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Effects of hybridization and life history tradeoffs on pathogen resistance in the Harvester ants (Pogonomyrmex)

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EFFECTS OF HYBRIDIZATION AND LIFE HISTORY TRADEOFFS ON PATHOGEN RESISTANCE IN THE HARVESTER ANTS (*POGONOMYRMEX*)

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ABSTRACT

A fundamental challenge faced by all organisms is the risk of infection by pathogens that can significantly reduce their fitness. The evolutionary dynamic between hosts and pathogens is expected to be a coevolutionary cycle, as pathogens evolve by increasing their level of virulence and hosts respond by increasing their level of resistance. The factors that influence the dynamics of adaptation by pathogen and host in response to one another are not well understood. Social insects live in dense colonies in high-pathogen soil environments, making them an ideal model system to study the factors influencing the evolution of pathogen resistance. In this thesis work, I investigated several alternative hypotheses to explain patterns of host resistance to entomopathogenic fungi in the harvester ant genus *Pogonomyrmex*: that high resistance is associated with high environmental pathogen loads, that local adaptation leads to increased resistance to coevolved pathogen populations, that life history tradeoffs increase allocation to resistance in harsher environments, and that increased genetic diversity caused by interspecific hybridization enhances inherent resistance. First, I characterized patterns of spatial variation in abundance and diversity of fungal pathogens among habitats of *Pogonomyrmex* species. I found 17 genera of fungi in the soil, six of which were entomopathogenic. Lower precipitation habitats, where *P. rugosus* occurs, had the lowest diversity, while the highest was experienced by the H lineage, one of two hybrid populations. When actual infection rates of field-caught workers were compared, the mesic-habitat *P. barbatus* was infected significantly more often. These results suggest that habitat does plays a role in fungal diversity, and that species are exposed to more entomopathogens may be more likely to get infected. Second, I tested experimentally whether hybridization and or habitat differences play a role in pathogen resistance by testing the effect of soil type and species identity on infection rates in pupae of the two species and their hybrids. This experiment showed *P. rugosus* ants had the highest inherent resistance to infection, supporting the life history tradeoff hypothesis. This suggest that *Pogonomyrmex* ants species are allocating their resources differently according to their environment, with more stressful environment leading to less investment in reproduction and more in protection against pathogens. Overall our study shows that environment plays a role in differences in infection risk, while genetic effects such as hybridization may not play a role in pathogen resistance.

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CHAPTER 1

COMPREHENSIVE LITERATURE REVIEW

Host-pathogen coevolution

Pathogens and their hosts are constantly evolving in response to one another, similar to the arms race scenario of the Red Queen hypothesis. Coevolution of host and pathogen should drive molecular evolution through continual natural selection and counter adaptation (Van Valen, 1973; Hughes and Boosma, 2004). In the case of pathogens, interaction with its host plays an important role in their evolutionary trajectory. There are a few common host-parasite interaction models used in the Red Queen hypothesis. One is the matching allele model, which occurs when the host can detect and eliminate parasites carrying alleles that differ from its own (Luijckx *et al.*, 2013). The second is the inverse matching alleles model, in which the host's alleles have the ability to recognize a particular allele in a parasite. Lastly, the gene-for-gene model suggests that a host can resist a parasite only if the host expresses a resistance allele, A, and the parasite expresses a noninfectious (avirulent) allele, A (Sasaki, 2000; Agrawal and Otto, 2006).

Pathogens adapt by increasing their level of virulence, which imposes selection on hosts to increase their resistance. This coevolutionary process can lead to strong differences in pathogen impact among individuals and populations, as pathogens adapted to infect one genotype in particular may not be able to infect other genotypes (Hughes and Boomsma, 2004). Similarly, hosts may vary in their resistance to infection when faced with local or novel pathogens. This hypothesis assumes that parasites genotypes

adapt to host when they become locally common (Jaenike, 1978). There are some costs, however, associated to the evolution of both virulence and resistance. When you have a highly virulent pathogen it can harm itself by reducing the population size of available hosts, therefore it might limit the transmission of the pathogen by limiting the movement of the host (Mosquera and Andler, 1998). Similarly, a highly resistant organism, especially one with many immune genes such as in some plants, can impact their reproductive fitness (Burdon and Thrall, 2003).

