors that influence insect behavior, physiology, and life history. Bacterial communities of eusocial insect species have been widely studied due to the economic impact their populations have, inherent of their altruistic and communal biology. For example, studies on symbiotic relationships of honey bee *Apis mellifera* (Hymenoptera: Apidae) pollinators (Crotti *et al.*, 2013; Kwong and Moran, 2016) or the diversity associated with *Cephalotes* (Hymenoptera: Formicidae) herbivorous ant species (Anderson *et al.*, 2012). In particular, focusing on the honey bee gut microbial community has given way to the characterization of determining factors, effects on bee health, and the identification of core microbiota for a host species. Martinson *et al.* (2011) studied *Apis* and *Bombus* microbiota and Cariveau *et al.* (2014) led comparisons of wild bumble bees *B. bimaculatus* and *B. impatiens* (Hymenoptera: Apidae) with pathogen infections, further describing distinctive gut communities as a result of pathogen infection and susceptibility. Caste-specific and developmental stage differences have also been noted in *Apis* microbial communities,

further describing transient flow of facultative bacterial in the colony and influencing the ultimate core taxa, with queen microbiomes enhancing energy conversion for egg production (Guo *et al.*, 2015; Hroncova *et al.*, 2015; Kapheim *et al.*, 2015). Physiological compartmentalization of gut microbes has been studied in *Cephalotes* ants (Lanan *et al.*, 2016), deeming the proventriculus in these specialized herbivorous feeders as filters that establish the host-microbe fidelity to the gut microbes present in the gut. Bacterial diversity has proven dependent on location and habitat, non-eusocial insect models compared across geographic regions. For example, the bacterial diversity of *Aedes albopictus* and *A. aegypti* (Diptera: Culicidae) is influenced by habitat and sex (Zouache *et al.*, 2011). In the beetle larvae of *Holotrichi parallela* (Coleoptera: Scarabaeoidea), bacterial diversity and community structure changed as a result of its environment and physiology, with diversity variations attributable to soil pH, organic carbon and total nitrogen, and climate factors of the sampling location (Huang and Zhang, 2013).

Revealing the structure of insect bacterial communities gives way to further ecological integration and knowledge of the biotic factors that influence insect behavior, physiology, and life history. Understanding the relationship between insects and their bacterial community diversity and distribution could thus impact how we study and approach entomological questions. The bacterial facet of insect biology can be used to expand resolutions that help native pollinators, inhibit vector competence, and mitigate invasive species. Novel management methods of targeted insect pests, via alteration of bacterial communities that target specific insect physiological processes or functions, such as vector capacity or insecticide resistance could be developed in the future (Crotti *et al.*,

2012). *Solenopsis invicta* Buren (Hymenoptera: Formicidae) could be such targeted insect species. *S. invicta*'s complex social structure, wide population distribution, and resilience in foreign environments deem it a worthy species to investigate. *S. invicta* is an omnivorous ant species that causes approximately $750 million in damage to agricultural assets, and about $5 billion in control expenditures, in the United States alone (McDonald, 2006). Environment disturbance and moisture are factors contributing to the impact *S. invicta* colonies will have in a region (LeBrun *et al.*, 2012), their regional dominance attributed to their aggressive foraging strategy and resilience in disturbed environments (LeBrun, 2005). These behaviors can be attributed to their eusociality and the interacting factors that impact such elicited behaviors, one possible factor being their bacterial community.

Previous bacterial studies involving *S. invicta* isolated and identified concomitant bacteria; however, most of these studies have focused on the establishment of endosymbiotic relationships. Studies focused on the isolation and characterization of culturable bacteria obtained from the midgut of the fourth instar *S. invicta* larva (Li *et al.*, 2003). These studies found bacteria limited to those capable of growing in culture, and no symbiotic relationships were determined in relation to the microorganisms and the fourth instar *S. invicta*. Through a culture-independent study, Lee *et al.* (2007) explored possible obligate and transient symbiotic relationships between *S. invicta* and associated bacteria. Chao1 estimate metric was used to calculate species diversity indices of the bacterial flora isolated in each sample colony and project 46-85% of the larval gut bacteria had been identified. Bacterial isolates obtained from the hemolymph of *S. invicta* fourth instars have

also been studied, by amplifying a partial sequence of the 16S rRNA gene using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis (Gunawan *et al.*, 2008). Ten species of bacteria were identified, more than half of these noted in previous studies. Ultimately, symbiotic associations between *S. invicta* and midgut bacteria were not established, leaving the nature of these bacterial associations open to further study. Ishak *et al.* (2011) compared the bacterial diversity of *S. invicta* and *S. geminata*, pooling species caste samples to compare workers, brood, and nest soil using DNA extractions and 454 pyrosequencing. Relatively, the communities of *S. geminata* and *invicta* had similar diversities with lower bacterial diversity attributed to the ant life stages, with minimal shared specific microbes across the two species. More recently, Woolfolk *et al.* (2016) conducted a study aimed to determine the microbial community structure of *S. invicta*, repeatedly collecting adult ant sterile workers from 5 colonies from 3 locations along a roadside in Mississippi, USA. Bacterial communities identified through a culture-dependent method and then sequenced isolates to confirm identities. No consistent trends in bacterial communities were found by comparing ants, location, or dates; and the limitations associated with culture-dependent methods produced a bias in the bacterial community described through this methodology. In particular, studies involving *S. invicta* have yet to capture the depth of information that could further develop knowledge about the success this highly successful invasive species. Functional category, environment, and colonies are adaption factors of bacterial diversity that warrant continued investigation.

In this study, we used next generation sequencing of the 16S rRNA gene v4 hypervariable region to explore the bacterial community of *Solenopsis invicta* Buren at the functional category, colony, and ecoregion levels to compare bacterial species associated with ant biology and behaviors leading to their survival. Further, we investigated differences in diversity associated with brood and adult caste-associated colony functions, colony differences across species, and ecoregion impacts across species. Specifically, we formulated a framework for the structure of this study using the following questions: (1) how do *S. invicta* bacterial communities differ in alpha and beta diversity, by various metrics/indices, among functional categories and between colonies in four ecoregions, (2) are there characteristic bacterial taxa attributable to distinct ecoregions, and how does the abundance of these dominant taxa compare across colonies?

Materials and Methods

Ecoregions

S. invicta samples and colonies were collected from four Texas ecoregions located in the Post Oak Savannah, Pineywoods, Gulf Coast Prairies and Marshes, and Blackland Prairies (Figure 2.1) (Gould *et al.*, 1960). These ecoregions were selected from ten described Texas ecoregions based on the climate of the region where the presence of *S. invicta* has been previously recorded. The Post Oak Savannah ecoregion is a forested area in west Texas that is part of a historic oak belt ecosystem extending south from Canada. This ecoregion receives approximately 102-122 cm of rainfall per year. The topography could be

described as bottomland where many areas are found in close proximity to streams and rivers. The soil ranges from clay loam and clay to sandy loam and sandy soil. The temperate coniferous ecoregion of the Pineywoods, or South Central Plains, is found on the east side of Texas and receives approximately 107-147 cm of precipitation annually. Sandy loam soils with moderate acidity occupy most of the Pineywoods soils. The sandy soil makes it easy for deep tunneling within colony mounds of the *S. invicta.* The Gulf Coast Prairies and Marshes ecoregion is found at the southwestern border of the state, adjacent to the Gulf of Mexico, and includes counties such as Fort Bend, Brazoria, Galveston, and parts of Houston. This region is characterized by sand-based soils where agriculture is prevalent; having an annual average precipitation of 58-147 cm per year. This subtropical grassland area is home to many endangered animals, including several migrating avian species. The Blackland Prairies are longitudinally extended from northern to central Texas and they receive an annual rainfall averaging 71-107 cm from north to south, respectively. This region contains a variety of soil types, ranging from marine chalks, limestone, and shale to black, heavy clay soils. These soils are considered some of the richest soil in the world such that major land use has been in crop agriculture. (Griffith *et al.*, 2007)

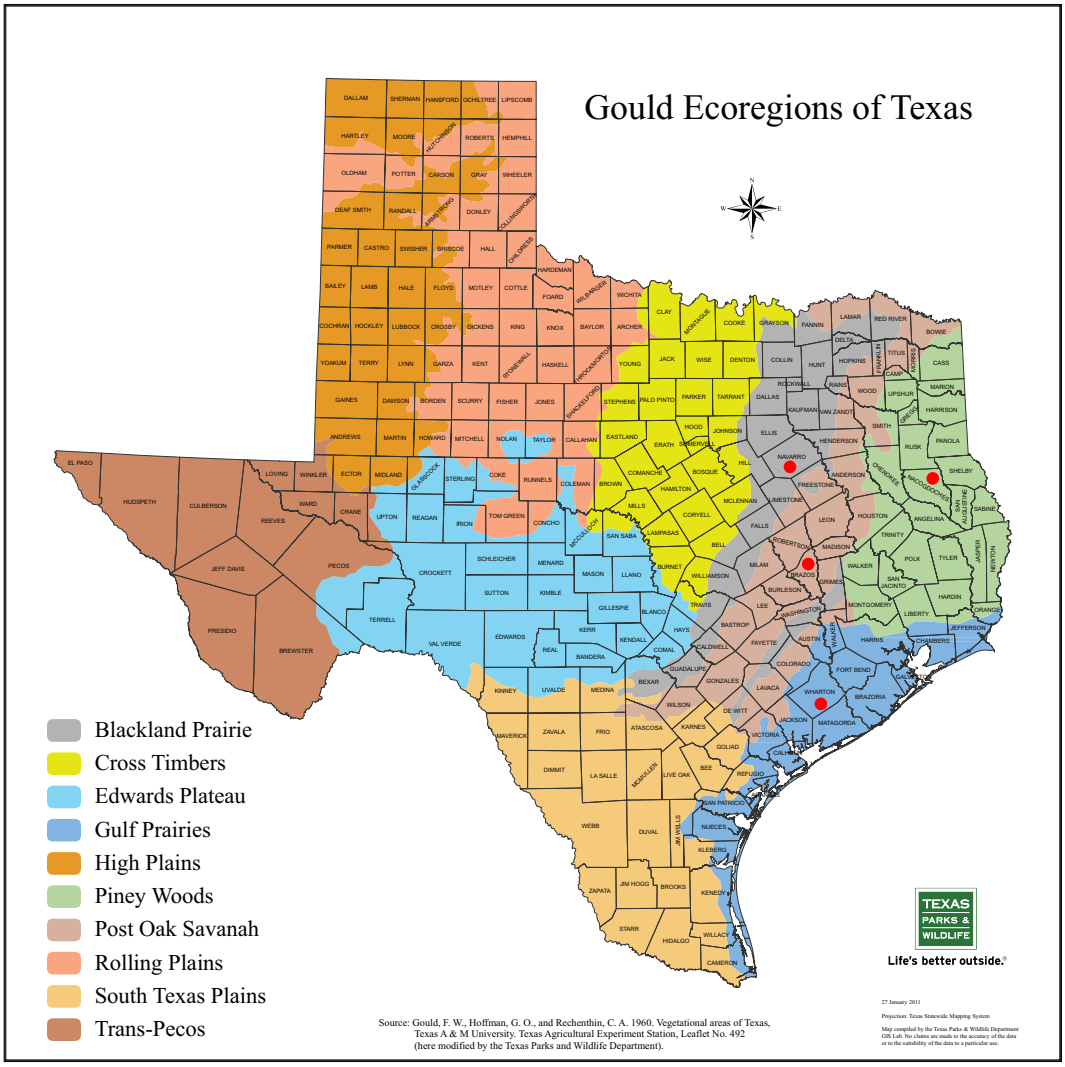

Figure 2.1. **Map of Texas Ecoregions.** Reprinted from Texas Parks and Wildlife Department (2011). Map of ecoregions of Texas based on Gould *et al.* 1960 "Vegetation areas of Texas" with red circles marking the county locations where *S. invicta* colonies were sampled for bacterial DNA 16S rRNA gene sequencing.

Colonies for Experiments

Three colonies were sampled from each ecoregion site, ranging from 67 to 6656 meters (1822.59 average) in distance from each other, during August 2014. A colony was selected if it was in a fresh mound constructed with loose soil, as a reference for activity. The nest tumulus was removed with a disinfected stainless steel shovel in order to expose the colony (Banks *et al.*, 1981). The shovel was decontaminated by disinfecting with Clorox wipes between sampling each colony use to prevent cross contamination of bacterial communities between colony samples. With the shovel, the top portion of the mound was removed to expose the larvae and pupae in the brood chamber. These collections were done between 0700 and 0900 hours, on days after a light rain to increase the likelihood of locating the brood. Brood was collected during exposure of the colony, finding brood pockets were the ants had collectively placed the larvae and using a sterile scoop (plastic spoon) (Wal-Mart Stores Inc. AR, USA) to place brood in a sterile container (condiment cups with cover) (Wal-Mart Stores Inc. AR, USA). Five to eight containers of brood per colony were collected, depending on colony abundance. Upon returning to the lab, brood was separated from any debris and isolated in a new sterile container, doing this under a biosafety cabinet with laminar flow. Colony workers were collected using sterile tongue depressors stored in aluminum foil. Tongue depressors were dropped in the colony immediately after nest tumulus removal to allow the workers to climb up the surface area of the tongue depressor. The tongue depressors were then collected and placed in a sterile plastic storage bag, collecting 5-8 bags of 200-500 workers per colony, depending on colony abundance. Colony brood was then collected using sterile scoops and placed in the

sterile containers. Whole colonies were collected and placed in a sweater box lined with Insect-a-Slip (BioQuip Products, Inc. CA, USA) to prevent escape and returned to the lab. Later in the lab, ant colonies were excavated for queens and virgin reproductives, while the rest of the ants were not fed for a week so that midden could be collected. Colony midden (dead ant refuse pile) was collected using sterile scoops and then placed in sterile containers. All colony samples were labeled and placed in a freezer at -20 °C until DNA extractions were performed.

Bacterial DNA Extractions

Modifications to the phenol chloroform extractions of darkling beetles (Crippen personal communication) were made to optimize DNA extractions from each functional category sample. Ant colony functional category samples consisted of four types: reproductives (female only), brood (larvae/pupae), workers (sterile adults), and midden. Pooled individuals were used for the extractions of three replicates from separate collected containers, each a sample functional category type per excavated colony of a given ecoregion, for a total of 144 sample extractions.

A standard phenol-chloroform bacterial DNA extraction was used for sample extractions, with modifications to centrifugation times, temperatures, and washes for all steps, specifically modified for *S. invicta* samples. All samples were pre-weighed to account for the necessary number of pooled individuals to perform extractions, this varied upon functional category type and was determined in preliminary study of sample weight to DNA concentration yields, namely: 0.5 g of brood, 0.5 g of workers, 0.35 g of midden,

and 10-15 reproductives. Samples were placed in 1.5 mL microcentrifuge tubes with zirconium beads and crushed with a new sterile plastic pestle used for each sample. Then, 5 µL of 15-mg/mL lysozyme, and 675 µL of Carlson Lysis Solution were added to the tubes before closing them tightly and placing them in the MP shaker (Fast Prep) for 40 seconds at 6 m/s for thorough mixing and rupturing sample contents. Tubes were taken out of the shaker and incubated for 1 hour at 37 °C in an incubator before adding 10 µL of 20 mg/mL proteinase K and 150 µL of 10% SDS were added, with worker and midden samples requiring an additional 150 µL of 10% SDS. The samples were then incubated at 65 °C for overnight in a heat block. Following incubation, a quick centrifugation of 1 minute at 13000 g was done to help with separation of fat and insoluble contents in the sample tubes. The liquid contents were then transferred to a new tube, with minimal insoluble material transferred to allow for cleaner aqueous washes to proceed. Equal volume of the phenol/chloroform/isoamyl alcohol (25:24:1) was added to each sample and the contents in the tube mixed on a rotator for 5 minutes. Tubes were then centrifuged in a temperature-controlled centrifuge at 13000 g for 6 minutes at 4 °C. The top aqueous phase was carefully transferred with a pipette into a new tube and other contents discarded, avoiding the interphase to obtain a cleaner aqueous sample free of phenol contamination or unwanted materials. The phenol/chloroform/isoamyl alcohol wash was repeated for the new tube contents, but only for brood and worker samples because the wash seemed to be harsher on reproductive and midden samples that typically contained less DNA. Next, the aqueous isolates in the new tubes were extracted using equal volume of chloroform/isoamyl alcohol and centrifuged at 13000 g for 6 minutes at 4 °C. Upon

centrifugation, the aqueous phase was transferred into a new tube. The aqueous phase was then used to precipitate DNA into a pelleted form. First, the contents were washed in an equal volume of room temperature isopropanol and centrifuged at 13000 g for 25 minutes at room temperature. After centrifugation, the supernatant was discarded, being extra careful to retain the pellet, by using a low volume pipette (200 µL) to slowly discard the liquid contents without causing forceful mixing within the tube that could result in dislodgment of the DNA pellet. More often than not, the DNA pellet was not visible at this step. If the DNA pellet was not visible, or only a small amount was visible, 100-500 µL of the bottom liquid contents was left in the tube, depending on starting aqueous sample volume in previous step, to make sure DNA remains in the sample tube for more washing. Isopropanol is extractable at the final supernatant removal, along with ethanol from the final wash so this did not inhibit final DNA collection. In the same tube, the DNA pellet or DNA liquid-content was washed with 800 µL of 70% ethanol and centrifuged at 17000 g for 25 minutes. This concentration of ethanol removed salt contaminants that may have precipitated with the DNA pellet. A single wash was generally sufficient to remove these kinds of contaminants but a second wash was done when needed, keeping in mind that with every wash some DNA will be lost. The supernatant was removed after each wash without disturbing the pellet and if no pellet was observed, an additional centrifugation was done. Finally, the DNA pellet was dissolved in 50 µL of nuclease-free water for 1-12 hours with constant mixing or rotating inversion. The integrity of the extracted DNA was then quantified by using micro volume spectrophotometry. DNA extraction products were stored at -20 °C until they were ready for further processing by next generation

sequencing. Additional samples were prepared to be stored for an extended period of time in an ultra-low temperature freezer at -80 °C, to serve as reference samples for future studies.

Bacterial DNA Sequencing and Analysis

DNA samples were packaged and submitted for sequencing by the Research and Testing Laboratory (Lubbock, TX, USA) using Illumina MiSeq 250x2 sequencing platform. DNA concentrations of samples ranged from 45-2765 ng/mL, and averaged 547 ng/mL, with 20-30 μL of DNA sent per sample. Bacterial amplicon sequencing was performed based on RTL protocols using 16S rDNA universal eubacterial primers 515 F (5' – TGCCAGCAGCCGCGGTAA) and 806 R (5' – ACGAGCTGACGACARCCATG), also known as the Earth Microbiome Project 16S rRNA amplicon eubacterial primers recommended for use of environmental samples sequencing (Gilbert *et al.*, 2014). Earth Microbiome Project primers were selected based on their success as degenerate primer sequences used in field sample studies, to account for the most bacteria in sequence amplification (Earth Microbiome Project, 2017).

Sequence data were processed using QIIME 1.9.1 (Caporaso *et al.*, 2010) following the standard procedure for Illumina sequencing data (Kuczynski *et al.*, 2011). Sequences containing chimeras were removed. The remaining sequences were matched against the 16S rRNA reference (RDP) database (Wang *et al.*, 2007). Sequences were then grouped into operational taxonomic units (OTU) with at least 97% similarity, and representative sequences were classified by matching against the nucleotide BLAST,

NCBI database. To investigate the similarity of bacterial communities across functional categories, colonies, and ecoregion samples, alpha diversity and beta diversity were compared using multiple indices in QIIME. All analysis of variance (ANOVA) statistics were performed in R version 3.1.2 (R Development Core Team, 2014). Dendrogram distances were generated using Bray-Curtis ordination technique and clustered by average linkage in R with the vegan library. The following alpha diversity measures/indices were generated for comparing diversity within individual samples: observed OTUs, phylogenetic diversity (PD) whole tree (Faith *et al.*, 2009), Shannon (Pielou, 1969), Simpson (Lande *et al.*, 1949), and effective number of species (ENS) (Jost, 2006). Factors associated with bacterial community diversities were compared among groups (functional category, colony, and ecoregion) using a PERMANOVA for weighted and unweighted UniFrac (Lozupone and Knight, 2005) beta diversity indices with QIIME scripts. Weighted UniFrac beta diversity matrices were generated to compare diversity among samples using principal coordinate analysis (PCoA) (Gower, 1966) and Unweighted Pair Group Method with Arithmetic mean (UPGMA) (Sokal and Michener, 1958) clustering with QIIME scripts.

Results

Summary of Sequences and Classification

After extractions and sequencing, a total of 139 samples had quality DNA to conduct analyses. A total of 5 samples were not processed due to low PCR yields, these samples

were 2 reproductive, 1 midden, and 1 worker sample from the Gulf Coast Prairies ecoregion, and 1 brood sample from the Blackland Prairies ecoregion. A total of 45,481 OTU observations and 5,201,387 OTU counts/sequences were retained after filtering. The median number of OTU counts/sequences per sample was 31,845 (mean 37,420 OTU counts/sequences per sample), with the minimum being 2,627 and the maximum 138,779. Rarefaction curves plateaued for most samples, indicating sufficient reads for quality bacterial community analysis. The number of sequences was significantly different between colonies (ANOVA $F_{11, 127} = 10.05$, $P < 0.05$) and between ecoregions (ANOVA $F_{3, 135} = 8.54$, $P < 0.05$), but not between functional categories (ANOVA $F_{3, 135} = 1.37$, $P = 0.25$). The number of observed OTUs was significantly different between functional categories (ANOVA $F_{3, 135} = 3.74$, $P < 0.05$), with workers having the most observed OTUs and brood having the least. For ecoregions, the number of observed OTUs was also significantly different among the four ecoregions (ANOVA $F_{3, 135} = 5.09$, $P < 0.05$), with the Post Oak Savannah ecoregion having the most observed OTUs and the Pineywoods ecoregion having the least. For colonies, the number of observed OTUs was also significantly different (ANOVA $F_{11, 127} = 4.73$, $P < 0.05$), with Post Oak Savannah Colony 1 having the most observed OTUs and Gulf Coast Prairies Colony 1 having the least. Samples with lower sequences counts, particularly those from the Gulf Coast Prairies, produced a lower number observed OTUs and shorter rarefaction curves indicating insufficient depth in sequencing. OTUs were classified into 48 taxonomic groups at the phylum level.