Selection for resistance may be particularly important for species that are highly social, because infectious diseases can potentially spread more easily between group members (Schmid-Hempel, 1994, 1998; Hughes and Boomsma, 2004; Cremer *et al.*, 2007). A great model system to understand disease transmission in societies is the social insects. Eusocial insect colonies are made up of highly related individuals that live in close proximity in the environment, and living in close proximity can facilitate the spread of infectious diseases like pathogens and parasites. Their cooperation also increases the efficiency in predation defenses. Social insect colonies can be infected by a great diversity of parasites and pathogens, including arthropods, helmith worms, fungi, bacteria, viruses and protozoa (Cremer *et al.*, 2007).

Pathogenic Fungi

Pathogenic fungi have a broad geographical distribution; however, their specific habitat type confines them. Habitat types are known to be important criteria for identification of insect-pathogenic fungal strains (Bidochka *et al.*, 1998; Bidochka *et al.*,

2002; Devi *et al.*, 2006). There are approximately 56 fungi genera known to be entomopathogenic (Tanada and Kaya, 1993). Entomopathogenic fungi are capable of infecting a wide range of hosts. For example, the entomopathogenic fungus *Beauveria bassiana* and *Metarhizium anisopliae* are known to infect over 200 species of insects but isolates of these two species have a higher degree of specificity (Maurer *et al.*, 1997; Devi *et al.*, 2006). Sanchez-Peña (2007) showed specificity of isolates when he used different isolates of *Beauveria bassiana* and was able to infect different insect pests: a greenhouse whitefly, a fall armyworm, and a potato psyllid.

Insects are usually infected by fungal propagules like spores or conidia, zoospores (motile asexual reproductive cells) and plantos or ascospores (Tanada and Kaya, 1993; Schmid-Hempel, 1998). Fungi can reproduce sexually and asexually. During asexual reproduction conidiophores release a conidium that lands on the cuticle of the host. Once the conidium gets in contact with the insects' surface, it can produce a secondary conidiophore, also known as the capilliconidium, which can become the source of the infection (Figure 1.1).

Once a conidium has encountered an insect host, there are three basic steps to fungal infection in insects. First, the formation of the infection structure occurs, and during this step the fungal spore penetrates the insect through the exoskeleton or cuticle. In some species of fungi they may also infect the insect through the tracheal opening such as in *Beauveria sp*. Fungal spore has to go through two layers, the outer epicuticle and procuticle. The conidium adheres to the surface of the insect, germinates, and often differentiates into an appresorium. Second, the fungi penetrate the cuticle, which

involves enzymatic degradation (chitinases and proteases) of the epicuticle and mechanical pressure (growing fungal tip) applied during the penetration of the procuticle. Lastly, the fungi penetrate the epidermis and enter the hemocoel where the hyphal body differentiates into blastospores; this helps with the dispersion and colonization of the haemocoel. Here fungi produce toxins that may cause death to the insect and it is attacked by the immune system and hemocyste, in defense fungi may or may not phagocytose and encapsulate itself (Hajek and St. Leger, 1994; Clarkson and Charnley, 1996; Castrillo *et al.*, 2005). Once a fungus kills the host, it continues to grow saprophytically and produces reproductive spores that are dispersed.