Bacterial Community Diversity Differences Within Samples

The relative abundance and diversity of bacterial communities of *S. invicta* varied by functional category, colony, and ecoregion, where alpha diversity indices used to determine difference all followed a similar pattern. Quantitative indices like the Shannon and Simpson diversity indices were calculated to identify bacterial species richness and dominance, respectively. Overall the worker and reproductive samples had a greater species richness and abundance, while the brood samples had the lowest overall average for both indices (Figure 2.2). Brood had more common taxa across all samples and the abundance of these taxa reaches similar quantities, which likely drives the Shannon index to be low, where a high number of sequences are representative of less taxa, thus less diverse. Colony comparisons showed the variation in diversity associated with individual colonies within the ecoregion groupings; colony 1-3 belong to the Gulf Coast Prairies ecoregion, colony 4-6 to the Blackland Prairies, colony 7-9 to the Post Oak Savannah, and colony 10-12 to the Pineywoods ecoregion. Colonies with the higher number of observed OTUs had the higher alpha diversity measures, specifically Simpson's diversity index measuring the dominance of taxa in the community (Figure 2.3). Most colonies had a sufficiently high diversity based on the Simpson index, with values ranging near 0.7 to 1, and the lower colonies being representative of the Gulf Coast Prairies ecoregion and the Blackland Prairies. However, the Shannon index, using both number of individuals and taxa to compare the bacterial communities, varied between 3-8 across all colonies. When comparing the Shannon index of colonies belonging to the same ecoregion, the index variation pattern is similar to the number of observed OTUs and even higher for colonies

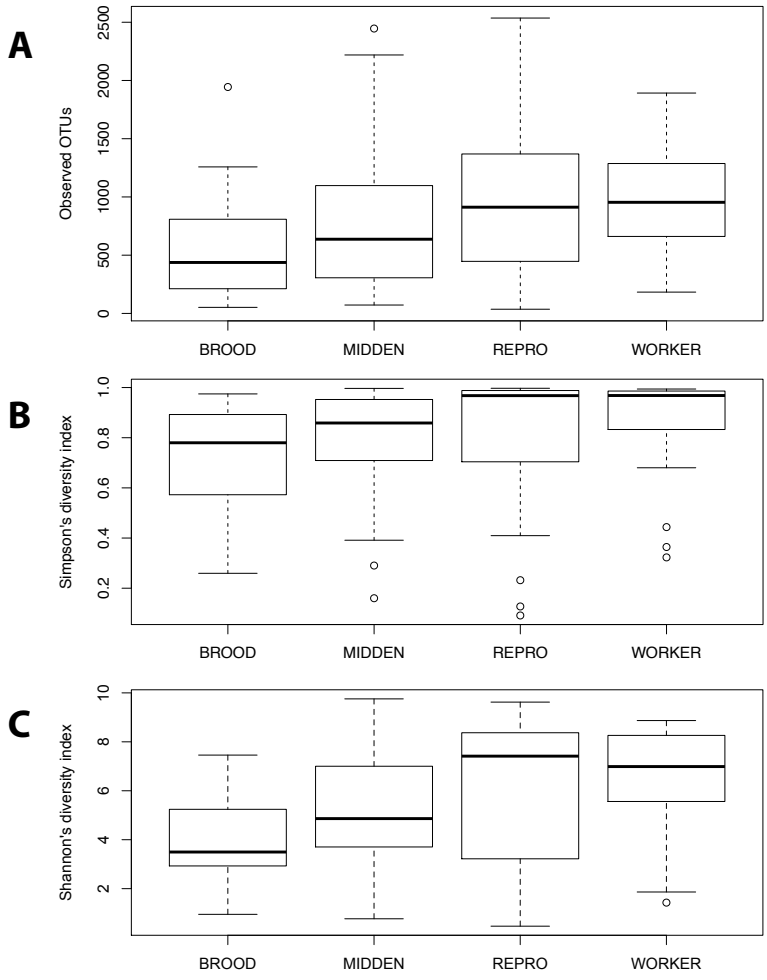

Figure 2.2. Functional Category Alpha Diversity Summary. Summary of alpha diversity indices based on OTU frequencies within colony functional category samples. Measures included: observed OTUs (A), Simpson's diversity index (B), and Shannon's diversity index (C).

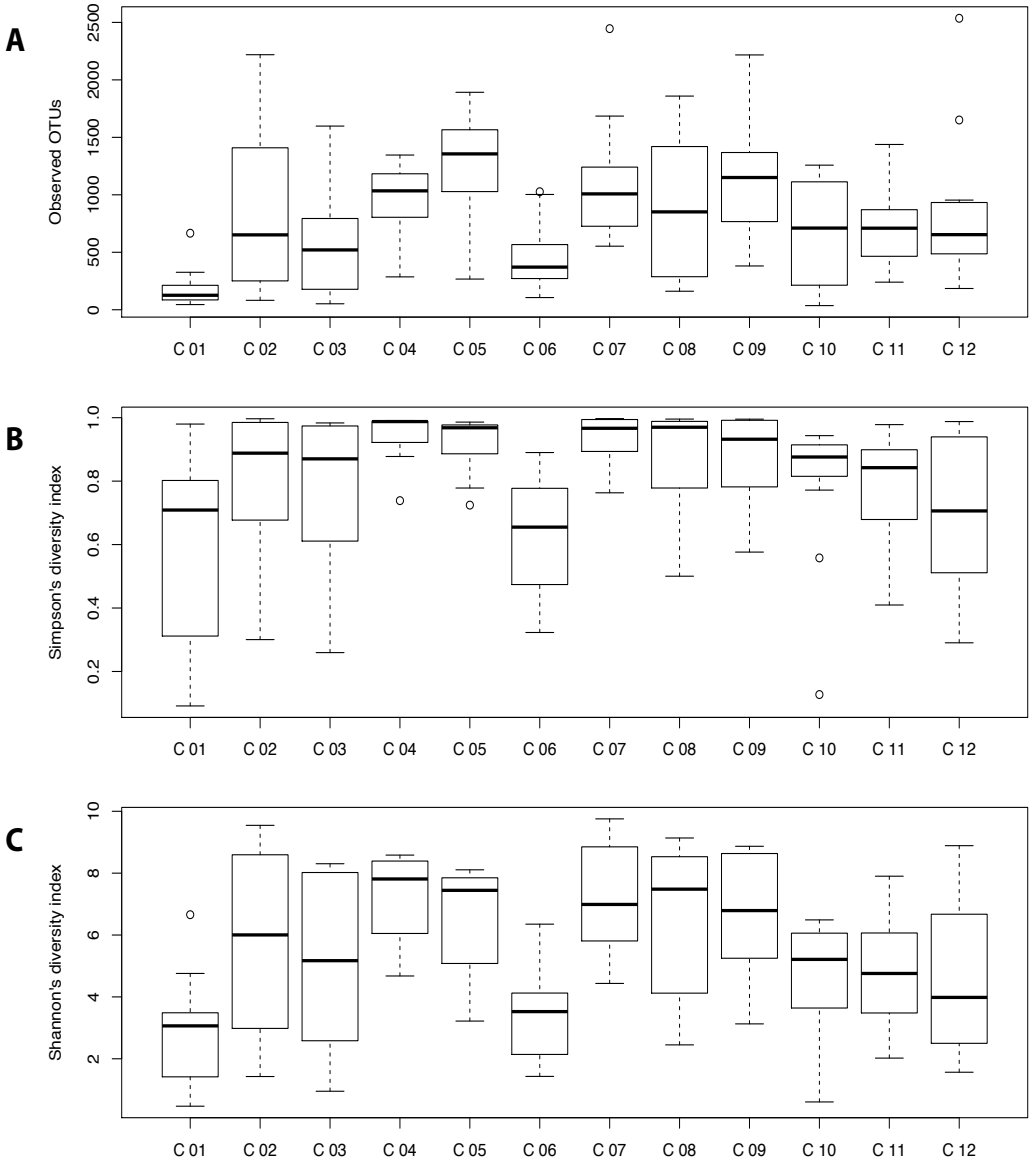

Figure 2.3. Colony Alpha Diversity Summary. Summary of alpha diversity indices based on OTU frequencies within individual colony samples. Measures included: observed OTUs (A), Simpson's diversity index (B), and Shannon's diversity index (C). Colony 1-3 belong to the Gulf Coast Prairies ecoregion, colony 4-6 to the Blackland Prairies, colony 7-9 to the Post Oak Savannah, and colony 10-12 to the Pineywoods ecoregion.

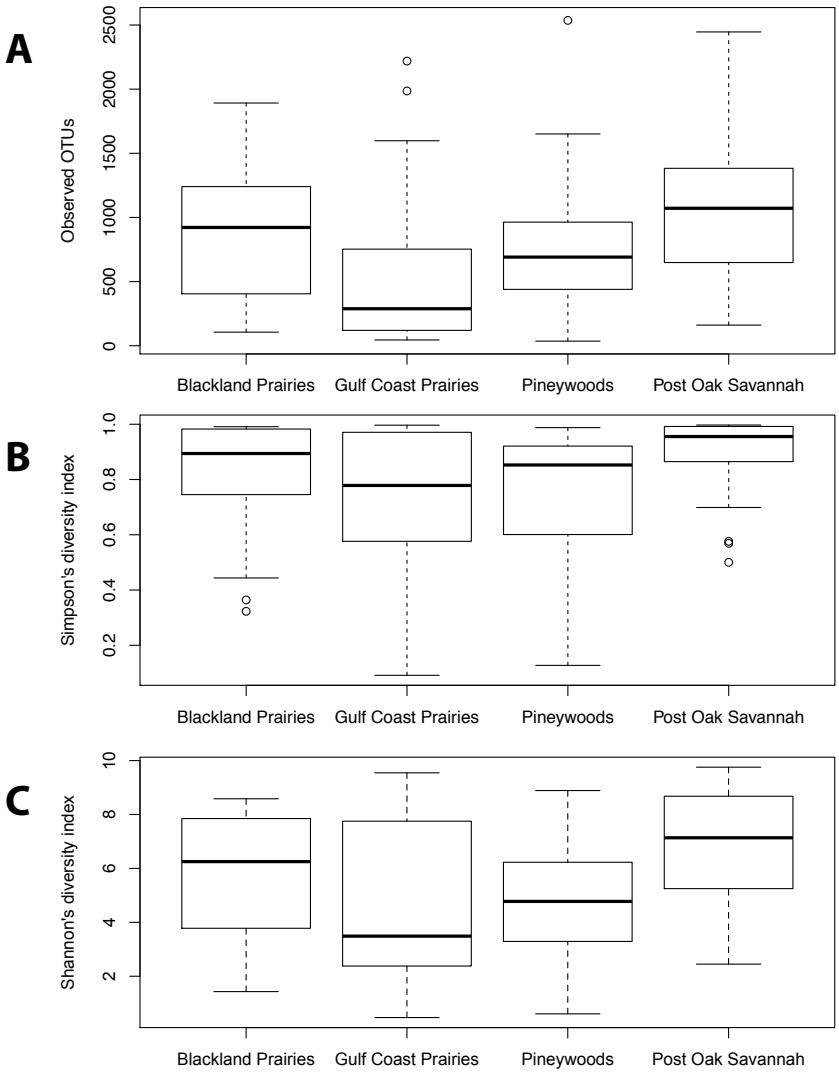

Figure 2.4. Ecoregion Alpha Diversity Summary. Summary of alpha diversity indices based on OTU frequencies within individual ecoregion samples. Measures included: observed OTUs (A), Simpson's diversity index (B), and Shannon's diversity index (C).

with lower observed OTUs, representative of similar richness and evenness for these low-sequence colonies. Based on ecoregion type, the patterns of bacterial diversity exhibited by samples grouped by this factor followed similar index averages for all alpha diversity measures. The Post Oak Savannah ecoregion had the greater diversity average indices. Gulf Coast Prairies ecoregion had the lowest measures, although some averages showed a wide variance also (Figure 2.4), but this was also the ecoregion with the lowest amount of sequences and OTUs observed which are likely attribute to the lower described alpha indices. A phylogenetic diversity (PD) regression analysis based on observed OTUs for each ecoregion, illustrates a comparison of branch lengths associated with the alpha diversity of samples within these groupings. Samples that fall below the pertaining ecoregion regression line are identified as having a lower phylogenetic diversity (Figure 2.5). The greatest phylogenetic diversity within samples is estimated for the Gulf Coast Prairies samples, relating the highest PD to OTU ratio. However, half of the samples determining this estimate were located at the base of the line and clustered, generating a more extreme extrapolation for PD estimates (Figure 2.5). Samples belonging to the Blackland Prairies had the lowest estimated phylogenetic diversity, with the lowest PD to OTU ratio; yet many of the points lie closely to the Blackland Prairies regression line, meaning there was likely more consistent taxa sampling and identification for the samples within this ecoregion (Figure 2.5).

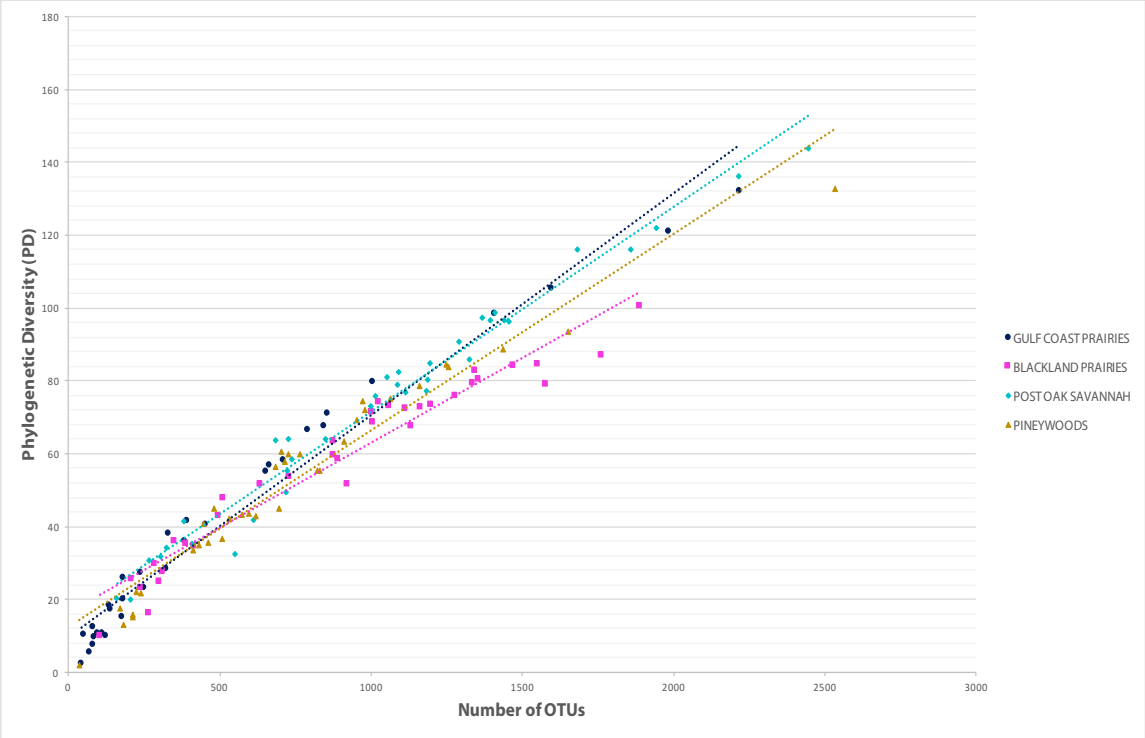

Figure 2.5. Phylogenetic Diversity Regression Analysis. Plot of the amount of branch length in the phylogenetic tree that leads to the community of the given sample's phylogenetic diversity (PD), vs. the number of observed OTUs that represent each sample.

Alpha diversity indices for each sample were further consolidated to obtain average and standard error values to be compare within ecoregion diversity across functional categories (Table 2.1). Comparison of consolidated samples by ANOVA indicated statistically significant differences across functional and ecoregion category groupings. Taxonomic diversity can be further assessed by comparing the average number of sequence and observed OTUs, where lower number of observed OTUs for brood across all ecoregions contribute to the higher, if not highest, number of average sequences per functional category (Table 2.1). Based on the average ENS diversity values, the diversity

		Sequences		Observed OTUs		Shannon's index		Simpson's index		PD Whole Tree		ENS	
		Average	*SE*	*Average*	*SE*	*Average*	*SE*	*Average*	*SE*	*Average*	*SE*	*Average*	*SE*
BLACKLAND PRAIRIES	Brood	56,795	8,562	734.7	196.4	4.323	0.801	0.819	0.078	51.672	10.993	7.317	19.646
	Midden	39,133	7,551	696.3	173.2	5.812	0.706	0.855	0.068	49.174	9.695	25.206	17.326
	Repro	56,306	7,551	1053.6	173.2	6.387	0.706	0.843	0.068	63.826	9.695	44.556	17.326
	Worker	44,528	7,163	1024.1	164.3	5.950	0.670	0.792	0.065	64.217	9.197	44.146	16.437
GULF COAST PRAIRIES	Brood	22,467	7,551	222.3	173.2	2.844	0.706	0.635	0.068	23.091	9.695	3.617	17.326
	Midden	28,055	8,009	749.5	183.7	4.658	0.749	0.705	0.073	49.955	10.283	55.709	18.377
	Repro	20,515	8,562	504.9	196.4	4.218	0.801	0.638	0.078	39.68	10.993	15.84	19.646
	Worker	16,126	8,009	648.8	183.7	6.410	0.749	0.886	0.073	54.964	10.283	33.3	18.377
PINEYWOODS	Brood	46,130	7,163	551.8	164.3	3.552	0.670	0.684	0.065	41.684	9.197	5.949	16.437
	Midden	37,968	7,551	603.6	173.2	4.203	0.706	0.731	0.068	42.799	9.695	6.614	17.326
	Repro	54,112	8,009	850.7	183.7	4.740	0.749	0.727	0.073	56.633	10.283	20.159	18.377
	Worker	31,775	7,551	970.3	173.2	6.230	0.706	0.887	0.068	69.014	9.695	12.289	17.326
POST OAK SAVANNAH	Brood	29,373	7,551	721.1	173.2	5.026	0.706	0.806	0.068	55.52	9.695	11.122	17.326
	Midden	43,269	7,551	1140.9	173.2	6.258	0.706	0.867	0.068	76.164	9.695	57.761	17.326
	Repro	37,483	7,551	1220.9	173.2	8.286	0.706	0.959	0.068	85.926	9.695	160.624	17.326
	Worker	31,925	7,551	1078.8	173.2	7.697	0.706	0.951	0.068	77.218	9.695	75.733	17.326
	F-stat	26.04		23.05		61.29		134.10		35.52		9.24	
	p-value	< 0.05		< 0.05		< 0.05		< 0.05		< 0.05		< 0.05	
	df	16, 123		16, 123		16, 123		16, 123		16, 123		16, 123	

Table 2.1. Summary of Alpha Diversity Indices. Summary of 16S rRNA gene amplicon analysis with estimator index averages and standard errors (SE) for *S. invicta* samples grouped by ecoregion and functional categories (brood, midden, reproductives, and workers). Alpha diversity indices include: Shannon's index, Simpson's index, phylogenetic diversity whole tree, and estimated number of species (ENS). In addition, average sequences and observed OTUs were included.

for brood was the lowest across all ecoregions, ranging from 6 to 18 times lower than the highest functional category diversity within its corresponding ecoregion (Table 2.1). The highest diversity was most often attributed to the reproductive samples, with the highest diversity found in the Post Oak Savannah samples. The Gulf Coast Prairies samples had the lowest diversities for brood and reproductive categories, but the midden and worker categories faired relatively well compared to other ecoregions, especially considering these functional categories had the lowest number of attributed sequences and observed OTUs. Post Oak Savannah samples had the overall greatest average alpha diversity across ecoregions and functional categories.

Bacterial Community Diversity Differences Between Samples

Pairwise comparisons of OTU variance between samples, based on unweighted UniFrac distance matrices, varied by functional category, colony, and ecoregion, with the greatest factor impacting variance being ecoregion (Table 2.2). Weighted UniFrac distances, accounting for observed OTUs and abundance, also proved all categorical factors, functional category, colony, and ecoregion to be significant in characterizing bacterial communities of *S. invicta* (Table 2.3). However, when abundance is accounted for as a quantitative factor of *S. invicta* bacterial communities, the functional category type becomes the most likely factor contributing the greatest impact on the composition of a given sample bacterial community.

Sample Category	Pseudo-F Statistic	P-Value
Functional	1.774	$P < 0.05$
Colony	4.393	$P < 0.05$
Ecoregion	16.935	$P < 0.05$
Functional x Colony	1.859	$P < 0.05$
Ecoregion x Functional	4.699	$P < 0.05$
Ecoregion x Colony	7.552	$P < 0.05$

Table 2.2. Unweighted UniFrac PERMANOVA Analysis. Results of PERMANOVA analysis for statistical significance of sample categories as factors determining beta diversity, namely functional, colony and ecoregion categories, using unweighted UniFrac distance matrix (accounting for OTUs only), determined using 999 iterations (sample size = 139).

Sample Category	Pseudo-F Statistic	P-Value
Functional	10.486	$P < 0.05$
Colony	2.821	$P < 0.05$
Ecoregion	6.639	$P < 0.05$
Functional x Colony	3.158	$P < 0.05$
Ecoregion x Functional	6.516	$P < 0.05$
Ecoregion x Colony	3.970	$P < 0.05$

Table 2.3. Weighted UniFrac PERMANOVA Analysis. Results of PERMANOVA analysis for statistical significance of sample categories as factors determining beta diversity, namely functional, colony and ecoregion categories, using weighted UniFrac distance matrix (accounting for OTUs and abundance), determined using 999 iterations (sample size = 139).

Continued analysis was organized based on PERMANOVA results, namely, with the functional and ecoregion categories as factors more likely to determine the bacterial community structure of *S. invicta* colonies. The OTUs representative of *S. invicta* bacterial communities by functional category showed the greatest separation by representative presence or absence of phylum-level taxa (Figure 2.6). Tenericutes and HK819 phyla were found in greater abundance in the reproductive functional category, versus all other functional categories. The brood functional category showed greater abundance across a more reduced phyla range, relative to the other functional categories.

Comparison of OTUs at the phylum level illustrate differentiation between the representative phyla that make up the composite bacterial communities of the different

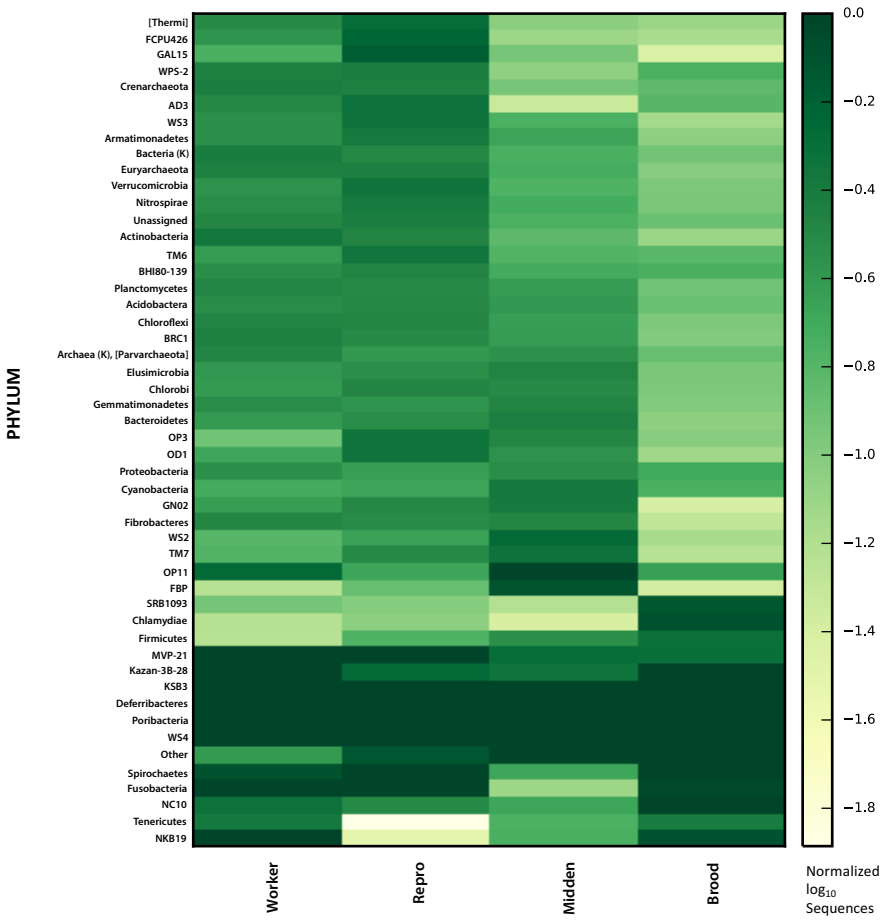

Figure 2.6. Phylum-Level OTU Heat Map for Functional Category. Phylum-level OTU heat map of sequenced samples grouped by functional category. OTU rows and columns are clustered by UPGMA hierarchical clustering based on presence and abundance within each sample category.

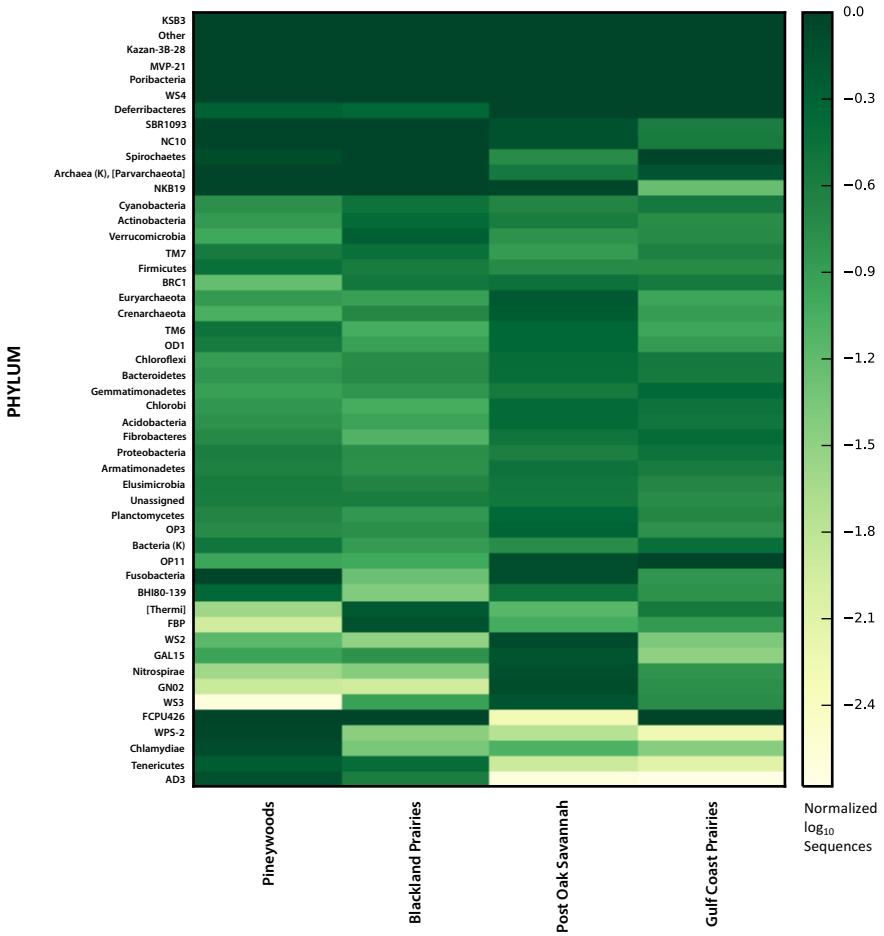

Figure 2.7. **Phylum-Level OTU Heat Map for Ecoregions.** Phylum-level OTUs heat map of sequenced samples grouped by ecoregion. OTU rows and columns are clustered by UPGMA hierarchical clustering based on presence and abundance within each sample category.

ecoregions (Figure 2.7). The Gulf Coast Prairies samples were more closely associated with the Post Oak Savannah, while the Pineywoods samples were represented by the most divergent bacterial community across all ecoregions. Unweighted and weighted UniFrac sample data was investigated by PCoA. Sample clustering according to ecoregion and functional category factors is illustrated in Figures 2.8-11. Unweighted UniFrac PCoA analyses heavily clustered by ecoregion, visible across all planes (Figure 2.8). Bacterial

communities cluster according to ecoregion when only OTUs identification, and not abundance, is accounted for. However, unweighted functional category data does not produce any form of structured clustering, instead the samples are disturbed throughout all planes (Figure 2.9). The PCoA analyses performed on the bacterial community weighted UniFrac beta diversity according to functional category illustrate a more pronounced demarcation of clustered samples, where the brood samples are the most separated from other functional categories (Figure 2.11, PC1 vs PC3). Furthermore, notable clustering among the different functional categories, particularly the brood and reproductive samples, corresponds to this factor being the strongest driver of OTU presence for *S. invicta.* Sample distribution according to weighted ecoregion data on the PCoA graph (Figure 2.10) is more distributed throughout the graphical space, not clustering along either of the axes.

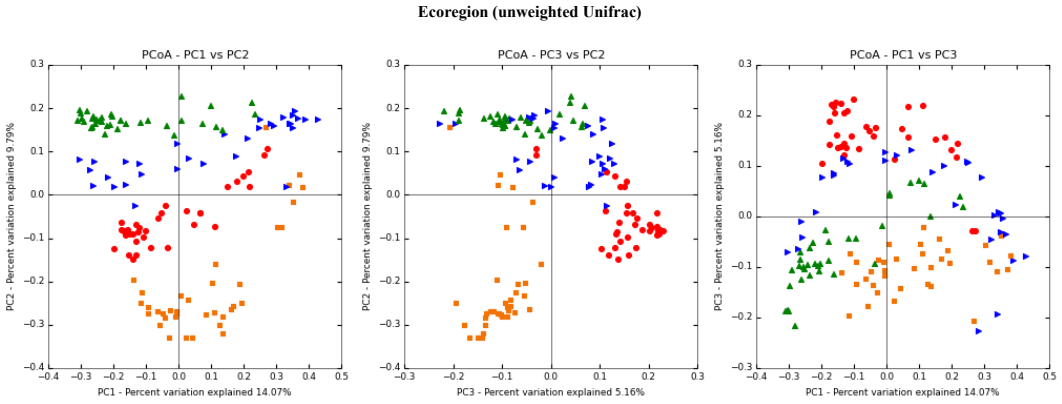

Figure 2.8. Unweighted PCoA Analysis for Ecoregions. Results of unweighted UniFrac PCoA analysis colored by ecoregion type. Each graph represents a different plane of the three-dimensional PCoA eigenvalue space, with each point representing one of the 139 sequenced samples. The samples are shaped/colored by the ecoregion type with the following corresponding shapes/colors: Blackland Prairies, red circles; Gulf Coast Prairies, blue side-point isosceles triangles; Pineywoods, orange squares; Post Oak Savannah, green up-point isosceles triangles.

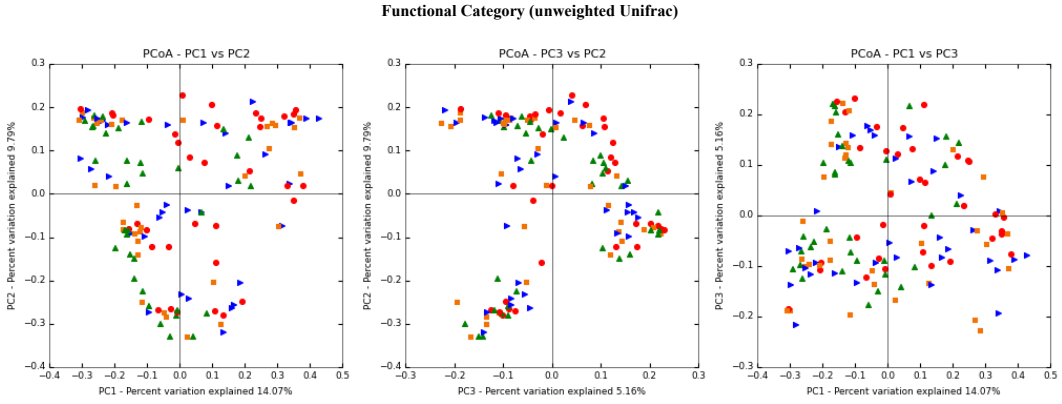

Figure 2.9. Unweighted PCoA Analysis for Functional Category. Results of unweighted UniFrac PCoA analysis colored by functional category type. Each graph represents a different plane of the three-dimensional PCoA eigenvalue space, with each point representing one of the 139 sequenced samples. The samples are shaped/colored by the functional category type with the following corresponding shapes/colors: brood, red circles; midden, blue side-point isosceles triangles; reproductives, orange squares; workers, green up-point isosceles triangles.

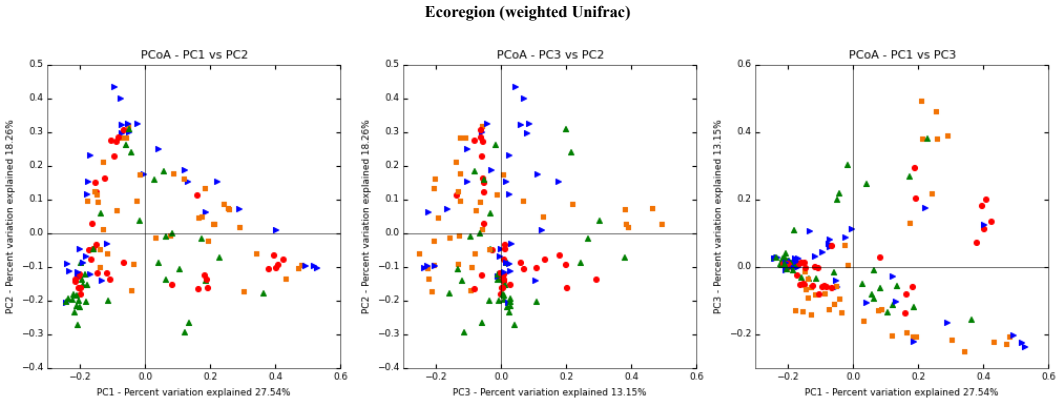

Figure 2.10. Weighted PCoA Analysis for Ecoregions. Results of weighted UniFrac PCoA analysis colored by ecoregion type. Each graph represents a different plane of the three-dimensional PCoA eigenvalue space, with each point representing one of the 139 sequenced samples. The samples are shaped/colored by the ecoregion type with the following corresponding shapes/colors: Blackland Prairies, red circles; Gulf Coast Prairies, blue side-point isosceles triangles; Pineywoods, orange squares; Post Oak Savannah, green up-point isosceles triangles.

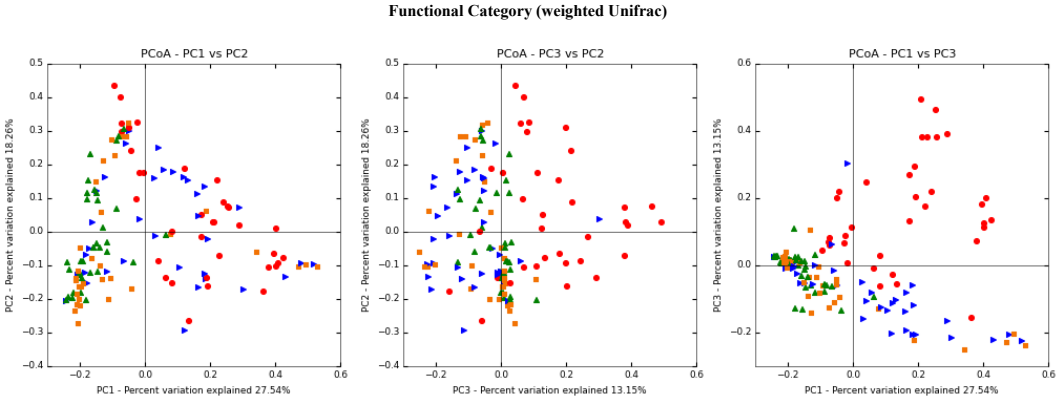

Figure 2.11. Weighted PCoA Analysis for Functional Category. Results of weighted UniFrac PCoA analysis colored by functional category type. Each graph represents a different plane of the three-dimensional PCoA eigenvalue space, with each point representing one of the 139 sequenced samples. The samples are shaped/colored by the functional category type with the following corresponding shapes/colors: brood, red circles; midden, blue side-point isosceles triangles; reproductives, orange squares; workers, green up-point isosceles triangles.

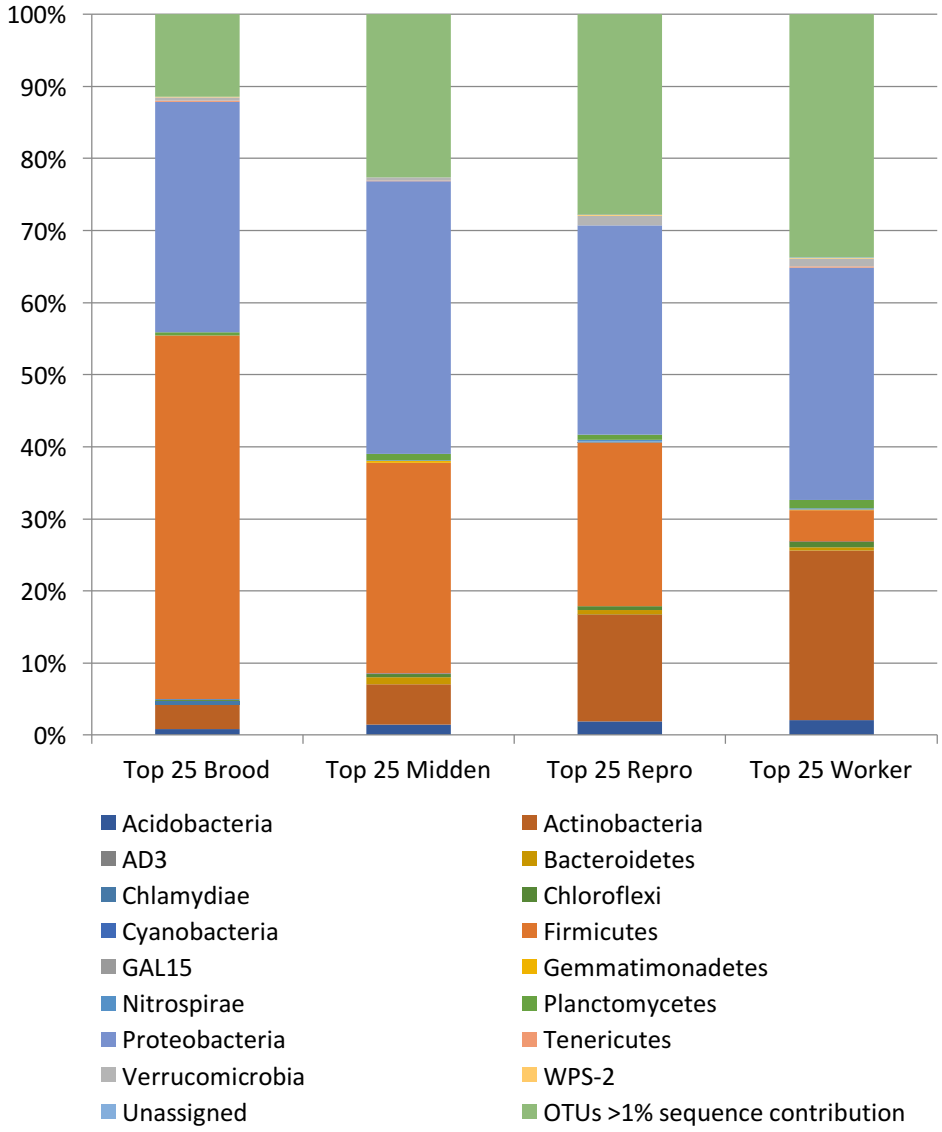

Figure 2.12. Top 25 OTUs Summary. Summary of cumulative phyla distribution for the Top 25 OTUs within colony x functional category factor groupings, based on 97% similarity OTU assignment and illustrated by functional category for comparison.

Top 25 OTUs

To assess the variability of dominant taxa of *S. invicta* bacterial communities, the 25 most numerous taxa, based on 97% sequence similarity of highest taxonomic level, were combined by colony, functional category sample replicates (Figure 2.12).

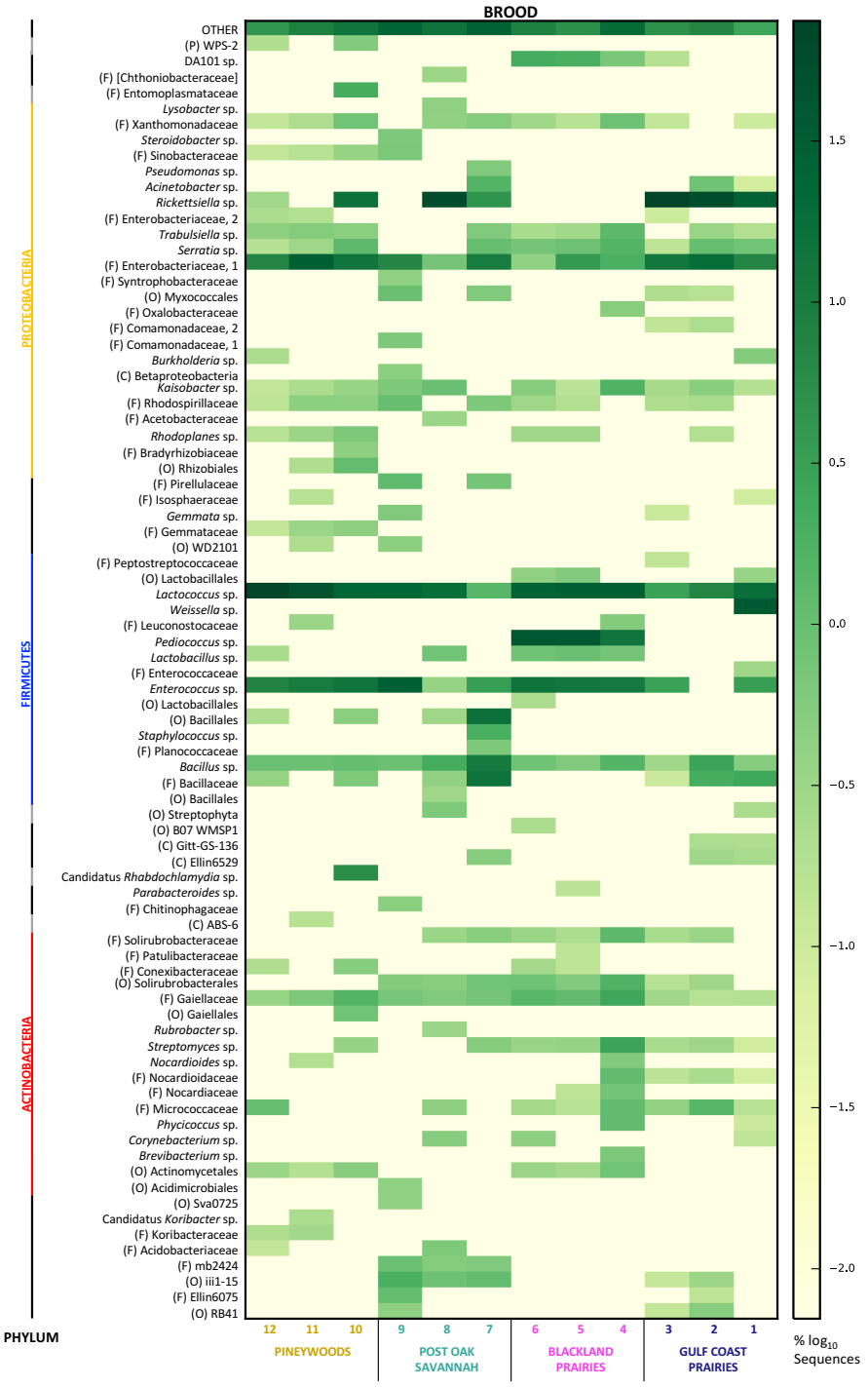

Figure 2.13. Brood Top 25 OTUs Heat Map. Top 25 OTUs heat map, with OTUs identified to the most specific taxa-level for the brood functional category 16S rRNA gene sequenced samples arranged by colonies. OTU rows are grouped by phylum, outlining the 3 most dominant phyla: Actinobacteria, Firmicutes, and Proteobacteria.

Figure 2.14. Midden Top 25 OTUs Heat Map. Top 25 OTUs heat map, with OTUs identified to the most specific taxa-level for the midden functional category 16S rRNA gene sequenced samples arranged by colonies. OTU rows are grouped by phylum, outlining the 3 most dominant phyla: Actinobacteria, Firmicutes, and Proteobacteria.

These top 25 OTUs were used to compare relative abundance within the ecoregion colonies, attributing to the taxa found in a particular functional category within a specific colony. The comparisons are best depicted in the Top 25 OTUs heat maps (Figures 2.13-16). The top OTU, of highest abundance, for brood in all colonies contributed 15.8-74.6% of the sequences for the brood functional category of the colony; for midden, the top OTU contributed 4.9-78.7% sequences; for reproductives, the top OTU contributed 4.2-95.6% sequences; and for workers, the top OTUs contributed 8.7-77.5% sequences for each sampled colony. The top 25 OTUs (rank 25) for brood in all colonies contributed 0.08-0.50% of the sequences for the brood functional category of the colony; for midden, the top 25 OTUs contributed 0.06-0.99% sequences; for reproductives, the top OTU contributed 0.02-1.01% sequences; and for workers, the top 25 OTUs contributed 0.1-0.95% sequences for each sampled colony. Based on these percentages, any OTU ranking higher than the 25th position in abundance of sequences for a colony functional category, contributed to less than 1.01% of that community's sequences. The overall bacterial community accounted by the top 25 OTUs differed across colony functional categories, with brood Top 25 OTUs attributing 75.3-97.4% of all sequences for the functional category sample conglomerations within a given colony. Midden, reproductive, and worker attributions had a wider range, 48.6-98.1%, 43-99.1%, and 53.9-95.7%, respectively. Figure 2.12 illustrates the phylum distribution of the top 25 OTUs when consolidated into functional categories, where the "OTUs with >1% sequence contribution" series describes the OTUs with lower sequence abundances ranking greater than the 25th OTU position and thus contributing to approximately less than 1% of the

Figure 2.15. Reproductive Top 25 OTUs Heat Map. Top 25 OTUs heat map, with OTUs identified to the most specific taxa-level for the reproductive functional category 16S rRNA gene sequenced samples arranged by colonies. OTU rows are grouped by phylum, outlining the 3 most dominant phyla: Actinobacteria, Firmicutes, and Proteobacteria.

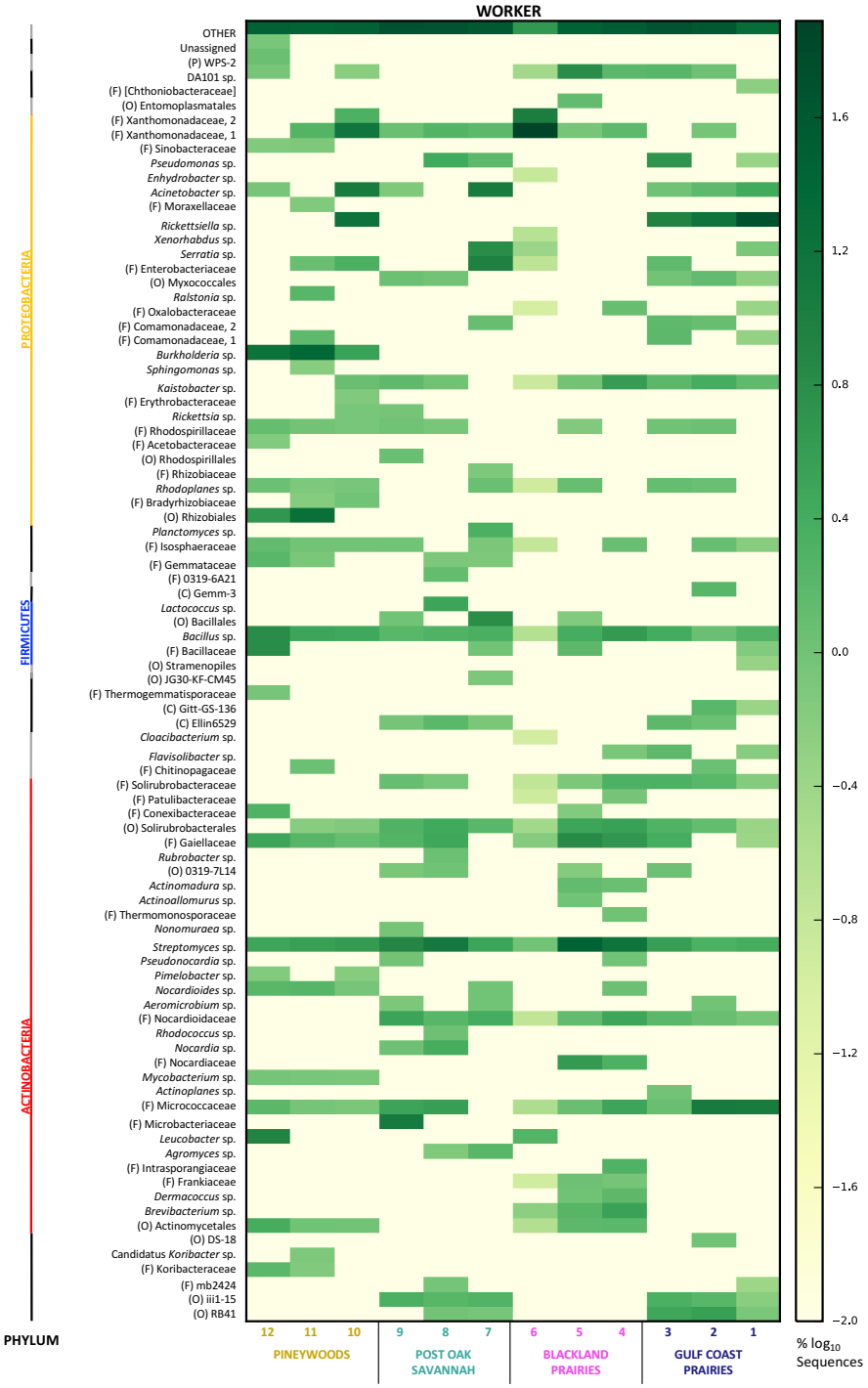

Figure 2.16. Worker Top 25 OTUs Heat Map. Top 25 OTUs heat map, with OTUs identified to the most specific taxa-level for the worker functional category 16S rRNA gene sequenced samples arranged by colonies. OTU rows are grouped by phylum, outlining the 3 most dominant phyla: Actinobacteria, Firmicutes, and Proteobacteria.

sequences within the colony functional category. The number of OTUs making up this series varies across colonies, but the greater the series contribution the higher likelihood of more numerous low-sequence-abundance OTUs making up the series. In essence, the worker functional category has the largest percentage of 33.75% making up the "OTUs with >1% sequence contribution" series, versus brood at 11.47%. The worker category has at least three times more OTUs contributing to this percentage assuming each OTU is contributing at most 1% sequences; if more OTUs are contributing even less than 1% for both categories the multiplicity factor would change.