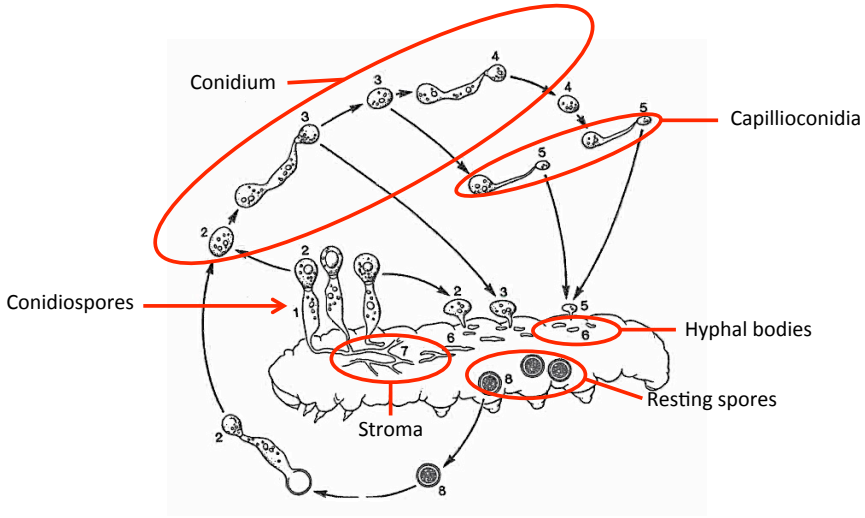

Figure 1.1. Typical life cycle of an fungus. The endostroma in the insect host produces conidiospores. A primary, secondary and tertiary conidium is formed when no further host are available. Capilliconidia are the major infective units. After successful infection, hyphal bodies are formed and that develop into mycelia and stroma, which also produce resting spores (Tanada and Kaya, 2003).

Study organism

The genus *Pogonomyrmex* is one of the most abundant genera of harvester ants located in semiarid regions of North America. The closely related species

Pogonomyrmex barbatus and *Pogonomyrmex rugosus* are the largest harvester ants found in the southwestern United States (Whitford *et al.*, 1975; Hölldobler, 1976). Hybridization between these two species of harvester ants has given rise to multiple hybrid populations ("G", "F", "H" and "J") that are highly ecologically successful (Helms Cahan and Keller, 2003; Anderson, 2006a,b; Schwander *et al.*, 2007a). Schwander and colleagues (2007a) found that F, G, and H lineages are derived from a single origin, while J lineage originated separately. F, G, and H individuals are similar in appearance to *P. rugosus,* while J individuals are more similar in appearance to *P. barbatus* (Anderson, 2006).

One population that shows a consistently high level of heterozygosity, especially when compared to closely related parental species, is the hybridizing harvester ants. Each population is composed of two genetically distinct lineages (H1 and H2 & J1 and J2) (Helms Cahan and Keller, 2003; Schwander *et al.*, 2007a). In contrast with the parental species, reproductive caste is genetically determined; mating with males of the same lineage produces daughter queens, while workers are only produced from mating with males of the opposite lineage. As a result, workers in the colony are significantly more heterozygous in relation to queens and males (Helms Cahan and Keller, 2003; Helms Cahan and Keller, 2004; Anderson *et al.*, 2006a,b; Schwander *et al.*, 2007a,b) (Figure 1.2). This system is specifically important to test whether the increased heterozygosity increases resistance in the hybridizing population.

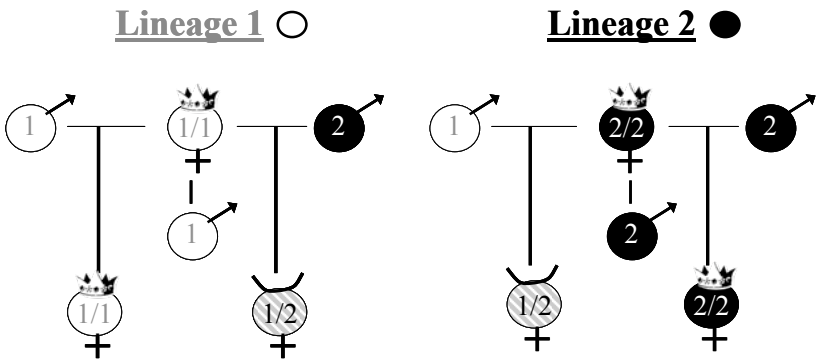

Figure 1.2. Schematic showing the genetic caste determination in hybridizing lineages. Each number represents the lineage of the individual. The crowns represent queens and workers are represented by the horns.