Actinobacteria, Firmicutes, and Proteobacteria were the most abundant phyla across all functional categories. The most abundant classes within these phyla were Actinobacteria, Bacilli, and Gamma-Proteobacteria. The genera forming the makeup of these abundant classes were used to compare presence in the functional categories (Figures 2.17-19). Bacilli genera made up more of the brood bacterial community, with *Lactococcus* sp. accounting for 25% of the brood bacterial community and a similar abundance not present in midden, reproductives or worker categories (Figure 2.17). The *Lactococcus* genus included unidentified species and *L. garvieae. Pediococcus* sp. and *Enterococcus* sp. made up 7.6% and 9.1% of the brood bacterial community, made up by unidentified species and *P. acidilactici, E. casseliflavus, and E. cecorum.* The *Bacillus* genus was also highly abundant, contributing to 11.8% of the bacterial community of the reproductive caste. The genus included some unidentified species and more prominent species *B. cereus, B. flexus,* and *B. humi,* found in all colony reproductives (Figure 2.17). Other *Bacillus* species included: *B. coagulans, B. firmus, B. foraminis, B. horikoshii, B.*

horti, B. muralis, B. selenatarsenatis, and *B. thermoamylovorans.* More genera, families, and orders comprising the Firmicutes can be noted in the top 25 OTUs functional category heat maps (Figures 2.13-16).

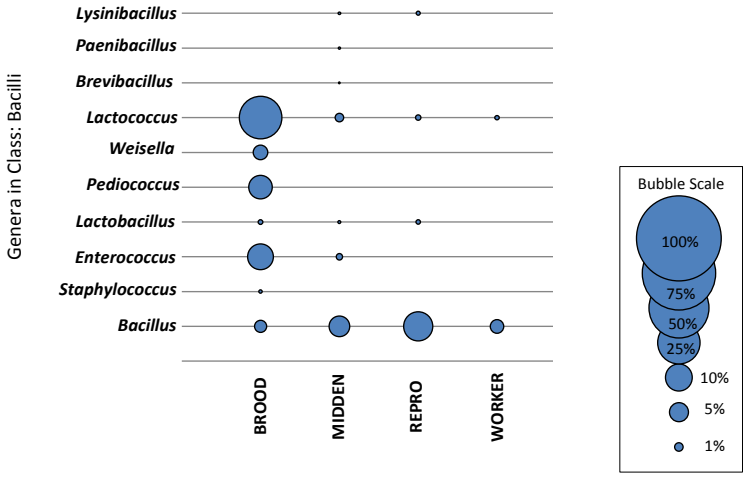

Figure 2.17. Genera in Class Bacilli. Abundance of dominant genera in Top 25 OTUs for class Bacilli in *S. invicta* based on 97% similarity OTU assignment.

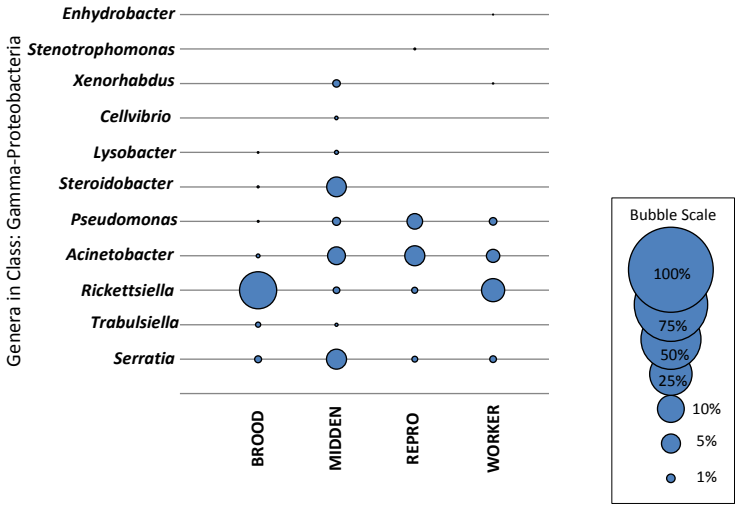

Figure 2.18. Genera in Class Gamma-Proteobacteria. Abundance of dominant genera in Top 25 OTUs for class Gamma-Proteobacteria in *S. invicta* based on 97% similarity OTU assignment.

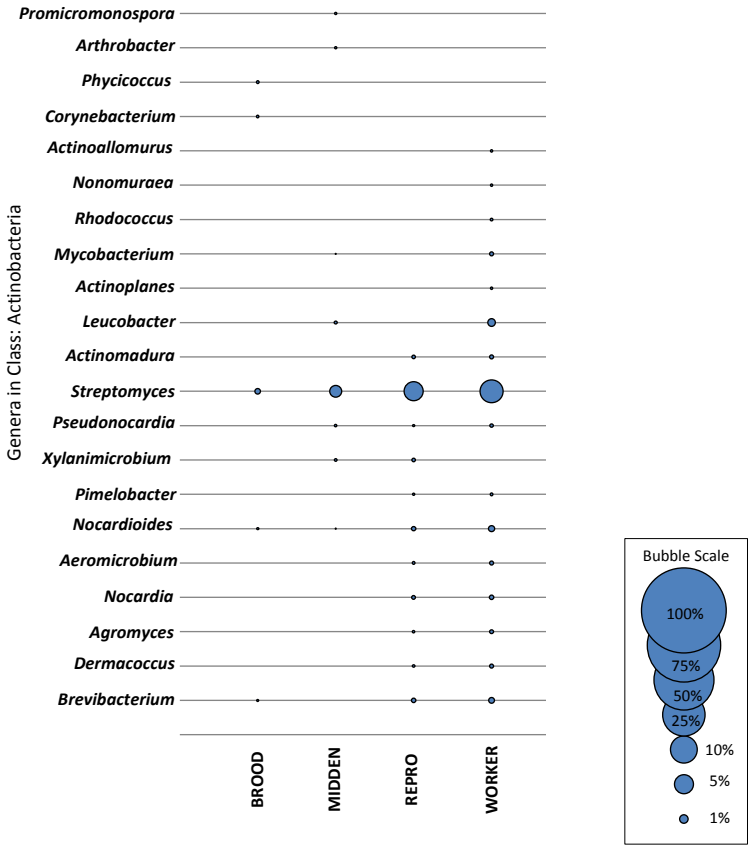

Figure 2.19. Genera in Class Actinobacteria. Abundance of dominant genera in Top 25 OTUs for class Actinobacteria in *S. invicta* based on 97% similarity OTU assignment.

Class Gamma-Proteobacteria made up approximately 30% of the brood bacterial community, *Rickettsiella* sp. contributed 19.4%. of the total sequences, most in Colony 12 of the Pineywoods ecoregion (Figure 2.18). *Rickettsiella* sp. was also abundant in worker bacterial communities, at a lower abundance of 7.49%, mostly associated with the colonies in the Gulf Coast Prairie (Figure 2.18) and Colony 10 of the Pineywoods ecoregions. Most of the Gamma-Proteobacteria genera were found in the midden samples, including *Serratia* sp. (5.5%), *Acinetobacter* sp. (4.4%), and *Steroidobacter* sp. (5.4%). The reproductive samples had the greatest abundance of *Acinetobacter* sp. (5.5%) and

Pseudomonas sp. (3.41%) presence. Genus composition varied with some unknown species, but identifiable species included: *Serratia marcescens, A. johnsonii, A. rhizosphaerae, Steroidobacter acidaminiphila, Pseudomonas nitroreducens, P. pseudoalcaligenes, P. stutzeri, P. umsongensis, P. veronii, and P. viridiflava.*

The top 25 genera in Class Actinobacteria were more diverse than class Bacilli and Gamma-Proteobacteria, accounting for a lower abundance of sequences for all functional categories but made up by more identifiable genera (Figure 2.19). The *Streptomyces* genus contributed to some unidentified species, those identified to species included: *S. aculeolatus, S. mashuensis, S. mirabilis,* and *S. radiopugnans*. Other identified species included: *Brevibacterium aureum, B. paucivorans, Corynebacterium kroppenstedtii, Arthrobacter woluwensis, Mycobacterium arupense, M. celatum, Rhodococcus fascians, R. ruber,* and *Pseudonocardia halophobica.*

Discussion

The key result to this study is that the bacterial community of *S. invicta* varied predominantly based on two factors, namely, ecoregion location and functional category grouping. Furthermore, the unweighted versus weighted UniFrac data determined the differentiation pattern of a given factor on beta diversity. The unweighted data accounting for OTUs presence/absence was more significantly associated according to ecoregion, these results shown via both PERMANOVA and PCoA analyses. Similarly, there was consensus in analyses results comparing weighted data accounting for sample OTUs and

relative abundance. Weighted UniFrac data was more significantly related by functional grouping. This means that, for example, brood bacterial communities were more similar in composition and abundance to other brood bacterial communities, regardless of colony and ecoregion. The same can be said about the other functional category groupings, midden, reproductives, and workers. The functional category with the greatest bacterial community alpha diversity depended on the ecoregion, with reproductive samples representing the greatest diversity in the Blackland Prairies and Post Oak Savannah, midden in the Gulf Coast Prairies, and workers in the Pineywoods. The degree of difference in the overall bacterial community composition of *S. invicta* was dependent upon the taxonomic level, making taxonomic resolution essential when comparing OTU relative abundance in samples. At the phylum level, most OTUs belonged to phyla Actinobacteria, Firmicutes, and Proteobacteria, with increasing abundance of Actinobacteria across functional categories where worker samples had the greatest abundance at 32.85% versus brood samples with 5.89%. The opposite was true for Firmicutes with worker samples composed of 6.31% abundance versus brood samples 51.40% of OTU abundance. The Proteobacteria phylum exhibited a similar range across all functional categories. OTU differences were detected when comparing the most abundant OTUs, i.e. top 25 OTUs, in the samples, grouped by functional categories within colonies. Nearly all of the OTUs making up the bacterial diversity of the brood functional category were accounted for in the top 25 most abundant OTU sequences. The brood functional category also always had the lowest alpha diversity by ecoregion, with a comparable amount of sequences belonging to only a relatively few OTUs.

These conclusions are derived from the analyses of PCR-generated 16S rRNA gene hypervariable amplicons, a next generation sequencing methodology subject to its own limitations. Caveats associated with generating data may arise from incomplete extraction of DNA or universal primer adherence, from bias against Gram-positive bacteria for example. Furthermore, interpretation of data and results can give rise to difficulties when comparing samples with sequencing depth variation, sparsity, nonconformity of read counts, and unbalanced study designs (Thorsen *et al.*, 2016). To minimize possible errors, DNA extractions were designed and tested to obtain Gram-positive bacteria sequences, such as those residing in phyla Firmicutes and Actinobacteria. Theorized sequencing depth, based on sequencing platform performance, was compared to the true post-hoc sequencing depth were the average number of expected reads were reached. Sequencing depth was sufficient for most of the sequenced samples; only 8 of 139 samples used in analyses had less than 10,000 sequences/sample. Rarefaction curves indicated sufficient sequencing for samples grouped by ecoregion, colony, and functional category. Further still, OTU count matrices used for comparisons in relative abundance excluded singletons and doubletons, and the comparison of only top 25 taxa inherently did not contain inflation produced by OTUs with minimal abundance. In terms of beta diversity comparisons, data was not transformed in any way to avoid skewed abundances and Euclidean distances were not used to avoid "double zero" problem in ecological distance measurements used to produce indices (Legendre and Legendre, 2012; Paulson *et al.*, 2013). Nevertheless, limitations remain, namely: undetected 16S sequences belonging to bacteria yet to be identified or included in the OTU sequence database and

greater differentiation beyond the genus taxonomic-level using the 16S rRNA gene v4 hypervariable region, with limited within-species separation and thus detection.

Acknowledging the previously stated conditions for sequence data interpretation, this study revealed patterns of diversity and identified taxa found in previously studied systems. First, the clustering of samples, based on unweighted UniFrac distance matrices, by ecoregion source illustrates the relatedness of *S. invicta* bacterial communities, is likely due to a founding effect. The presence of the environmental bacterial communities present in the varying environments will dictate the possible bacterial variety to be encountered by the colonies in a particular region, and thus the founding bacteria inhabiting *S. invicta*. Bacterial diversity is likely to vary across spatial scales, here ecoregions, due to variances in factors that form more or less conducive environments for bacteria survival, in the words of Dutch microbiologist Martinus Wilhelm Beijerinck "everything is everywhere, but the environment selects" (O'Malley, 2008). *S. invicta* colonies inhabit soil environments, complex matrices with high species level bacterial diversity that are phylogenetically similar (Lozupone and Knight, 2007). Strong predictive patterns of environmental bacterial diversity were likely to reflect the taxa diversity found on *S. invicta* colonies. A higher number of OTUs were found in the Blackland Prairies and Post Oak Savannah ecoregions, ecoregions with lower acidity and mixture-based soil, not purely sand or clay. Additionally, soil texture plays an important role in the ability of soils to hold nutrients, retain water, and allow for air flow (Pepper *et al.*, 2015). Soils with a mixture of sand, silt, or clay offer a more favorable environment for bacteria to grow because there is a higher number of retained nutrients and moisture, making the microbial

communities of these soils likely to have a greater bacterial abundance (Pepper *et al.*, 2015). The functional categories can also be considered as part of a spatial scale characterized as microenvironments existing within the ecoregion, the colony, and further the particular organism. Space in this case is described by the functional isolation of bacteria attributed to the organism responsible for carrying out a specific role or function for the colony. Organisms with different bacterial communities can be shaped by physiology, diet, and behavior, all factors attributable to the holometabolous insects and the polyphenism of social insects like *S. invicta.*

In the case of brood, the community structure relates to both digestive roles and immobile state in the more secluded brood cavities of the *S. invicta* subterranean colony. The bacterial community of brood were very predictable at high-taxonomic levels, with class Bacilli and Gamma-Proteobacteria dominating much of the consistency in this functional group, particularly lactic acid bacteria genera *Lactococcus, Enterococcus, Pediococcus,* and *Weissella. Pediococcus* and *Weisella* had not previously been detected in *S. invicta* larva. These bacteria are far from unique to digestive functions of *S. invicta*; however, and are likely using anaerobic respiration to aid digestion as they do in other animals. Lactic acid bacteria have long been described as part of the gut flora of many species, including other insects (Cariveau *et al.*, 2014; Huang and Zhang, 2013), fish (Ringø and Gatesoupe, 1998) and humans (Heilig *et al.*, 2002). *Ricketsiella* was another genus never previously detected in *S. invicta* larva. These bacteria either develop as a facultative pathogen or interact with facultative symbionts involved in digestion, as is the case with aphid symbiont *Hamiltonella* (Tsuchida *et al.*, 2014). Further studies should

explore the functional activities dominant bacteria are partaking in as part of the brood bacterial community. Of particular interest, would be *Lactococcus,* because it dominated the brood community across all colonies and regions. Strains of *Lactococcus* are better metabolizers of complex sugars like fructose, which dominated the composition of honey, for example (Barriere *et al.*, 2005). It is likely that similar strains have long been established and associated with the diet of *S. invicta,* where workers more actively forage for high carbohydrate resources, exploiting those with complex and simple sugars alike (Howard and Tschinkel, 1981).

Many *Streptomyces* sequences where accounted for in worker samples. Of the strains identified to species, all produce streptomycin and other antibacterial metabolites. *Streptomyces* are bacteria found universally in soil, giving soil the "earthy" smell. Because workers are more active and constantly interacting with their soil environment, they are likely to encounter many bacterial taxa characteristic of soil bacterial communities. However, *Streptomyces* was still more dominantly present in workers and reproductives, with brood having less, and further studies exploring this prevalence pattern could provide insight on worker adaptations to deal with harsher, exposure to environmental pathogens, for example.

S. invicta serves as a model of an invasive eusocial species occurring throughout a wide range of diverse ecological regions. We found that diversity indices of the bacteria communities differed between ecoregions, but more significantly across functional groupings. The bacterial community structure and species of *S. invicta* functional categories are likely shaped by their contributions to the colony and is likely to be less

variable across all colonies, regardless of origin. So regardless of the foundational bacterial community established in an ecoregion, specific bacterial taxa will characterize the functional categories of any colony found in any ecoregion. These same bacterial taxa likely to facilitate the functional role in the life history of the fire ant, from brood to adult castes, which in part lead to the success of these species in the disturbed habitats.

In addition, *Proteus* bacteria was found present in all but three colonies, namely, colony 2, 3 and 10, in all colony functional categories, and accounted for in all ecoregions.

CHAPTER III

SOLENOPSIS INVICTA BUREN NESTMATE INTERACTIONS WITHIN SUBCOLONIES IN THE PRESENCE OF SWARMING BACTERIA *PROTEUS MIRABILIS*

Introduction

Nestmate recognition in ants exists because of the cost and benefits that come with cooperation and shared activities, the investment of time and energy for the good of all in the altruistic ant colony. Excluding nestmates that are not part of the colony limits the benefits to only those ants working for the good of the colony, the individuals sharing genetic makeup. This shared genetic makeup is quantified as the degree of relatedness, and is used to define the threshold necessary for the altruistic interaction to occur amongst kin. As described by the inclusive fitness theory, individuals must share at least 50% of their genetic makeup for a kin selection strategy to drive altruistic relationships in species groups (Hamilton, 1964a). Social hymenoptera have been used over and over again to study the dynamics of such a theory, using haplodiploidy sex determination and the resulting gene distribution among female and male progeny (Hamilton, 1964b). Hamilton's law is satisfied when social hymenoptera colonies in which the queen is the only reproductive member produce sterile female offspring that share three quarters of their genetic makeup. The relatedness between individuals is greater than the cost to benefit ratio of an individual's behavior, increasing the fitness of the colony as a whole in

expense of the decreased fitness of the individual actor. This inclusive fitness theory (sometimes termed kin selection theory) is a much-discussed theory with various contentions on how it is used and how the factors of altruism are quantified, namely the cost of the bearer, the benefit to the recipient, and the relatedness of both (Nowak *et al.*, 2010). In addition to the genetic factors driving nestmate interactions, ecological factors impacting eusociality and altruistic behavior should also be considered when studying social organisms.

In ants, like other social hymenoptera, nestmate recognition and foraging ant behaviors are driven by the chemical communication of individual ants within the colony (Vander Meer *et al.*, 1988). Notable chemicals include pheromones and cuticular hydrocarbons, which alongside sensory systems and receptors found on the ant, constitute evolutionary signals established to convey particular messages (Vander Meer and Lofgren, 1989). These interpretive signals then provide direction for the behavior of *S. invicta*, affecting not only the individual ant, but also the function of the colony the individual belongs to. Take for example the aggressive behavior when protecting the colony from intruders. For aggression to ensue, contact with any part of the intruder's body or foreign colony individual usually takes place, implying that the factor driving the aggressive behavior is likely a non-volatile chemical cue distributed throughout the ant body (Tschinkel, 2006). A widely accepted hypothesis in ant recognition studies is that the colony exudes a characteristic odor produced by the ants, a kind of odor profile established by both heritable and environmental components (Hölldobler and Wilson, 1990; Obin, 1986).

Colony odor is thought to be shared by all colony nestmates via constant interaction and close proximity. Although the exact sensory mechanism and physiology of nestmate recognition is not clearly understood, nestmate interactions based on nestmate recognition of chemical cues are dependent on the colony odor formation. Cuticular hydrocarbon compounds and contents in the postpharyngeal glands of various ant species are known as contributors to the synthesis of colony odor, according to several studies where these components affected nestmate interaction behavior (Hefetz *et al.*, 1996; Lahav *et al.*, 1999; Soroker *et al.*, 1994). Cuticular hydrocarbons are compounds found universally on the surface of all insects to help prevent cuticular water loss (Blomquist and Dillworth, 1985).

The purpose of postpharyngeal gland contents is not clearly known, but they do coincide with some of the makeup of major cuticular hydrocarbons found on *S. invicta* queens (Thompson *et al.*, 1981). These postpharyngeal gland contents were compared between queens belonging to different colonies, and although they contained chemically different cuticular hydrocarbons, no discernible effect on colony odor effect was determined (Nelson *et al.*, 1980; Thompson *et al.*, 1981). Further, *S. invicta* studies where cuticular hydrocarbons were extracted from different colonies and mortality due to aggression was compared between colonies, do not support a strong role in nestmate recognition via these chemicals (Tschinkel, 2006).

Obin (1986) noted that *S. invicta* colonies that have been maintained in a laboratory for longer periods of time are generally less aggressive than colonies newly collected from the field environment. In these experiments, aggression between ants from different lab-

maintained colonies caused lower mortalities than lab-maintained versus field colony individuals. Obin (1986) discussed that this may be due to environmental components introducing greater chemical differences between non-nestmates, and thus a greater aggression towards a non-nestmate intruder. Diet as an environmental factor contributing to nestmate recognition was then considered in experiments comparing moth-fed and cockroach-fed subcolonies from a single lab colony (Obin and Vander Meer, 1989). After several months of separation and consuming different diets, the introduction of former nestmates increased the aggression between the two diet-differing nestmate subcolonies. In another study comparing *S. invicta* nestmate interactions, subcolonies housed in their colony soil were introduced in the following combinations: monogyne to monogyne, polygyne to polygyne, monogyne to polygyne, and *S. richteri* Forel to *S. invicta* subcolonies (Morel *et al.*, 1990). Among all introduced interactions, ant residents from monogyne colonies reacted with the highest levels of aggression towards other monogyne non-nestmates and polygyne ants, while any heterospecific interaction resulted in high aggression. This study described polygyne ants as less aggressive towards conspecific non-nestmates, likely due to the movement of ants between nearby mounds. A more recent study conducted on the monogyne and polygyne *S. invicta*, and *S. geminata* (Fabricius), monogyne *S. invicta* also had the highest levels of aggression of all ants (Lai *et al.*, 2015). A laboratory study using monogyne and polygyne *S. invicta* fed some colonies different diets over a long period of time and then introduced polygyne intruders to resident monogyne ants (Obin *et al.*, 1993). Attacks and mortality more commonly occurred when the interacting ants had been fed different diets, even monogyne versus polygyne

introductions fed the same diet showed decreased aggression. These findings depict a variety of environmental factors, namely soil environment and diet breadth, that likely contribute to the cues mediating *S. invicta* nestmate or non-nestmate recognition (Tschinkel, 2006). More importantly, these experiments set a precedent for *S. invicta* nestmate interaction studies that deviate from the hypotheses which limited the dependence of dynamic ant interactions to only heritable, or genetic, cues like cuticular hydrocarbons.

Heritable cues proved to have little impact on nestmate recognition and behavior when environmental factors like diet were taken into account. Still, there are many environmental factors that could likely play a role in *S. invicta* nestmate recognition. Particularly, there is an abundance of players in the environment continuously contributing to various dynamic chemical processes. Further, there are copious molecules being used as nutrients and catalyzers of biogeochemical processes carried out by the abundant diversity of microorganisms found in soil. The microbial fauna plays the role of decomposers, recycling nutrients in the soil and plants, and often times becoming closely associated with other organisms sharing the same environment. The second chapter of this dissertation was dedicated to the comparison of bacterial communities associated with *S. invicta* colonies across various ecoregions. Worker caste colony members had the greatest diversity of bacteria found on them, on both the external and internal ant body. Based on the pronounced abundance of bacteria on *S. invicta,* and in the *S. invicta* environment, namely, the soil matrix that readily promotes the propagation of many bacterial species,

bacteria are likely to contribute to the environmental factors or chemicals associated with *S. invicta* nestmate profiles.

Proteus mirabilis is a swarming quorum-sensing bacterium known for producing by-products, such as volatile organic compounds (VOCs), that serve various purposes in the biology of other organisms. For example, *P. mirabilis* can be found in decomposing carcasses, serving as ephemeral nutritional resources to many flies looking to consume protein for egg production and then lay eggs for successful development through their larval stages (Ma *et al.*, 2012). *P. mirabilis* produces chemicals like ammonia, phenol and lactic acid which not only induce the swarming capability of the bacterial cells but also attract flies. These compounds are not only a signal to swarming bacteria, but a cue recognized by blow flies and used to identify resources for reproduction. *P. mirabilis* has thus been recognized as a bacterium influencing insect behavior and interactions. *P. mirabilis* was also found in several *S. invicta* colonies from the bacterial community studies conducted as a part of this dissertation. For these reasons, the experiments described in this chapter used *P. mirabilis* in *S. invicta* lab colonies to determine whether the external presence of this bacteria affected the interaction between nestmate and non-nestmate ants. Experiments were developed to represent realistic means of bacterial exposure in the natural environment of *S. invicta.*

The purpose of this study was to determine if an increased presence versus an absence of *P. mirabilis* can be differentiated by nestmates in *S. invicta* laboratory colonies to investigate how differentiating bacterial communities impact nestmate interactions. Differentiating bacteria presence served as a proxy for bacterial community differences

established by the increased abundance of a different, dominant bacteria. Essentially, *P. mirabilis* will represent a dominant bacterial community component and environmental factor that is not found as abundantly present in the environment and bodies of the host colony.

Materials and Methods

Colony Maintenance

S. invicta colonies were collected during early mornings in November of 2016. All *S. invicta* colonies were polygyne colonies with multiple queens. *S. invicta* ant colony mounds were excavated from pecan (*Carya* species) cultivars at the USDA-ARS-SPARC Crop Germplasm Research laboratory (Somerville, Texas). Each colony mounds were placed in different 18.9-liter buckets and returned to the Rollins Urban and Structural Entomology Facility at Texas A&M University (College Station, Texas). Ant colonies were separated from the mound soil using the drip-method (Banks *et al.*, 1981) and placed in sweater boxes (39.7 cm x 33.3 cm x 17.1 cm) (The Container Store® Inc. CA, USA) inner-coated with Insect-a-slip (BioQuip Products, Inc. CA, USA) to prevent their escape. The queen and brood were kept in a formicary constructed from a 150 x 50 mm petri dish (Thermo Fisher Scientific MA, USA) half filled with Castone® Dental Stone (Dentsply PA, USA) to retain moisture, with two holes made on the petri dish cover to allow entrance and exit of worker ants. Ant colonies were provided water and 15% honey water solution ad libitum, apple (0.5-g) and 4-6 medium crickets (PremiumCrickets.com GA, USA)

every other day. Ant colonies were consistently fed until before the initiation of the experiment.

Bacterial Treatment Preparation

A bacterial glycerol stock of wild-type *P. mirabilis* described in Tomberlin *et al.* (2012) was maintained by a co-author at the United States Department of Agriculture (USDA-ARS-SPARC) Food and Feed Safety Research laboratory (College Station, Texas) and used for experiments. The Office of Research Compliance and Biosafety at Texas A&M University did not require a permit because research was conducted on an arthropod species. *P. mirabilis* concentration for treating soil environment and exposure to *S. invicta* workers and brood were prepared using ten-fold serial dilutions in phosphate-buffered saline (PBS). The starting inoculum concentration of wild type *P. mirabilis* used in all experiments was 1 x 10^9 CFU (Colony Forming Units) /mL, as an ecologically relevant high concentration an insect might encounter in the environment. Tryptic soy agar (TSA) (Bio-Link Scientific TX, USA) treatment plates (100 x 15 mm petri dish) and two-times Charcoal agar (2xCHR) (Bio-Link Scientific TX, USA) count plates (100 x 15 mm petri dish) were inoculated to count bacterial colonies and determine bacterial concentrations of the introduced ants before introduction to the host colony, and host colony ants after the 7 h interaction observation period.

Determination of Bacterial Load on Soil, Workers, and Brood

In order to determine the necessary time ant workers and brood needed to be exposed in order to be inoculated with bacterial cells from the *P. mirabilis* soil environment, a preliminary study determining the load of bacteria concentration transferred from soil environment to worker and brood bodies was conducted. Soil from a location different than that of the ant colonies was collected, this was outside the Rollins Urban and Structural Entomology Facility at Texas A&M University (College Station, TX, USA). This soil was novel to all the ant colonies to prevent any bias or predetermined conditioning in association to the introduced or host soil environment. Over 50 g of soil were collected and autoclaved three times at 121°C for 30 minutes to kill all present microorganisms, specially bacteria in the soil (Kästner *et al.*, 1998). The soil was dried in a dehydrator for 24 hours between each autoclaving. Soil was then re-weighed and 33 g of sterile soil were collected to be treated with bacteria. All experimental treatments and assays were conducted at the USDA-ARS-SPARC Food and Feed Safety Research laboratory. The sterile soil was thoroughly mixed with 3.3. mL of 10^8 CFU/mL *P. mirabilis* and 1.2 mL of sterile water to achieve the ideal water holding capacity (WHC) of ant colony soil, predetermined to be at approximately 60%. *P. mirabilis* treated soil was then weighed out into 11 g parts, and partitions separately placed into a petri dish to serve as the formicary housing an *S. invicta* queen, 500 workers, and 100 brood. The formicaries were placed inside a 946-mL wide mouth autoclaveable jar (Consolidated Plastics OH, USA) inner-coated with Insect-a-slip (BioQuip Products, Inc. CA, USA) to prevent ant escape. Three formicaries were prepared; each formicary would be used to expose ants for

24 hours and 48 hours. At the end of the 24-hour and 48-hour exposure periods, 100 workers, 20 brood, and 1 g of soil were collected from each formicary to determine *P. mirabilis* concentration load and persistence on each substrate. *S. invicta* workers were collected by allowing them to climb onto a disposable sterile plastic spatula (Thermo Fisher Scientific MA, USA) and using sterile soft forceps to them place them inside a 15-mL conical tube (Thermo Fisher Scientific MA, USA) filled with 5 mL of phosphate-buffered saline (PBS). *S. invicta* brood were collected using sterile soft forceps to pick up brood and place in 15-mL conical tube filled with 5 mL of PBS. Formicary soil was collected using sterile soft forceps and placing contents on a scale to obtain 1 g of soil without any ant workers or brood, and then placed in 15-mL conical tube filled with 5 mL of PBS. A total of 9 tubes were collected for each time exposure. The contents of each tube were homogenized separately, using a new autoclaved homogenizing tip for each to prevent cross-contamination between samples (Crippen *et al.*, 2012). Ten-fold serial dilutions were carried out for the contents of each homogenizing tube, and plated on two-times Charcoal agar (2xCHR) (Bio-Link Scientific TX, USA) count plates (100 x 15 mm petri dish) to obtain counts for determining bacterial concentrations. Bacterial nutritional enrichment incubations were carried out for the contents of the remaining homogenates, adding 5 mL of tryptic soy broth (TSB) to ensure the capture of *P. mirabilis* cell presence in the sampled substrates (Crippen *et al.*, 2012). Serial dilution inoculated plates and enrichments were placed incubated for 24 hours at 37°C to facilitate bacterial growth and obtain *P. mirabilis* colony counts the next day.

HOST colony	INTRODUCED workers/brood	Sterile Soil Treatment	Code
Colony 1	Colony 1	*P. mirabilis* on INTRO-1	N [H-/I+]
Colony 2	Colony 2	*P. mirabilis* on INTRO-2	N [H-/I+]
Colony 3	Colony 3	*P. mirabilis* on INTRO-3	N [H-/I+]
Colony 1	Colony 4	*P. mirabilis* on INTRO-4	nn [H-/I+]
Colony 2	Colony 5	*P. mirabilis* on INTRO-5	nn [H-/I+]
Colony 3	Colony 6	*P. mirabilis* on INTRO-6	nn [H-/I+]
Colony 1	Colony 4	*P. mirabilis* on HOST-1 and INTRO-4	nn [H+/I+]
Colony 2	Colony 5	*P. mirabilis* on HOST-2 and INTRO-5	nn [H+/I+]
Colony 3	Colony 6	*P. mirabilis* on HOST-3 and INTRO-6	nn [H+/I+]
Colony 1	Colony 1	No *P. mirabilis* on INTRO-1	N [H-/I-]
Colony 2	Colony 2	No *P. mirabilis* on INTRO-2	N [H-/I-]
Colony 3	Colony 3	No *P. mirabilis* on INTRO-3	N [H-/I-]
Colony 1	Colony 4	No *P. mirabilis* on INTRO-4	nn [H-/I-]
Colony 2	Colony 5	No *P. mirabilis* on INTRO-5	nn [H-/I-]
Colony 3	Colony 6	No *P. mirabilis* on INTRO-6	nn [H-/I-]

Table 3.1. Host-Introduced Ant Interaction Soil Treatments. Six different *S. invicta* colonies (colonies 1-6) were collected from the same field location and used as source colonies to populate subcolonies in interaction experiments. Various host colony vs. introduced ant combinations and *P. mirabilis* soil treatments were used. Identification codes associated with each treatment type are listed. N, nestmates (same source colony). nn, non-nestmates (different source colony). H, host colony. I, introduced ants. +, sterile soil environment in formicary treated with 10^8 CFU/mL of *P. mirabilis*. -, sterile soil environment in formicary not treated with any bacteria.

Host-Introduced Ant Interaction Experiments

Upon determining the soil exposure time necessary for transmission of *P. mirabilis* cells to ant and brood bodies, host-introduced ant interaction experiments were planned and executed. The host colony was composed of 500 workers, 100 brood and one queen. Queens were introduced to each experimental host colony because the presence of the *S. invicta* queen primer pheromone increases worker sensory differentiation between colony nestmates and non-nestmate conspecific intruders (Obin and Vander Meer, 1989; Vander Meer and Alonso, 2002). This additional queen element is important when considering factors determining nestmate interaction of *S. invicta* in their natural environments.

Introduced ants would need to be identified upon introduction to the host colony so they were marked using Testor's paint (Fluorescent Sky Blue) (The Testor Corporation, Rockfor, IL, USA) (Bhatkar *et al.*, 1991). A disposable plastic atomizer was used to spray paint onto ants from a 45-cm distance; paint was allowed to dry for 20 minutes before ants were placed into their introduction treatment formicaries. Introduced ant formicaries consisted of 100 ants and 20 brood, to be transferred after the 24-hour *P. mirabilis* soil exposure treatment. Soil treatment and ant transfer was carried out as described before. Table 3.1 lists the five different treatments used in this study, based mainly on presence or absence of *P. mirabilis* in formicary soil of introduced ants, and one treatment with both host colony and introduced ants exposed to *P. mirabilis.* Three substrate replicates were collected prior to introduction to determine available *P. mirabilis* concentration load on introduced ants at the beginning of the experiment. Host and introduced ants were observed for a 7-hour period, taking observations at different time intervals to assess host and introduced ant engagement behavior with each other. Host colony chambers housing the formicary and ant activity were video recorded for 30-60 seconds at 1 minute, 10 minutes, 30 minutes, 60 minutes (1 hour), 120 minutes (2 hours), 240 minutes (4 hours) and 420 minutes (7 hours). Video recordings were later reviewed to assess engagement activities between host and introduced ants, recording the number of host colony ants engaging introduced ants and assigning an aggression score to the host colony ants at each observation time point. *S. invicta* aggression scores were defined based on the work of Morel *et al.* (1990), where she provided a range of 10 scores (0-9) describing *S. invicta* interactions with newly introduced ants (Table 3.2). In this study, the use of score 9 was

excluded because the first initial interaction of host ants was not monitored, as observations began after 1 minute of introduction. At the end of the 7-hour observation period, again, three substrate replicates were collected to determine the amount of *P. mirabilis* transferred to host colony members interacting with *P. mirabilis* introduced ants. Substrate samples, colony counts and enrichments were performed, following steps as previously described.

Score	Behavior
8	Opponent surrounded and "held" in mandibles, appendages pulled or bitten off, eventual stinging
7	Opponent held (as in 8) but released, biting, abdomen-curling (stinging position) but not stinging
6	As in 7, but no abdomen curling
5	Alarm (running, abdomen elevation and vibration) and recruitment
4	Mandible gaping, rapid antennation, "sliding" (maintaining a lateral orientation to and slowly circling opponent).
3	Rapid antennation with antennae extended for more than 2 seconds
2	Antennation for less than 2 seconds. If mobile, opponent is followed slowly for several centimeters; if opponent stationary, worker stops.
1	As in 2, but opponent does not induce following or stop.
0	No interactive behavior displayed.

Table 3.2. Behavioral Aggression Scores. Adapted from Morel *et al.* (1990). Behavioral units and aggression scores used to assess nestmate recognition as described by Morel *et al.* (1990), with aggression score 9 removed due to ant interaction observations beginning after 1 minute and not at the initial moment of introduction to the host colony.

Experimental Design and Statistical Analysis

A total of six different field colonies were used to perform host-interaction experiments. Colonies 1-3 were consistently used in all experimental treatment replicates to provide each trial with the same repeated *S. invicta* colony-associated variances. Nestmate treatment trials introduced ants from colonies 1-3, with respective subcolonies belonging to the same source colony. Non-nestmate trials introduced ants from colonies 4-6, consistently using colony combinations 1 vs. 4, 2 vs. 5 and 3 vs. 6. After *S. invicta* host-introduced subcolony interaction experiments, and behavior observations were assessed prior to reporting treatment label in order to conduct studies blindly and avoid confirmation bias, as recommended by (van Wilgenburg and Elgar, 2013). ANOVA and Tukey HSD tests for multiple comparisons statistics were then performed to determine significant ($P < 0.05$) difference in aggression scores across treatments and across colonies. All statistical testing was performed in R version 3.4.0 (R Development Core Team, 2016).

Results

Bacterial Load Concentrations from Soil Exposure

Sterile soil retained the degree of *P. mirabilis* concentration used to initially inoculate it, going from an inoculation concentration of 8.10×10^8 CFU/mL to 4.78×10^8 CFU/mL after 24 hours, and 4.92×10^8 CFU/mL after 48 hours of inoculation. This was a decrease in 50 % of the total inoculation of CFU/mL, but maintained overall concentration in the

hundred million counts. *S. invicta* worker and brood substrates became inoculated with *P. mirabilis* present in the soil, via exposure to the bacteria treated soil environment in the formicary. The concentration of bacteria found on both worker and brood was much higher by 100-fold CFU/mL for ants exposed for 48 hours, versus 24 hours (Table 3.3). The 24-hour exposure period was ultimately selected to proceed with host-introduction experiments as this was a sufficient time period for ants to become inoculated with *P. mirabilis*.

Initial *P. mirabilis* Inoculum Concentration (CFU/mL)		
8.10×10^8		
***P. mirabilis* Load Mediums**	**Post 24-hour Exposure *P. mirabilis* Concentration (CFU/mL)**	**Post 48-hour Exposure *P. mirabilis* Concentration (CFU/mL)**
Soil (1 g, n=3)	4.78×10^8	4.92×10^8
Worker (100, n=3)	4.72×10^3	1.34×10^5
Brood (20, n=3)	2.96×10^3	5.01×10^5

Table 3.3. Initial Determination of Exposure Time and Bacterial Load. Initial determination of exposure time and bacterial load of *P. mirabilis* on substrates exposed to sterile soil *P. mirabilis* environment for 24 hours and 48 hours. Substrates include sterile soil, *S. invicta* worker ants and brood.

Aggression Scores

Aggression scores assigned to the host colony at every observation time point were collected and mean average aggression scores over time determined for each host-introduced ant treatment (Figure 3.1). Aggression scores were significantly different according to treatment (ANOVA $F_{4, 100} = 122.5$, $P < 0.05$). Treatments with non-nestmate

introduced ants had significantly higher aggression scores than treatments where the host colony was exposed to nestmate ants (Tukey HSD, $P < 0.05$). There was no significant difference in aggression scores across host colonies (ANOVA $F_{3, 102} = 0.96$, $P = 0.39$). Mean cumulative mortality was also compared across all treatment trials, with statistical differences in mortality associated with nestmate or non-nestmate presence (ANOVA $F_{4, 10} = 12.69$, $P < 0.05$), following the aggression score patterns.

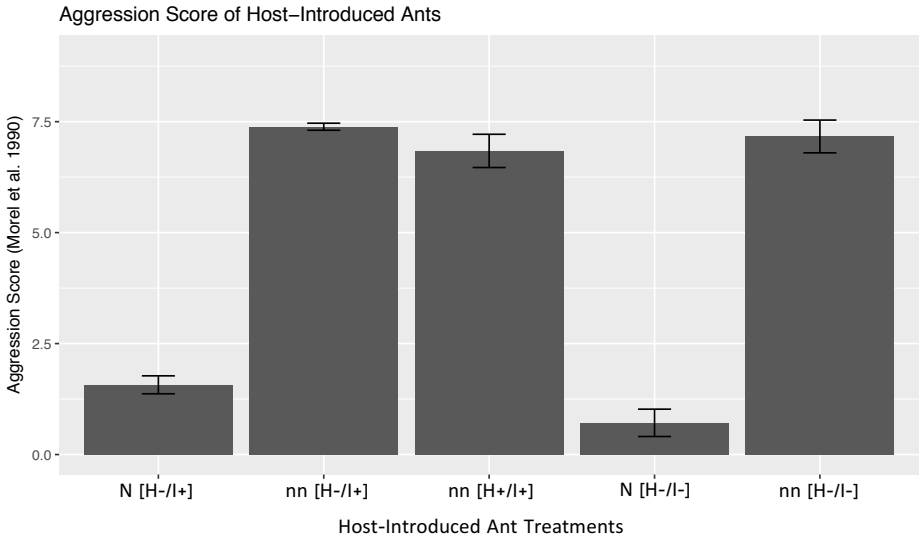

Figure 3.1. Mean Aggression Scores. Mean aggression scores for host-introduced ant trials over 7-hour interaction period. N, nestmates (same source colony). nn, non-nestmates (different source colony). H, host colony. I, introduced ants. +, sterile soil environment in formicary treated with 10^8 CFU/mL of *P. mirabilis*. -, sterile soil environment in formicary not treated with any bacteria.

Host-Introduced Ant Engagement Ratios

Engagement ratios in non-nestmate treatments interactions were always of the aggression engagement type, where aggression scores were high and the number of host to introduced ants was also high (Figure 3.2). Minimal host and introduced interactions were noted in nestmate interaction treatments. Introduced ants in these formicary chambers quickly became a part of the formicary colony, contributing to tasks in the ant chamber. Introduced ants were also noted entering the formicary soil and caring for brood of the host colony. Non-nestmate introduced ants, on the other hand, were immediately surrounded and aggravated by host colony ants. No non-nestmate introduced ants were seen inside the host formicary soil, or contributing to host colony tasks, such as moving around soil or grooming brood.

Bacterial Load Concentrations of Introduced Substrates Pre-Experimentation

Pre-introduction bacterial loads were used to estimate the average amount of *P. mirabilis* found on ants exposed in *P. mirabilis* treated sterile soil, and then introduced to host colonies for interaction observations. The pre-introduction bacterial loads of all substrates were similar to those determined in the preliminary 24-hour soil exposure, as expected (Table 3.4). However, the worker and brood *P. mirabilis* concentrations did show an average 10-fold CFU/mL increase relative to the concentrations determined in the preliminary study.

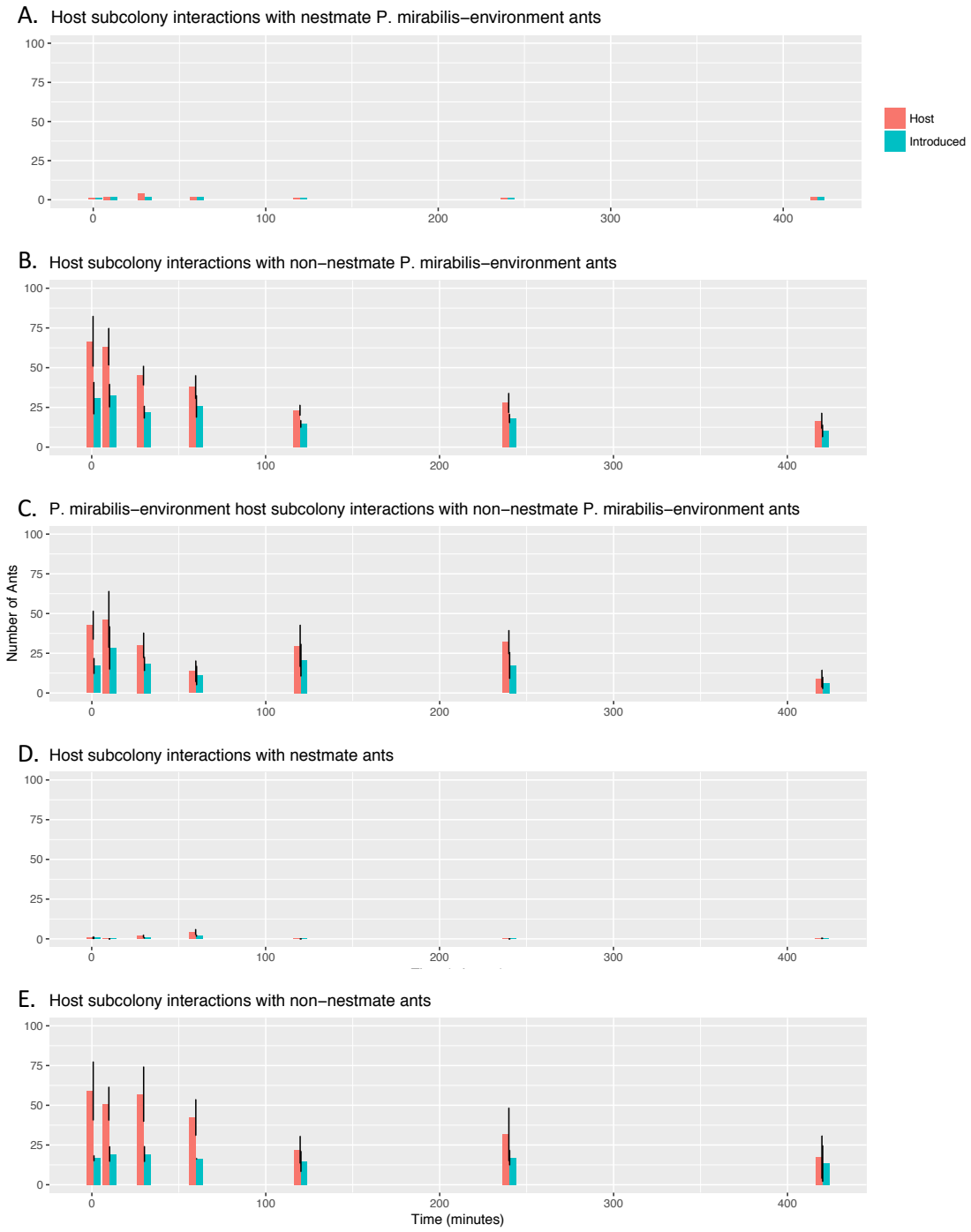

Figure 3.2. Host-Introduced Ant Engagement Ratios. The number of ants engaged in interactions used to determine aggression scores at the different time observation periods. N, nestmates (same source colony). nn, non-nestmates (different source colony). A. N [H-/I+], B. nn [H-/I+], C. nn [H+/I+], D. N [H-/I-], E. nn [H-/I-]. H, host colony. I, introduced ants. +, sterile soil environment in formicary treated with 10^8 CFU/mL of *P. mirabilis*. -, sterile soil environment in formicary not treated with any bacteria.