There is evidence that some of the subpopulations of the harvester ants have different levels of heterozygosity. This allows us to test for correlation in pathogen resistance among these hybrid populations. Nuclear markers suggest *P. rugosus* has a higher contribution to the lineages H1 and J1 than *P. barbatus*. *P. barbatus* exclusively contributes to H2 and J2 (Helms Cahan and Keller, 2003) (Figure 1.3). The *cox*1 mitochondrial haplotypes show J lineage as a paraphyletic group, where J1 is more closely related to *P. rugosus* and J2 is more closely related to *P. barbatus* (Helms Cahan and Keller, 2003). Schwander and colleagues (2007a) looked at the distribution of the lineages and suggested that the genetic caste determination might have originated in eastern New Mexico or west Texas, and small founder populations radiated west (H and G lineage pair diverge from F pair) where the lineages' genetic caste determination evolved by introgressive hybridization between *P. barbatus* and *P. rugosus* (Schwander *et al.*, 2007b). As a result of these small founder populations, there was a loss of genetic variation in these populations. Both phenotype and genotypes are distinctively different from the parental populations.

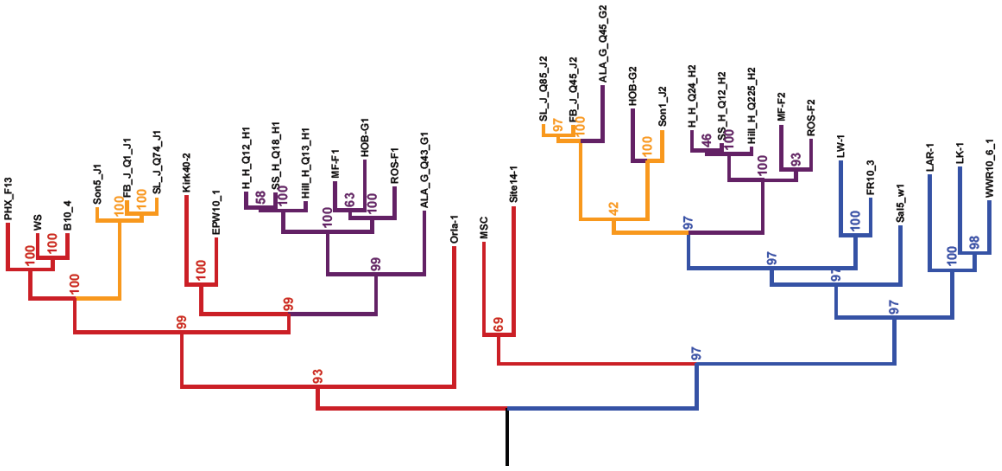

Figure 1.3. Maximum-likelihood consensus tree. Nuclear markers show the relationship between parental species and hybrids. Each interbreeding lineage is derived from a different parent species. Red branches represent *P. rugosus* species, yellow branches represent J1/J1 lineages, purple branches represent H1/H2 lineages and blue represents *P. barbatus* species. Tree constructed by Andrew Nguyen.

Project Goals

My thesis work aims to assess the role of hybridization on pathogen resistance in the ant genus *Pogonomyrmex*: (1) determine abundance and diversity of fungi among habitats of *P. barbatus*, *P. rugosus*, and two lineages of hybrid origin and (2) test experimentally whether hybridization and/or habitat differences play a role in pathogen resistance; To accomplish Objective 1, I characterized entomopathogenic fungi in *Pogonomyrmex* species and compare abundance and diversity of pathogenic fungi between habitats. To be able to make better predictions on the effects of pathogens on our species we first need a survey of the different fungal pathogens they encounter in their natural habitats. To accomplish Objective 2, I exposed ant pupae to their different soil habitats and compare the infection frequency of fungi for each species and hybrid lineage.