Initial *P. mirabilis* Inoculum Concentration (CFU/mL)	
1.39×10^9	
P. mirabilis **Load Mediums**	**Pre-Introduction *P. mirabilis* Concentration (CFU/mL)**
Soil (1 g, n=3)	1.01×10^8
Worker (100, n=3)	3.44×10^4
Brood (20, n=3)	1.20×10^4

Table 3.4. Pre-Introduction Bacterial Load. Pre-introduction bacterial load of *P. mirabilis* on substrates exposed to sterile soil *P. mirabilis* environment for 24 hours and to be introduced into host colony formicary chamber. Substrates include introduced ant sterile soil, *S. invicta* introduced worker ants and introduced brood.

Bacterial Load Concentrations of Host Colony Substrates Post-Experimentation

Post-experiment bacterial loads were calculated for host colony substrates only, to determine if interactions with *P. mirabilis* inoculated, introduced ants caused inoculation of *P. mirabilis* on *S. invicta* in the host colony, via ant to ant contact. *P. mirabilis* was only found in host colonies where the sterile soil environment had been treated with *P. mirabilis*, the host ants were also exposed for 24 hours (Table 3.5). All other treatments trials did not have any *P. mirabilis* found on any substrate (soil, worker, or brood). Treatments where the host colony with a sterile soil environment interacted with introduced ants, nestmates and non-nestmates, with *P. mirabilis* concentrations of about 10^4 CFU/mL were not inoculated with *P. mirabilis.* Also, all enrichment results were negative for these treatments. Enrichment results from these same treatment trials indicate

the highly unlikely probability that even a single *P.* mirabilis cell was transferred from ant-to-ant contact.

Initial *P. mirabilis* Inoculum Concentration (CFU/mL)
1.39×10^9

Post-Introduction and 7-hour Aggression Observation Experiments *P. mirabilis* Concentration (CFU/mL)					
***P. mirabilis* on Host Colony Load Mediums**	**N [H-/I+]**	**nn [H-/I+]**	**nn [H+/I+]**	**N [H-/I-]**	**nn [H-/I-]**
Soil (1 g, n=3)	*enrich* – , 0	*enrich* – , 0	1.42×10^8	0	0
Worker (100, n=3)	*enrich* – , 0	*enrich* – , 0	2.90×10^2	0	0
Brood (20, n=3)	*enrich* – , 0	*enrich* – , 0	6.64×10^4	0	0

Table 3.5. Post-Introduction Bacterial Load. Post-introduction and 7-hour interaction observation experiment bacterial load of *P. mirabilis* on host colony substrates exposed to introduced ants in the host colony formicary chamber. Substrates include host colony sterile soil, *S. invicta* host colony worker ants and host colony brood.

Discussion

Aggressive interactions between host and introduced ants were determined by whether or not the subcolonies interacting belonged to the same source colony; essentially, whether ants were originally nestmates or non-nestmates. Additionally, *P. mirabilis* bacteria was not transferred via ant-to-ant contact when interacting with ants with a bacterial load concentrations at approximately 10^4 CFU/mL *P. mirabilis.* At the core of this whole study, was the conceptual proof and quantification of transfer of bacteria in a

soil environment transferred to *S. invicta* ant workers and brood. To my knowledge, this is the first study to quantify the transfer of bacteria from soil-to-ant and ant-to-ant.

The nestmate interactions of non-nestmate *S. invicta* were not affected by the shared presence of *P. mirabilis* in the soil environment and subcolony members. These results follow typical *S. invicta* behavior, where worker caste individuals will readily defend and attack foreign ants, even conspecific ants that do not belong to their own colony (Morel *et al.*, 1990; Obin *et al.*, 1993). Polygyne colonies used in this experiment showed high degrees of aggression, with scores consistently averaging above 7 in non-nestmate interactions and aggression ratios between host and introduced ants were consistent over time. The number of ants engaged in aggression, however, were lower as time went by, because less and less introduced ants remained active or alive, with most aggressive interactions resulting in the death of the introduced ants. *P. mirabilis* exposed brood transferred into host colonies were often killed and eaten by the host colony ants, this particular behavior was not consistent with previous studies. Brood are generally recognized as brood and accepted by colony members from foreign conspecific colonies (Tschinkel, 2006). Previous studies have suggested that this may be due to the lack of antagonistic behavior, as brood are not mobile and will not react negatively to worker interactions (Morel and Vander Meer, 1988). Nestmate brood that had not been exposed to *P. mirabilis* were readily accepted by worker ants, worker ants having been observed picking up the brood and taking them inside the formicary with other brood, typical behavior. Future studies should focus on brood-associated worker caste behavior in the presence of bacteria. An experiment designed to properly observe these interactions with

brood and account for their location in the host colony would help to further explain the unusual behaviors observed in the experimental trials.

The mechanism of direct transfer from soil environment to ant body has been previously suggested in many *S. invicta*-bacterial studies (Allen and Buren, 1974; Ishak *et al.*, 2011; Jouvenaz *et al.*, 1977; Woolfolk *et al.*, 2016a; Woolfolk *et al.*, 2016b). Direct mechanical transfer has always been highly likely, especially with other models of ant-microbial interactions indicating direct transfer and persistence of microbes on the ant integument (Andersen *et al.*, 2015). In the leaf-cutter ant model, naïve leaf-cutter ant colonies depend on the transfers of fungi between well-established colonies and incipient colonies to initiate the fungal farms that will provide sustenance to the colony. Before this study, direct transfer quantification of the number of bacteria transferred via this mechanism had not been performed for ant-bacterial interactions. Further, this study quantified the transfer from soil environment to ant body for two exposure periods, at 24 hours and 48 hours, and for three different substrates, soil, worker caste pooled individuals, and brood pooled individuals.

Particularly to polygyne colonies, the ability to differentiate between true nestmates and reject non-nestmates comes at a price that can be costly to a foreign individual ant looking to contribute to the wrong colony. Throughout this chapter, *S. invicta* aggressive behaviors as a result of the differing presence and absence of bacteria on nestmate and non-nestmates were discussed. Bacterial abundance differences likely bear no impact on these interactions, especially in reference to *P. mirabilis* bacteria and *S. invicta* ants.

CHAPTER IV

SOLENOPSIS INVICTA BUREN FORAGING IN THE PRESENCE OF QUORUM SENSING BACTERIA *PROTEUS MIRABILIS*

Introduction

Various aspects of *Solenopsis invicta* foraging behavior have been well studied, including search time, foraging rate, recruitment time, transportation, polymorphic worker distribution associated with foraging tasks, and worker foraging response to various food types, in terms of quantity, quality and nutritional value. High carbohydrate diets tend to benefit overall colony mass (Wilder *et al.* 2011), and are the primary energy resource that the limited-diet adult worker ants can consume due to their constricting physiology (Vinson 1983). Food consumption and transfer differ on the basis of caste polyethism, where the reserve subcastes and brood exploit proteinaceous resources at a higher rate than the other forager and nurse worker castes (Sorensen *et al.* 1983). Abiotic factors like weather and seasons also affect *S. invicta* foraging behavior; lower temperatures inhibit overall activity, and high soil surface temperatures drive foraging activity to deeper underground tunneling (Porter and Tschinkel 1987). The wealth of information available on *S. invicta* provides a model organism for which additional factors impacting foraging behavior may be assessed, such as developing a better understanding of interactions with environmental factors and organisms in their shared habitat. Trophic interactions with aphids in agricultural systems have added depth to the nutritive model and additional

resource stipulations impacting *S. invicta* foraging behavior (Eubanks 2005). Beyond arthropod interactions, microbial interactions contributing to the trophic pyramid of any system remain less explored and likely integral to the foraging behavior of this omnivorous Myrmicine ant.

Nestmate recognition and foraging are ant behaviors driven by chemical communication within the colony (Vander Meer *et al.* 1988). Notable chemicals include pheromones and cuticular hydrocarbons, which give way to interpretive signals (Vander Meer *et al.*, 1989). These interpretive signals then provide direction for the behavior of the insect, affecting not only the individual, but also the function of the associated colony. Chemically derived messages drive the behavioral ecology of the colony. Microbes, specially bacteria, may well constitute another chemical communicator that affects fire ant behavioral ecology.

Previous *S. invicta*- bacterial studies have isolated and identified concomitant bacteria. However, most studies focused on the establishment of endosymbiotic relationships, using models of well-known bacteria-insect mutualism to interpret results. Early efforts focused on the isolation and characterization of culturable bacteria obtained from the midgut of the fourth instar *S. invicta* Buren larva, focusing on the stage responsible for the digestion of solid food resources (Li *et al.*, 2005; Peloquin and Greenberg, 2003). Unfortunately, these studies determined that culturable bacteria had no symbiotic relationships with the fourth instar *S. invicta.* While these studies were inconclusive in regards to the authors' specific hypotheses, testing did not address the possibility of other ecological roles these bacteria play with *S. invicta*.

Previous studies with other organisms determined animal behavior is partly regulated by the associated microbiome (Ezenwa *et al.*, 2012). Multiple host-behavior-and-microbiome interactions were discussed, such as the possible role bacteria play in the determination of cuticular hydrocarbons of fruit flies (Sharon *et al.*, 2010). The implication of this effect marks microbiomes as drivers of speciation, contributing to the mating preference of fruit flies. Still, these studies only begin to explore animal behavior and the role microorganisms play in the ecology of these animals. The classifications of microbe interactions with associated host organisms are yet to be determined, in many cases. Delving into the complexity of this relationship raises several questions: Are these interactions beneficial or detrimental to the host organism? Is this a single or multiple microbial species effort? Does behavior shape the microbiome, or does the microbiome shape behavior?

Bacterial quorum sensing (QS) could serve as the mechanism regulating RIFA behavior. QS is a system of gene expression response correlated with the population density of bacteria, where harmonized gene expression occurs once the local bacterial population achieves an indicative density threshold (Miller and Bassler, 2001). The bacteria are able to sense population abundance in their immediate surroundings and releases proteins, such as autoinducer molecules or volatiles, to further stipulate gene expression, interaction, and behavior in other bacterial cells. These signaling molecules have facilitated cross-signaling communication between prokaryotes and eukaryotes. Various prokaryotic signaling molecules involved in QS are recognized by eukaryotic organisms, such as blow flies (Diptera: Calliphoridae), mammals, and plants (Hughes and

Sperandio, 2008; Ma *et al.*, 2012; Miller and Bassler, 2001; Tomberlin *et al.*, 2012). As an example, *Proteus mirabilis* is a Gram-negative bacterium with a quorum sensing gene (Schneider *et al.*, 2002; Stankowska *et al.*, 2012). A *P. mirabilis* strain has been isolated from *Lucilia sericata* (Diptera: Calliphoridae) salivary glands, and has been used to study attraction and oviposition of *L. sericata* (Ma *et al.*, 2012; Tomberlin *et al.*, 2012). *P. mirabilis* provided a swarming phenotype, producing indole cues used by *L. sericata* for locating food and oviposition sites. Researchers found that the presence of swarming *P. mirabilis* attracted fly oviposition, and supplements used to replenish non-swarming mutant strains produced the same attraction. Interkingdom cues between the swarming bacterial cells translated to the induced behavioral attraction of an insect species. In addition, *P. mirabilis* has previously been isolated from *S. invicta* (Chadee and Le Maitre 1990) and was also isolated in studies conducted for in the second chapter of this dissertation.

Indole, a metabolite derived from the amino acid tryptophan, often serves as an environmental cue to organisms localizing a beneficial resource, and could thus be a cue to organisms searching for such a resource (Tomberlin *et al.*, 2017). As described earlier, swarming *P. mirabilis* is known to produce the indole cue recognized by flies associated with ephemeral resources and decomposition (Liu *et al.*, 2016). Essential amino acids are necessary for the growth and reproduction of organisms unable to synthesize these amino acids themselves. Resources containing the essential amino acid concentrations necessary for growth and reproduction are likely to be attractive to such an organism. *S. invicta* are known to readily consume high carbohydrate resources, especially sucrose rich nectars

from plants with readily available amino acids, including tryptophan (Lanza *et al.*, 1993). Tryptophan is an alpha amino acid used for the biosynthesis of proteins, often an essential amino acid, and widely found in various nutritional resources used by and experimentally tested for *S. invicta* nutrient consumption (Wills *et al.*, 2015). Tryptophan was also found in early studies determining the composition and amino acid components of *S. invicta* venom, albeit contributing only small amount, specifically 2.7 of 64 μg of estimated protein content (Baer *et al.*, 1979). In spite of low protein components, *S. invicta* venom allergens are still known to have a great reactionary effect in people with hypersensitive reactions to their sting (Stafford, 1996) and tryptophan may contribute to the synthesis of the venom proteomic library (dos Santos Pinto *et al.*, 2012) and the efficacy of this defense strategy. Indole produce by swarming *P. mirabilis* could thus contribute to the environmental cues *S. invicta* recognize when foraging for a nutritional resource. Based on the results from the previous chapter, *P. mirabilis* was present in the bacterial communities of 75% of the colonies samples, and in all ecoregions.

The experiments described in this chapter aimed to answer the following questions (1) do varying concentrations of *P. mirabilis* bait treatment have an effect on *S. invicta* foraging behavior, and (2) does *P. mirabilis* bait treatment affect the foraging responsiveness, specifically *S. invicta* foraging rate and bait removal, and how does this behavior vary across different baits and colonies. *S. invicta* foraging behaviors in the presence of growing bacteria, and more specifically quorum-sensing bacteria *P. mirabilis,* have not been studied previously. These experiments explore the impact that the presence of a bacterial microbe has on the foraging behavior of an invertebrate species, and further,

how the cellular density and phenotypic changes in bacteria affect the nutritional opportunity another organism exploits.

Materials and Methods

The methods of this research were based on previous ant foraging bioassays and the behavioral insect studies with *P. mirabilis* described in Tomberlin *et al.* (2012). A glycerol stock of wild-type *P. mirabilis* described in Tomberlin *et al.* (2012) was maintained by a co-author at the United States Department of Agriculture (USDA-ARS-SPARC) Food and Feed Safety Research laboratory (College Station, Texas) and used for experiments. The Office of Research Compliance and Biosafety at Texas A&M University did not require a permit because research was conducted on an arthropod species.

Colony Maintenance

S. invicta colonies were collected during early mornings in May-June of 2015 and 2016. All *S. invicta* colonies were polygyne colonies with multiple queens. *S. invicta* ant colony mounds were excavated from soil in pecan (*Carya* species) cultivars at the USDA-ARS-SPARC Crop Germplasm Research laboratory (Somerville, Texas). Each colony mound was placed in different 18.9-liter buckets and returned to the Rollins Urban and Structural Entomology Facility at Texas A&M University (College Station, Texas). Ant colonies were separated from the mound soil using the drip-method (Banks *et al.*, 1981) and placed in sweater boxes (39.7 cm x 33.3 cm x 17.1 cm) (The Container Store® Inc. CA, USA)

inner-coated with Insect-a-slip (BioQuip Products, Inc. CA, USA) to prevent their escape. The queen and brood were kept in a formicary constructed from a 150 x 50 mm petri dish (Thermo Fisher Scientific MA, USA) half filled with Castone® Dental Stone (Dentsply PA, USA) to retain moisture, with two holes made on the petri dish cover to allow entrance and exit of worker ants. Ant colonies were provided water and 15% honey water solution ad libitum, apple (0.5 g) and 4-6 medium crickets (PremiumCrickets.com GA, USA) every other day. Ant colonies were starved for 36 hours prior to experimentation.

Bait Preparation

As ant foraging of a given bait can vary depending on protein to carbohydrate ratio, three baits were selected to monitor the foraging responsiveness of *S. invicta*: a high carbohydrate resource of 15% honey water solution, a high protein resource of pork and chicken hot dog, and a balanced nutrient resource consisting of a granular diet (21% carbohydrate, 21% protein) designed for *S. invicta* foraging field studies (Cook *et al.*, 2010). The baits were prepared by sterilizing them first. Baits were autoclaved for 30 minutes at 121 °C to prevent contamination and effect from non-target microbes in the study. Baits were then aseptically pre-weighed to 0.5 g or 500 μL, and stored in a sterile 56.7-g container (hot dog and granular bait) or immediately used in experiment (honey water solution).

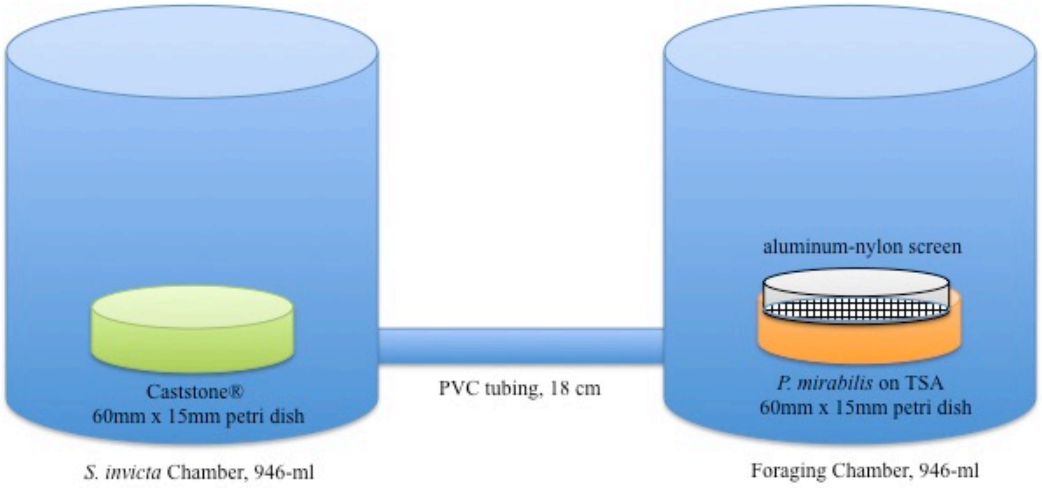

Figure 4.1. Single-Choice Foraging Experimental Arena. *S. invicta* experimental arena for single-choice foraging responsiveness experiments consisting of the "*S. invicta* chamber" housing the experimental colonoid (brood and worker ants) within a Castone® formicary, from the start to end of the 24-hour experiment duration, connected via a sterile PVC tube to the "Foraging chamber" where the bait was placed and worker ant activity observed, specifically, the number of ants every 30 minutes for a 6-hour period upon exposure to bait treatment.

Single-Choice Foraging Arena

The foraging arena consists of two 946-mL wide mouth autoclaveable jars (Consolidated Plastics OH, USA) inner-coated with Insect-a-slip (BioQuip Products, Inc. CA, USA). The first jar labeled the "*S. invicta* chamber" contained a small formicary constructed in a 60 x 15 mm petri dish (Thermo Fisher Scientific MA, USA), with Castone®. Worker ants were introduced into the arena by placing them in the formicary with 20 larval colony members to motivate foraging (Figure 4.1). The second jar is labeled the "foraging chamber." The bait treatment plate was placed in this foraging chamber. Both chambers had a 12.7 mm diameter hole closed with a rubber stopper to connect a piece of 18 cm long, 9.52 mm x 12.7 mm autoclaveable clear PVC tubing (VWR International PA, USA) to allow foraging across chambers. The 18-cm tunneling length was selected based on the

lasting limits of the scout recruitment trail pheromone, estimating an approximate 20 cm recruitment distance limit from tunnel entrance to food source on a glass surface (Dhami, 2008; Tschinkel, 2006). The tube material used in this experiment was clear PVC versus glass used to determine pheromone persistence, and although the porosity of the materials differ, the trail pheromone persistence determined using a glass surface marks the more limiting distance due to the non-porous nature of glass. Thus, using the limiting distance to determine a length for the foraging tunnel allowed for an experiment where foraging *S. invicta* ants would be at an optimal length for detection and response to the trailing pheromones or the perception of other cues in the experimental arena. All materials used for the experimental arena were sterilized in an autoclave for 30 minutes at 121 °C to prevent contamination and effect from non-target microbes in the study.

Foraging Responsiveness Experiments

There were two sets of foraging responsiveness experiments conducted in this study, bait foraging I and bait foraging II. Each bait trial from the bait foraging I experiments was carried out separately, testing the controls and multiple *P. mirabilis* concentration treatments over the course of three days for replication. Nine different colonies (Colonies A-I) were used, three for each bait experiment (hot dog, honey water solution, and granular diet). Bait trials for bait foraging II experiments were also carried out separately, testing all replications of control and a single *P. mirabilis* concentration treatment in a single day, using the three same colonies (Colonies J-L) for each bait experiment (hot dog and honey water solution).

Bait foraging I experiments, testing *S. invicta* colonies A-I, consisted of exposing baits to bacteria by placing the bait on top of an agar plate inoculated with *P. mirabilis.* Colonies A, B, C were used for the hot dog bait experiments; colonies D, E, F were used for the honey water solution experiments; and colonies G, H, I were used for the granular diet experiments. Approximately 183 worker ants (MEAN= 183.60, SE = 2.69 worker ants, N = 90) were collected from colonies A-I by placing a sterile tongue depressor and allowing the ants to climb onto the surface to be transferred into experimental arena *S. invicta* chamber. Twenty brood were added to each chamber to motivate worker foraging behavior.

P. mirabilis treatment concentrations were prepared using ten-fold serial dilutions in phosphate-buffered saline (PBS). The starting inoculum concentration of wild type *P. mirabilis* used in all experiments was 1 x 10^9 CFU/mL, as an ecologically relevant high concentration an insect might encounter in the environment. Tryptic soy agar (TSA) (Bio-Link Scientific TX, USA) treatment plates (60 by 15 mm petri dish) and two-times Charcoal agar (2xCHR) (Bio-Link Scientific TX, USA) count plates (100 x 15 mm petri dish) were inoculated. Each treatment plate was fitted with an aluminum-nylon screen atop the agar to prevent worker ants from destroying the agar, but still allowing exposure of the swarming bacterial cells through the nylon screen. The aluminum-screens were also placed atop control treatment exposures plates with no agar. A total of ten treatment exposures were used to study *S. invicta* foraging to the each given bait, treatments and reference abbreviations are listed in Table 4.1. Four control treatments were incorporated in the study: two consisting of no agar media, and two with the TSA (agar) media and PBS

used to suspend *P. mirabilis*. Six bacterial treatments increasing by 100-fold CFU/mL were used to study the foraging responsiveness with increasing *P. mirabilis* concentrations. The "WT 10^7 ONLY" treatment concentration is relevant to bacterial concentrations used in other insect-bacterial behavioral studies (Table 4.1) (Ma *et al.*, 2012; Vriesekoop and Shaw, 2010; Zhang *et al.*, 2015). Treatment plates were then placed in an incubator at 37 °C for 30 minutes to allow *P. mirabilis* optimal conditions to enter their growth phase. The bait was then placed on treatment plates and into the foraging arena for the experiment to begin.

***P. mirabilis* Bait Treatments**	**Associated Bait Treatment Identifier**
Nothing, No Bait, Bacteria Level 0	Nothing (Control)
Nothing, Bait, Bacteria Level 0	Nothing + Bait (Control)
Agar and PBS, No Bait, Bacteria Level 0	Agar ONLY (Control)
Agar and PBS, Bait, Bacteria Level 0	Agar + Bait (Control)
Wild-type *P. mirabilis*, Bait, Bacteria Level 1 35 µL of 1 x 10^1 CFU/mL *P. mirabilis*	WT 10^1
Wild-type *P. mirabilis*, Bait, Bacteria Level 2 35 µL of 1 x 10^3 CFU/mL *P. mirabilis*	WT 10^3
Wild-type *P. mirabilis*, Bait, Bacteria Level 3 35 µL of 1 x 10^5 CFU/mL *P. mirabilis*	WT 10^5
Wild-type *P. mirabilis*, Bait, Bacteria Level 4 35 µL of 1 x 10^7 CFU/mL *P. mirabilis*	WT 10^7
Wild-type *P. mirabilis*, No Bait, Bacteria Level 4 35 µL of 1 x 10^7 CFU/mL *P. mirabilis*	WT 10^7 ONLY (Control)
Wild-type *P. mirabilis*, Bait, Bacteria Level 5 35 µL of 1 x 10^9 CFU/mL *P. mirabilis*	WT 10^9

Table 4.1. Bait Foraging I Experimental Treatments. *P. mirabilis* bait treatment concentration descriptions for *S. invicta* foraging responsiveness experiments, including the various components of each treatment and indicating bait treatment identifiers for future reference.

Hot dog baits were placed directly atop the bacteria agar plate while the honey water solution and the granular diet baits were placed in a mini weigh-boat atop the bacteria agar plate. Upon placement of the bait on the treatment plate, the rubber stoppers were removed from the chambers, the tubing was used to connect the two chambers, and experimental observations began. Ants in the foraging chamber were recorded every 30 minutes for the 15-30 seconds over a 6-hour period to obtain ant counts. A final recording at the 24-hour mark from the start of the experiment completed the observation period. The exposed bait was collected from the foraging arena to obtain a post-weight to determine bait removal. Each bait experimental treatment was replicated three times, running single replicates for each colony with each treatment on three different days.

Colonies J, K, L were used for bait foraging II experiments where *P. mirabilis* was thoroughly mixed with two of the three baits, directly inoculating the hot dog bait and the honey water solution to obtain a bacteria-bait mix. Approximately 370 worker ants (MEAN= 370.50, SE = 8.75 worker ants, N = 72) were collected from colonies J-L by placing a sterile tongue depressor and allowing the ants to climb onto the surface to be transferred into experimental arena *S. invicta* chamber. Forty brood were added to each chamber to motivate worker foraging behavior. *P. mirabilis* treatment concentrations were prepared using ten-fold serial dilutions in phosphate-buffered saline (PBS). Four total treatments were used for bait foraging II experiments (Table 4.2). The first consisted of no bait or nothing, and only sterile water in the honey water bait experiment (Nothing).

P. mirabilis Bait Treatments	Associated Bait Treatment Identifier
Nothing, No Bait, Bacteria Level 0	Nothing (Control)
Nothing, Bait, Bacteria Level 0	Bait ONLY (Control)
Wild-type *P. mirabilis*, Bait, Bacteria Level 4 35 µL of 1 x 10^7 CFU/mL *P. mirabilis* (3.5 x 10^5 CFU)	WT 10^7 + Bait
Wild-type *P. mirabilis*, No Bait, Bacteria Level 4 35 µL of 1 x 10^7 CFU/mL *P. mirabilis* (3.5 x 10^5 CFU)	WT 10^7 ONLY (Control)

Table 4.2. Bait Foraging II Experimental Treatments. *P. mirabilis* bait treatment concentration descriptions for *S. invicta* foraging responsiveness experiments, including the various components of each treatment and indicating bait treatment identifiers for future reference.

The second consisted of the control non-treated bait (Bait ONLY). The third consisted of a bacteria-treated bait (WT 10^7 + Bait). Finally, the fourth treatment consisted of only the bacteria (WT 10^7 ONLY). In the hot dog bait experiment, filter paper was used in all treatments to suspend the bacteria-only treatment on the filter paper. In the honey water bait experiment, sterile water was used in all treatments to suspend the bacterial-only treatment in the sterile water. The *P. mirabilis* concentration for the treatment of baits was selected based on foraging results from the bait foraging I experiments. The bacteria concentration with the greatest effect on foraging responsiveness, bait removal and foraging rate was chosen to test the impact of treating bait directly with the given *P. mirabilis* colony forming units.

Hot dog mix baits were placed directly atop a piece of autoclaved filter paper in the base of a sterile petri dish (60 mm x 15 mm), while the honey water solution baits were placed directly in the base of a sterile petri dish. Upon placement of experimental baits in the foraging chamber, the rubber stoppers were removed from the chambers, the tubing

was used to connect the two chambers, and experimental observations began. Ants in the foraging chamber were recorded every 30 minutes for the 15-30 seconds over a 12-hour period to obtain ant counts. A final recording at the 24-hour mark from the start of the experiment completed the observation period. The bait was then collected from the foraging arena to obtain a post-weight to determine the amount of bait removed. Each bait experimental treatment was replicated three times, running all three replicates for each colony with each treatment on a single day.

Experimental Design and Statistical Analysis

The data obtained from the foraging responsiveness assays were analyzed using an analysis of covariance (ANCOVA) to determine covariates and factors affecting the total percentage ant activity, i.e. foraging rate, in the foraging chamber. Factors were then used to block the data and analyze according to the experimental design, namely by baits, by colonies within the bait experiments, and by treatment across single colonies. Post experiment bait weight was used to assess bait removal as proxy of attractive bait removed for consumption, taking into account moisture content effect. ANOVA and Tukey HSD and Dunnett's test for multiple comparisons test statistics were then performed to determine significant ($P < 0.05$) difference in bait removal across treatments and across colonies.

Foraging rate was analyzed by organizing the percentage ant counts over the foraging period in a time series and fitting the series to a generalized linear model (GLM). To further assess the effect of *P. mirabilis* concentration treatments on *S. invicta* foraging

rates, the data was logarithmically transformed to linearize the multiplicative characteristic of ant foraging activities (Nestel and Dickschen, 1990). Using the one-parameter logistic model, namely:

$$f(t) = \frac{ae^{\beta t}}{1 + e^{\beta t}} - \frac{a}{2}$$

where $f(t)$ is mean cumulative percent ants and β is the parameter denoting the rate of exponential growth in foraging activity. Following the logistic transformation using the properties implied by the natural logarithm, the exponential growth of foraging *S. invicta* were estimated using:

$$y(t) = \beta t$$

a linear regression model to solve for β, the foraging rate. R-squared values and parametric ANOVA model statistic values were obtained for the foraging rate time series linear regression models.

Results

Explanatory model testing for bait foraging I (BF-I) experiments using the ANCOVA approach designated the most significant factors of *S. invicta* foraging rate to be time point, colony, and a covariance factor between the colony and treatment effects for the hot dog bait (ANCOVA $F_{41,\ 1128} = 17.14$, $P < 0.05$), 15% honey water bait (ANCOVA $F_{41,\ 1128} = 13.8$, $P < 0.05$), and the granular diet bait (ANCOVA $F_{41,\ 1479} = 20.84$, $P < 0.05$). Based on these determinations, treatment differences proceeded to be

analyzed separately within each colony. The data of two treatments was also removed from further bait foraging I experiment analyses, namely "Nothing" and "Nothing + Bait" because there was an agar effect on *S. invicta* overall activity. Treatments with agar had a substantial increase in the moisture of the foraging arena and thus changed the environment of the experiment. Data for foraging rate comparison was analyzed separately by bait and then separately by colony, based on ANCOVA results on total percentage ant activity in the foraging chamber. Foraging rates (mean percentage ants / 30-minute observation interval) for the various agar *P. mirabilis* treatments were analyzed by colony and compared using ANOVA and the Tukey HSD. Overall, foraging rates followed a similar bacteria density dependent effect across some colonies, although this pattern was also dependent on the type of bait being tested. The highest foraging rates for all colonies had an adjusted R squared value greater than 0.5 and were statistically significant ($P < 0.05$).

BF-I: Hot Dog (High Protein) Bait Experiments

Hot dog bait removal was not significantly different among colonies (ANOVA $F_{2, 15} = 0.83$, $P = 0.45$) or treatments (ANOVA $F_{5, 12} = 1.39$, $P = 0.29$) (Figure 4.2). Treatment comparisons were not significant although means of bait removal for WT 10^5 *P. mirabilis* treatment showed the lowest relative bait removal with closely grouped replicate. Overall, however, the bait removal varied widely across all treatments. The foraging rates across colonies varied in activity over time, Colony C consistently having more active foragers among the three colonies. Figure 4.3 illustrates the percent increase of foraging rates

across increasing *P. mirabilis* concentration bait exposure. The highest foraging rate among agar-based treatments varied by colony, but were achieved by ants in the lower *P. mirabilis* treatments, either WT 10^3 or WT 10^5. Colony B had minimal percent increase or decrease across *P. mirabilis* concentrations. Colony A had the lowest foraging rate across all hot dog bait exposure treatments at WT 10^7, the second highest concentration (Figure 4.3). Here, the foraging rate was 0.07, equivalent to an average 7% growth increase of percent ants per observation period. This was also the greatest decrease in foraging rate across *P. mirabilis* concentrations present in colony A. The rate decreased by 10% as the bacteria concentration increased 100-fold. The highest overall foraging rate was reached by colony C at the WT 10^3 *P. mirabilis* concentration, increasing an average of 22% more per observation than the "Agar" bait control.

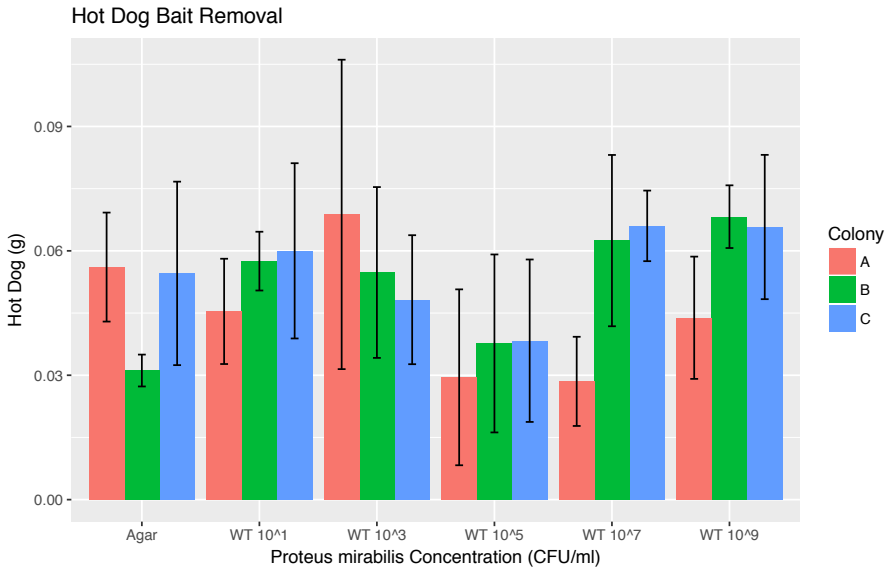

Figure 4.2. BF-I Bait Removal for Hot Dog Bait. Bait removal of 0.5 g hot dog for all colonies (A, B, C) at the end of the 24-hour foraging period in bait experiments where agar and hot dog bait was present, namely, Agar, WT 10^1, WT 10^3, WT 10^5, WT 10^7, and WT 10^9.

***S. invicta* Foraging Rate in Presence of *Proteus mirabilis* Concentration Bait Treatments, Pr(>|t|)**

	Agar ONLY	Agar	10^1 CFU/mL	10^3 CFU/mL	10^5 CFU/mL	10^7 CFU/mL	10^7 CFU/mL ONLY	10^9 CFU/mL
Hot Dog								
Colony A	0.11	0.15	0.13	0.16	0.17	0.07	0.16	0.18
Colony B	0.22	0.13	0.15	0.12	0.12	0.11	0.14	0.12
Colony C	0.23	0.08	0.26	0.30	0.21	0.21	0.22	0.20

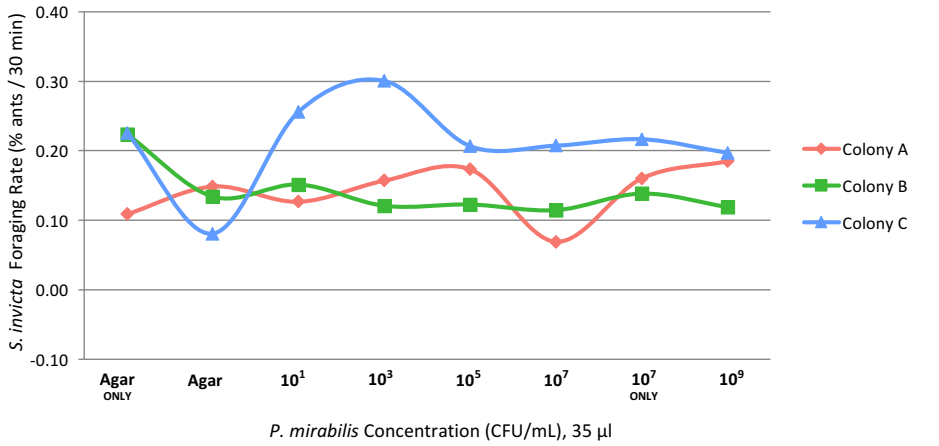

Figure 4.3. BF-I Foraging Rates for Hot Dog Bait. *S. invicta* foraging rate calculated from the mean percentage ants in the hot dog bait foraging chamber. Foraging rate includes data from three replicates over the 6-hour observation period at the initiation of exposure to the bait. The foraging rates are organized by colony (Colony A, B, C) and treatment (Agar ONLY, Agar, WT 10^1, WT 10^3, WT 10^5, WT 10^7, WT 10^7 ONLY, and WT 10^9). Foraging rates plotted onto graph illustrate the increasing and decreasing change in foraging activity across increasing *P. mirabilis* concentration treatments.

BF-I: Fifteen Percent Honey water (High Carbohydrate) Bait Experiments

Honey water bait removal was significantly different among colonies (ANOVA $F_{2, 15} = 10.63$, $P < 0.05$), with Colony E having overall lower honey water bait removal (Tukey HSD $P < 0.05$) (Figure 4.4). Bait removal according to treatment comparisons were not significant, and instead consistent in means and variances (ANOVA $F_{5, 12} = 0.29$, $P = 0.91$). The degree of foraging rates across colonies varied, however, there was a consistent increase in foraging rate at the WT 10^5 *P. mirabilis* bait exposure treatment for all colonies (Figure 4.5). Foraging rates at this moderate concentration, relative to the others, ranged from 0.13 to 0.19 percent ants per observation. The greatest range of increase happened

for colony D, drastically increasing by the growth rate by 22%. All colonies also exhibited a drastic decrease in foraging rate for the higher concentrations, at either WT 10^7 or WT 10^9 (Figure 4.5). Oscillatory patterns followed the increasing presence of *P. mirabilis* concentrations, with a relatively moderate foraging rate in the absence of the bacteria, followed by small changes between each concentration amount, decreasing, then increasing, and finally decreasing again at the highest concentration treatment, WT 10^9. Colony E did have a final increasing oscillation represented by a 13% higher percentage growth in foraging activity for the WT 10^9 *P. mirabilis* concentration exposure treatment.

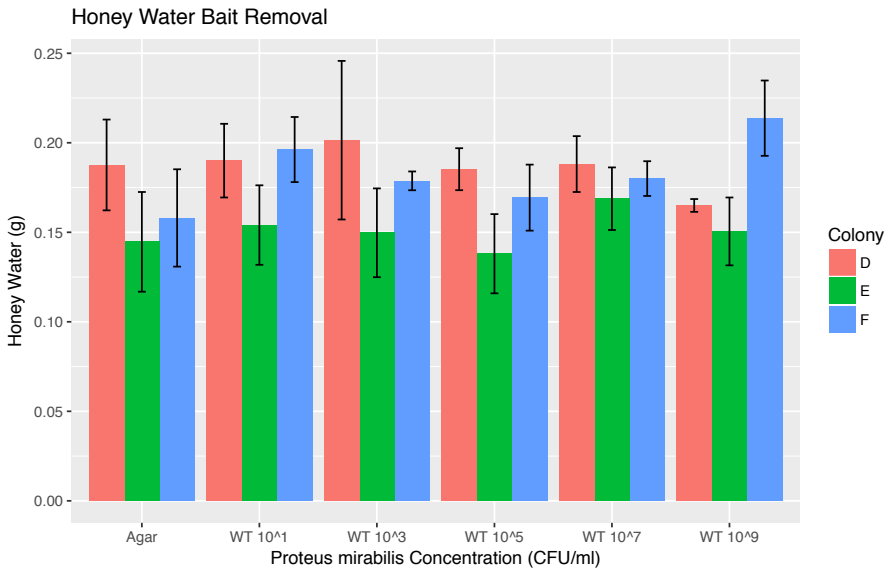

Figure 4.4. BF-I Bait Removal for Honey Water Bait. Bait removal of 0.5 g of 15% honey water for all colonies (D, E, F) at the end of the 24-hour foraging period in bait experiments where agar and honey water bait was present, namely, Agar, WT 10^1, WT 10^3, WT 10^5, WT 10^7, and WT 10^9.

***S. invicta* Foraging Rate in Presence of *Proteus mirabilis* Concentration Bait Treatments, Pr(>|t|)**

	Agar ONLY	Agar	10^1 CFU/mL	10^3 CFU/mL	10^5 CFU/mL	10^7 CFU/mL	10^7 CFU/mL ONLY	10^9 CFU/mL
Honey Water								
Colony D	0.05	0.02	-0.03	-0.03	0.19	0.00	-0.08	-0.07
Colony E	0.13	0.11	0.06	0.10	0.13	-0.02	0.08	0.11
Colony F	0.03	0.07	0.05	0.03	0.18	0.19	0.09	0.08

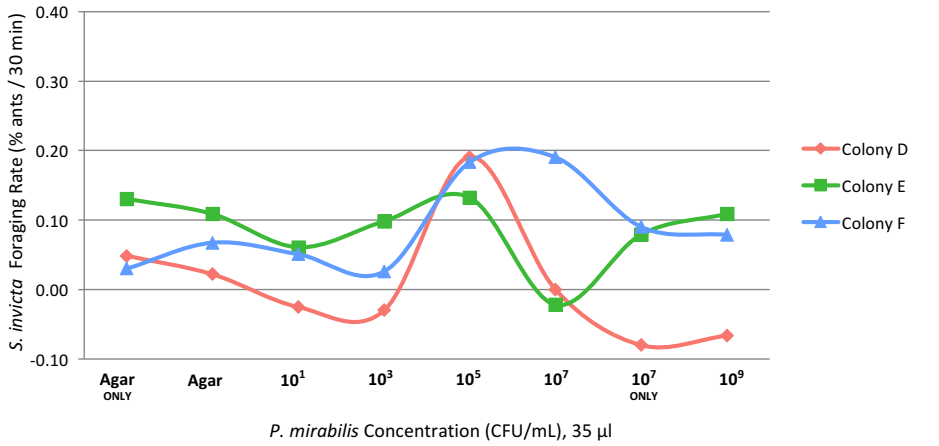

Figure 4.5. BF-I Foraging Rates for Honey Water Bait. *S. invicta* foraging rate calculated from the mean percentage ants in the honey water bait foraging chamber. Foraging rate includes data from three replicates over the 6-hour observation period at the initiation of exposure to the bait. The foraging rates are organized by colony (Colony D, E, F) and treatment (Agar ONLY, Agar, WT 10^1, WT 10^3, WT 10^5, WT 10^7, WT 10^7 ONLY, and WT 10^9). Foraging rates plotted onto graph illustrate the increasing and decreasing change in foraging activity across increasing *P. mirabilis* concentration treatments.

BF-I: Granular Diet (Balanced) Bait Experiments

Granular diet bait removal was not significantly different among colonies (ANOVA $F_{2, 15} = 0.87$, $P = 0.43$) or treatments (ANOVA $F_{5, 12} = 1.45$, $P = 0.28$) (Figure 4.6). Treatment comparisons were not significant although mean of bait removal for WT 10^3 *P. mirabilis* treatment showed the lowest relative bait removal with closely grouped replicate. Granular diet foraging rates varied across colonies, as with the other tested baits, with increasing and then decreasing oscillating changes in patterns of increase or decrease across treatments. The higher *P. mirabilis* concentration bait exposure treatments had some of the lowest foraging rates, but this varied by colony. Colonies G and I had the lowest

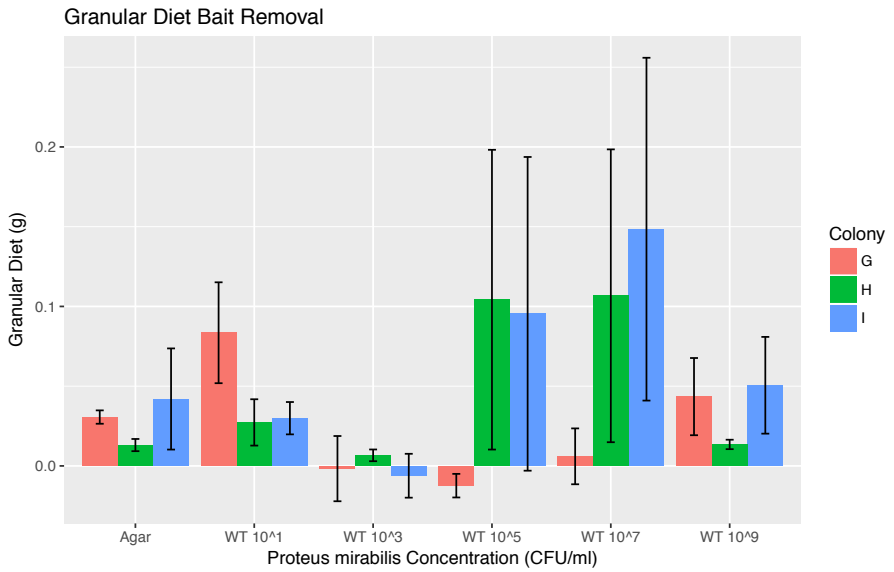

Figure 4.6. BF-I Bait Removal for Granular Diet Bait. Bait removal of 0.5 g of granular diet for all colonies (G, H, I) at the end of the 24-hour foraging period in bait experiments where agar and granular diet bait was present, namely, Agar, WT 10^1, WT 10^3, WT 10^5, WT 10^7, and WT 10^9.

***S. invicta* Foraging Rate in Presence of *Proteus mirabilis* Concentration Bait Treatments, Pr(>|t|)**

	Agar ONLY	Agar	10^1 CFU/mL	10^3 CFU/mL	10^5 CFU/mL	10^7 CFU/mL	10^7 CFU/mL ONLY	10^9 CFU/mL
Granular Diet								
Colony G	0.07	0.03	0.04	0.13	0.09	0.11	0.01	0.07
Colony H	0.05	0.04	0.13	0.05	0.09	0.08	0.09	0.09
Colony I	0.12	0.15	0.05	0.12	0.07	0.01	0.20	0.07

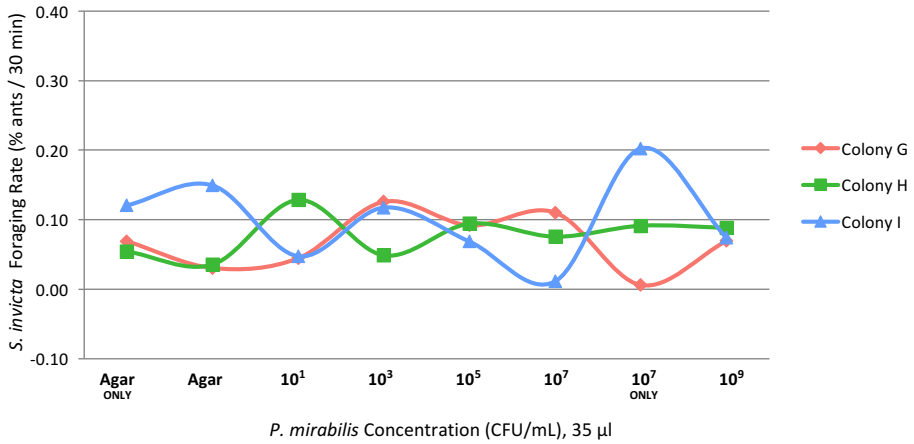

Figure 4.7. BF-I Foraging Rates for Granular Diet Bait. *S. invicta* foraging rate calculated from the mean percentage ants in the granular diet bait foraging chamber. Foraging rate includes data from three replicates over the 6-hour observation period at the initiation of exposure to the bait. The foraging rates are organized by colony (Colony G, H, I) and treatment (Agar ONLY, Agar, WT 10^1, WT 10^3, WT 10^5, WT 10^7, WT 10^7 ONLY, and WT 10^9). Foraging rates plotted onto graph illustrate the increasing and decreasing change in foraging activity across increasing *P. mirabilis* concentration treatments.

foraging rate attained by exposure to the 10^7 CFU/mL *P. mirabilis* concentration. For all colonies, the second lowest foraging rates were reached for the "Agar" bait treatment, where no bacteria were present, or the lowest *P. mirabilis* concentration at 10^1 CFU/mL. Colony I, however, also had the highest foraging rate at treatment with no granular bait present and exposure to 10^7 CFU/mL (R-squared = 0.62, $P < 0.05$). There was a 19% foraging rate difference between the same concentration treatment where granular diet was present (Figure 4.7). Colonies G and K had the highest foraging rates at the lower *P. mirabilis* concentrations 10^3 CFU/mL and 10^1 CFU/mL, respectively. At most treatments, colony H foraging rates indicated an opposite foraging behavior response than that of colonies G and I.

Upon review of the foraging rate and bait removal results from the BF-I experiments, the hot dog bait and the honey water solution bait were selected to move onto the next phase of these experiments. These baits were selected because there was less variability in foraging responsiveness, relative to the granular bait, with active *S. invicta* workers collecting the bait and responding similarly to *P. mirabilis* concentration patterns. By selecting these two baits, the consistency of the bait would not be altered through the bacteria-mix treatment. In addition, the TSA agar plate would no longer be needed, and thus the agar moisture effect avoided. *P. mirabilis* at the 10^5 CFU/mL had the greatest affect in foraging rate for both hot dog and honey water baits. In terms of bait removal, *P. mirabilis* 10^5 CFU/mL concentration increase bait removal activity for the hot dog bait while the 10^7 CFU/mL concentration exposure caused a decrease in honey water solution bait removal. The 10^7 CFU/mL *P. mirabilis* concentration was used to treat the bait-

bacteria mixtures and achieve the 10^5 CFU concentration to be used in the BF-II experiments.

Explanatory model testing for bait foraging II (BF-II) experiments using the ANCOVA approach designated the most significant factors of *S. invicta* foraging rate to be time point, colony, and a covariance factor between the colony and treatment effects for the hot dog bait (ANCOVA $F_{36,\ 899} = 30.66$, $P < 0.05$) and 15% honey water bait (ANCOVA $F_{36,\ 899} = 52.04$, $P < 0.05$). Based on these determinations, treatment differences proceeded to be analyzed separately within each colony. Data for foraging rate comparison were analyzed separately by bait and then separately by colony, based on ANCOVA results on total percentage ant activity in the foraging chamber.

BF-II: Hot Dog (High Protein) Bait Experiments

There were no significant differences of bait removal between colonies J, K, and L (ANOVA $F_{2,\ 33} = 1.43$, $P = 0.25$). There was also no significant difference in bait removal according to bait treatment, the differences in this test where attributable to the treatments with no baits only (ANOVA $F_{3,\ 32} = 21.43$, $P < 0.05$) (Figure 4.8). Interaction between the treatment and colony factors were also tested for treatments where bait was made available, but no statistical significant difference was found (ANOVA $F_{5,\ 12} = 2.99$, $P = 0.06$). The foraging rates of treatments without the presence of bait were the lowest, while those with the bait present were the highest (Figure 4.9). The hot dog-bacteria mix bait had increased foraging rates for colonies J and L, but not for K, where the highest foraging rate was observed in the absence of bait and bacteria, at the "Nothing" treatment.

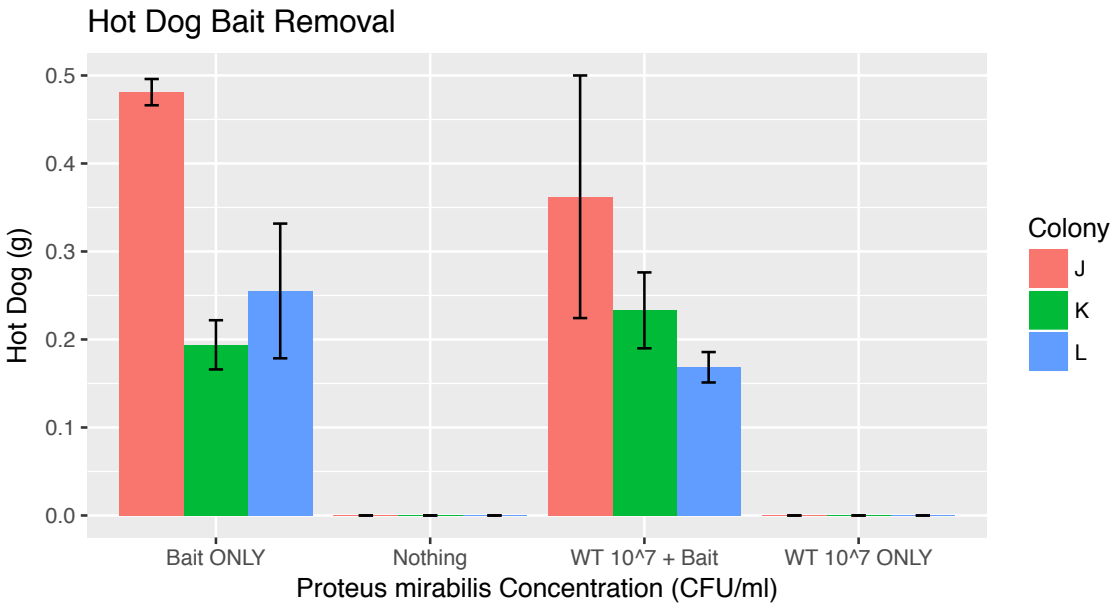

Figure 4.8. BF-II Bait Removal for Hot Dog Bait. Bait removal of 0.5 g of hot dog bait for all colonies (J, K, L) at the end of the 24-hour foraging period in bait experiments with two bait treatments, namely, Bait ONLY (filter paper control and hot dog bait), Nothing (filter paper), WT 10^7 + Bait (*P. mirabilis* bacteria-hot dog bait mix), and WT 10^7 ONLY (filter paper and *P. mirabilis*).

***S. invicta* Foraging Rate in Presence of *Proteus mirabilis* Treated Baits, Pr(>|t|)**

	Nothing	Bait ONLY	WT 10^7 + Bait	WT 10^7 ONLY
Hot Dog				
Colony J	0.05	0.01	0.03	0.01
Colony K	0.04	0.11	0.12	0.09
Colony L	0.14	0.12	0.14	0.07

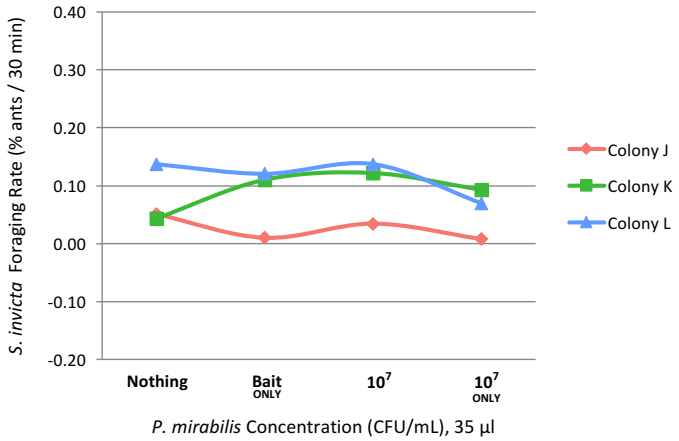

Figure 4.9. BF-II Foraging Rates for Hot Dog Bait. *S. invicta* foraging rate calculated from the mean percentage ants in the hot dog-bait foraging chamber. Foraging rate includes data from three replicates over the 12-hour observation period at the initiation of exposure to the bait. The foraging rates are organized by colony (Colony J, K, L) and treatment (Nothing, Bait ONLY, WT 10^7 + Bait, and WT 10^7 ONLY). Foraging rates plotted onto graph illustrate the increasing and decreasing change in foraging activity across bait-*P. mirabilis* bacteria mix combinations.

BF-II: Fifteen Percent Honey Water (High Carbohydrate) Bait Experiments

Bait removal did not differ based on colony (ANOVA $F_{2, 33} = 0.43$, $P = 0.65$). Treatment of baits with *P. mirabilis* did not have an effect on bait removal, although overall comparison of treatments showed a significant difference in bait removal (ANOVA $F_{3, 32} = 21.43$, $P < 0.05$), with all mean differences attributable to bait presence in the treatment (Figure 4.10). Interaction between the treatment and colony factors were also tested for treatments where bait was made available, but no statistically significant difference was found (ANOVA $F_{5, 12} = 2.08$, $P = 0.13$). The foraging rate of treatments without the presence of bait were the lowest once again, while those with the bait present were the

highest, except for colony K which seemed to forage actively in the absence of bait in the "Nothing" treatment consisting of only sterile water (Figure 4.11).

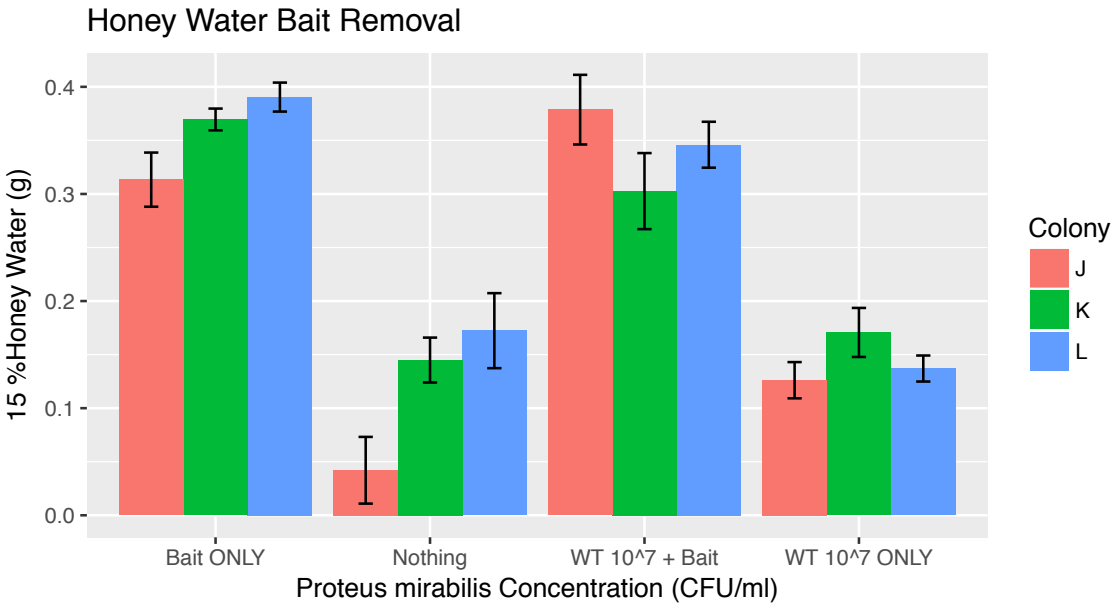

Figure 4.10. BF-II Bait Removal for Honey Water Bait. Bait removal of 0.5g of 15% honey water solution bait for all colonies (J, K, L) at the end of the 24-hour foraging period in bait experiments with two bait treatments, namely, Bait ONLY (sterile honey water solution bait), Nothing (sterile water), WT 10^7 + Bait (*P. mirabilis* bacteria-honey water solution bait mix), and WT 10^7 ONLY (sterile water and *P. mirabilis*).

***S. invicta* Foraging Rate in Presence of *Proteus mirabilis* Treated Baits, Pr(>|t|)**

	Nothing	Bait ONLY	WT 10^7 + Bait	WT 10^7 ONLY
Honey Water				
Colony J	0.02	0.02	0.01	0.04
Colony K	0.05	0.04	0.02	0.06
Colony L	0.03	0.04	0.04	0.03

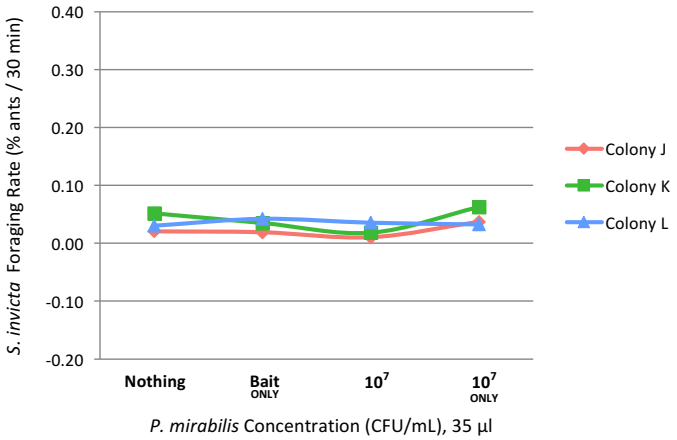

Figure 4.11. BF-II Foraging Rates for Honey Water Bait. *S. invicta* foraging rate calculated from the mean percentage ants in the honey water solution-bait foraging chamber. Foraging rate includes data from three replicates over the 12-hour observation period at the initiation of exposure to the bait. The foraging rates are organized by colony (Colony J, K, L) and treatment (Nothing, Bait ONLY, WT 10^7 + Bait, and WT 10^7 ONLY). Foraging rates plotted onto graph illustrate the increasing and decreasing change in foraging activity across bait-*P. mirabilis* bacteria mix combinations.

Discussion

The foraging responsiveness of *S. invicta* for baits in the presence of *P. mirabilis* varied by colony and *P. mirabilis* concentration. Foraging rates were compared by colony because the colony foraging intensity towards a bait varied for each bait experiment where *P. mirabilis* was inoculated onto a TSA agar plate. A comparison of foraging rates across these increasing-concentration *P. mirabilis* exposure treatments displayed patterns of a bacteria density dependent effect on foraging rate, with the lowest and highest concentration of *P. mirabilis* having the lowest foraging rates. The strongest predictive

response was attributed to the *P. mirabilis* 10^5 CFU/mL concentration, particularly in the high carbohydrate bait experiments, namely the honey water bait. The best bait to use for predictive response experimentations was the high carbohydrate bait. Bait removal, as a proxy measure for likely consumption by *S. invicta*, was not significantly affected by the presence of *P. mirabilis.*

Although comparisons across bait removal data were not statistically significant, the hot dog and granular diet bait experiment bait removal means were consistently lower for all colonies at 10^5 CFU/mL and 10^3 CFU/mL, respectively. There was a high amount of variance in bait removal, this variation is likely a result of within-colony variation associated with the different colonies tested. Exploration activities such as foraging, recruitment and exploration vary according to *S. invicta* colony lineage and are likely heritable traits under evolutionary selection pressures (Bockoven *et al.,* 2015). Despite all colonies used in this study being collected from the same regional area, colony-level variation in foraging responsiveness likely contributed the variance in the foraging results of this study. Future bacteria-interaction experiments monitoring the foraging rate or bait removal of *S. invicta* should quantify and compare *S. invicta* colony foraging competencies across colonies. This information could then be used to assess similarly competent colony behavior.

The two experiments, bacteria foraging I and bacteria foraging II, tested two different ways *P. mirabilis* could affect the foraging responsiveness of *S. invicta.* First, testing the exposure of the bait to the continual growth of *P. mirabilis* on the TSA agar plate (bait foraging I), where the bacteria multiplied due to the favorable environment and

reach the densities necessary for the quorum sensing phenotype to be expressed by the bacterial cells, i.e. swarming, established the quorum sensing effect variable in the experiment. *P. mirabilis* concentration treatments accounting for lower CFU/mL do not express the quorum sensing phenotype, i.e. swarming, because they do not have the sufficient cell density to activate the expression. Thus, the increasing concentrations of *P. mirabilis* treatments were tested to determine whether there was a *S. invicta* foraging responsiveness effect across the presence of an increasing gradient of quorum sensing phenotypic expression. Upon determining the presence of some effect, the *P. mirabilis* concentration (bacterial cell density) treatment associated with the greatest effect was selected for the bacterial foraging II experiments. The bacterial foraging II experiments used the selected concentration treatment to determine the *P. mirabilis* presence effect on *S. invicta* foraging responsiveness. Essentially, bacterial foraging II experiments contain a fixed *P. mirabilis* bacterial cell density, where the rate of bacterial cell density was more controlled and not increasing on an optimal media for growth. This fixed bacteria density was directly added and thoroughly mixed with the baits to isolate the particular 10^5 CFU *P. mirabilis* density within the bait. Mean bait removal for *P. mirabilis* mixed baits was overall lower, but not statistically significant due to variability in foraging behavior of the different colonies. All replicates for all treatment trials were carried out in the same day, imposing less variance in *S. invicta* foraging performance across different days, but within-colony variance was still present.

The foraging rate for the BF-I honey water bait experiments seemed the most consistent in comparison across the colonies tested with that bait. Here, the bacteria

density dependence pattern was present, with increasing and decreasing foraging rates of *S. invicta* across increasing concentrations of *P. mirabilis*. *P. mirabilis* 10^5 CFU/mL concentration had the highest foraging rates in colonies with hot dog and honey water solution baits. However, the rate and pattern was not indicative of the 24-hour period of bait removal treatment effects. This means that although the initial observed foraging activity may have been higher for some treatments and colonies than others, at the end of a 24-hour period, the bait removal performance outcome was close to equal for all colonies. Bait removal in all tested baits followed this same pattern. This leveling amount of bait removal could have occurred because the experiments were single choice bioassays and did not provide an alternative option for food to the ants. The foraging rate, accounting for the initial discovery and attraction to resource, is likely more representative of *S. invicta* association with a *P. mirabilis* inoculated resource. This is because ants initially recruit other ants if the forager determines the bait/resource to be attractive (Vander Meer *et al.*, 1988). Over time, however, the ants will determine that this is the only resource available in the environment and exploit their only food source until all colony members are satiated (Cassill and Tschinkel, 1999a; Howard and Tschinkel, 1980).

In the natural environment, a eukaryotic organism might perceive a localized, high abundance of bacteria on a food source as a threat to its own fitness, the bacteria can be both pathogens or a resource competitors (Janzen, 1977). In such a case, the organism might choose to risk consuming the nutritional resource or avoid it. This however may depend on an organism's typical habitat and whether a high bacteria density is characteristic of that environment. In the case of *L. sericata*, a bacteria-dense environment

does not affect the survival or fitness of individuals (Barnes and Gennard, 2011). Eggs develop into adults in an environment highly populated with microbes, and commonly pathogenic bacterium *Staphylococcus aureus* does not present a detrimental effect in the survival of adults. Carrion-feeding insects specialized in exploiting ephemeral environments with dense bacterial populations likely favor such interactions and come with little cost. Such is the reasoning behind *L. sericata* and *P. mirabilis* interkingdom interactions, where the fly is able to detect a cue that enables successful breeding and nutrition (Ma *et al.*, 2012; Tomberlin *et al.*, 2017). However, high levels of bacteria can be toxic or deadly to other insect species not commonly associated with such bacterially dense environments (Barribeau *et al.*, 2014). Such may be the case for *S. invicta,* the subterranean-nest dwelling ant commonly found grooming itself and its nestmates (Cassill and Tschinkel, 1999b). *S. invicta* are generalist, less consistently associated with putrefying resources rich with microbes, as is the case with Calliphoridae flies. One could hypothesize that *S. invicta* are less likely to interact with such an environment, having alternative resources to exploit and not confined, as a specialist, to bacterially dense environments. The exposure of *S. invicta* to microbes has been described before (Jouvenaz *et al.*, 1977). More recent studies have focused on identifying *S. invicta* internal microbes, fungi and bacteria specifically. Methodologies included disinfecting the external body of the ant, and thus exclude the identification of microbes likely encountered by ants in their environment (Woolfolk *et al.*, 2016a; Woolfolk *et al.*, 2016b). To better understand possible bacteria interactions affecting the foraging responsiveness of *S. invicta*, we need to first be able to identify and quantify bacteria present in their habitat. Based on the results

from Chapter I, *P. mirabilis* was found in several colonies of *S. invicta,* but at a very low abundance. *P. mirabilis* is likely a facultative bacterium in the ecology of *S. invicta,* encountered in smaller quantities and constantly ubiquitous in the environment. This study monitored *S. invicta* foraging behavior where food is in close proximity to high density abundances of *P. mirabilis*. The bacteria association had minimal impact on *S. invicta* foraging efforts, further exemplifying the resilience and adaptability of *S. invicta* to a wide variety of environments, even those with dense bacteria populations.

In summary, the present study provides evidence that *P. mirabilis* does not consistently play a significant role in the foraging responsiveness of *S. invicta.* Bacteria density dependent patterns were noted when assessing the foraging rate to a honey water bait exposed to *P. mirabilis* at 10^5 CFU/mL. However, within colony variances in foraging strategies make quantification of such patterns difficult. In addition, the study presented a model for quantifying the behavioral impacts of multiple quorum sensing bacteria concentrations on an invasive ant species. This model can be used in the future to assess a more pertinent bacterium associated with the behavioral ecology of *S. invicta* or another ant species where similar foraging behavior and resource discovery takes place.

CHAPTER V

SUMMARY AND CONCLUSIONS

The studies described in this dissertation used interdisciplinary tools in entomology and microbiology to study a putative interkingdom interaction between quorum sensing bacteria *P. mirabilis* and the red imported fire ant *S. invicta.*

First, the bacterial communities associated with *S. invicta* needed to be identified and assessed according to ecological regions, separate colonies, and among the various colony members which contribute diverse functions to the whole. To date, *S. invicta* bacterial studies had mainly focused on bacteria of a particular caste or colony member, and collected in a single region. Without the sampling of *S. invicta* in different regions, we were continuing to selective define bacteria associated with this invasive ant species. This study represents a valid assessment of alpha and beta diversity across ecoregions that vary in abiotic and biotic factors, for example, temperature, rainfall, plant and animal fauna. Ecological niches associated with the ecoregions samples were the defining structure for the construct of bacteria associated with *S. invicta.* More importantly, further assessment revealed the shared patterns of alpha and beta diversity across colony functional grouping, primarily defined by adult castes, immature brood, and dead midden. Not only did the colony functional groupings share dominant bacterial taxa across ecoregions, but also phylogenetic diversity pattern likely established to their environmental exposure and function within the ant colony. Further studies are essential to determine the underlying regulatory mechanism and biological functions of key

identified bacteria. Why do these bacteria dominate the community? What are these bacteria doing in the community?

By identifying *S. invicta* bacterial communities, OTUs referenced as taxa belonging to the genus *Proteus* were also identified. *Proteus* bacteria was found present in all but three colonies, namely, colony 2, 3 and 10, and in all colony functional categories, and were accounted for in all ecoregions. With this discovery, behavioral experiments used to identify the possible interactions between *P. mirabilis* and *S. invicta* followed. Based on *S. invicta* biology, nestmate recognition and foraging behaviors where olfactory reception and interaction with the environment are vital for individual and colony survival. These were selected as behaviors to test alongside *P. mirabilis* quorum sensing presence. Insects that readily forage on honey dew and nectars, rely on honest signals and cues from plants to consume these resources (Raguso, 2008). Similarly, environmental cues associated with nutritional resources and feeding, whether it be to a host-feeding insect, a parasitoid, or a scavenger, should activate some feeding behavior response (Cotes *et al.*, 2015; Cusumano *et al.*, 2015). However, foraging behavior of *S. invicta* did not exhibit a significant difference in response to the presence of *P. mirabilis,* specially for high protein and balanced baits. Ants removed little bait from the granular diet exposed at 10^3 CFU/mL. Still, due to high variances in bait removal of other concentrations, these results were not significant. An identified aversion to the presence of bacteria when exploiting a nutritional resource, would coincide with studies conducted in the past. Previous studies observed ant adverse responses to microbial parasites, specifically, fungi (Tranter *et al.*, 2015). *Polyrhachis dives* Southeast Asian weaver ants,

Messor barbarous European seed-harvesting ants, *Acromyrmex echinatior,* Panamanian leaf-cutting ants, and *Formica rufa* European wood ants all showed some detection of fungal threat, reducing travel around fungi-contaminated sites and grooming more often. Similar studies involving *S. invicta* exposure to fungi, noted behavioral changes in alkaloid substance exposure, a self-medication response by infected ants (Qiu *et al.*, 2014; Qiu *et al.*, 2016).

The profile structure of signals used by ants to recognize conspecific nestmates continue to be unknown. *P. mirabilis* did not affect the response of nestmate interactions. Exposing two non-nestmates to the same bacteria-treated environment which established the same bacterial profile via the treated-soil exposure, did not sufficiently mask or establish a nestmate recognition cue in *S. invicta.* This study did, however, provide an ecologically relevant laboratory model of the inoculation of bacteria that likely takes place in the natural environment. The bacterial load concentrations and transfer protocol can be used to further understand the potential role of *S. invicta* and other ants as mechanical vectors of pathogenic bacteria causing foodborne illness and nosocomial infections.

Information about the behavioral ecology of the red imported fire ant *S. invicta* will continue to be explored. Bacteria identified from the bacterial community study can serve as a guide for the selection of important bacteria related to *S. invicta* biology. New *S. invicta*-bacteria models will provide more explanations driving behavioral mechanism and adaptations contributing to the success of this ant in novel environments.

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