CHAPTER 2

DETERMINING ABUNDANCE, DIVERSITY AND COMPOSITION OF FUNGI IN *POGONOMYRMEX*

Abstract

Fungi are important pathogens of insects that are ubiquitous in soil environments. In social insects, especially ants, little is known on fungal diversity and composition in their environment. In this I study wanted to characterize and identify fungal diversity and composition across the *Pogonomyrmex barbatus* complex of harvester ants, which includes two species whose ranges differ in precipitation and soil moisture and two populations of hybrid origin that occupy a climatically intermediate zone. I hypothesized that precipitation is a driver of fungal diversity and composition, where higher precipitation habitats support a higher diversity and abundance than lower precipitation habitats. Using soil, ant and queen samples collected in the field, I isolated fungi and identified cultured colonies to genus. Diversity, abundance, composition and infection frequency were determined for each of the species/lineages. A total of 17 genera were found, which six were entomopathogenic. Abundance and richness did not differ significantly across the species' ranges, but Simpson's index of diversity did reveal some differences in the expected direction, where the desert-occupying *P. rugosus* had the lowest diversity relative to the other taxa, although regression of diversity against putative environmental variables did not indicate a significant relationship. For infection frequency, *P. barbatus* workers were more likely to be infected than *P. rugosus*, while H lineage, one of the two hybrid populations, had the lowest fungal spores in workers. Our results showed that habitat does plays a role in fungal composition but precipitation may not be a reliable predictor of abundance and diversity of fungi.

Introduction

Eusocial insect colonies are composed of highly related individuals that live in close proximity in the same environment. Living in groups increases the efficiency of brood care, foraging, and predation defenses; however, it also poses a tradeoff as infectious diseases can potentially spread more easily between individuals (Cremer *et al.*, 2007; Schmid-Hempel, 1994, 1998; Hughes and Boomsma, 2004). Because they nest predominantly in microbially-rich environments such as soil, ants are particularly likely

to face the challenge of living with a high density of pathogens. Indeed, the shift to a subterranean lifestyle in ants is hypothesized to have been facilitated by the evolution of the metapleural glands, paired structures situated at the posterolateral corners of the mesosoma that secrete chemical compounds that act as antimicrobial agents (Schild-Knecht and Koob, 1970, 1971; Do Nascimento *et al.*, 1996).

One important class of pathogens that can infect ants is the entomopathogenic fungi. Fungi are ubiquitous in the soil environment, and many opportunistically or obligately infect juvenile and adult insects (Branco, 2011). Insects are usually infected by fungal spores or conidia (Tanada and Kaya, 1993; Schmid-Hempel, 1998), which are most commonly found in soil but can also disperse through cadavers and wind currents (Meyling and Eilenberg, 2007). There are 700 species of entomopathogenic fungi known, although very few have been studied in any detail (Hajek and St. Leger, 1994). Just two of these, *Beauveria bassiana* and *Metarhizium anisopliae,* are known to infect over 200 species of insects, although specific isolates can have a higher degree of specificity (Maurer *et al.*, 1997; Devi *et al.*, 2005). Once a conidium has encountered an insect host, it forms an infection structure with which it penetrates the insect through the exoskeleton or tracheal opening through a combination of enzymatic degradation and mechanical pressure. In most insect orders, the nymphal or larval stages are infected more often than the egg, pupal and adult stages (Naug and Camazine, 2002). Even within the larval stage, resistance to fungal infection may vary over time. In the potato beetle, *Leptinotarsa decemlineata*, younger larval instars are more susceptible to infection with *B. bassiana* (Scheffenberg, 1957), while for the corn earworm, *Helico verpazea*, larvae are more

susceptible to *Nomuraea rileyi* in the third and fifth instar (Mohamed *et al.*, 1985). Enhanced resistance to fungal disease in the pupal stage may be associated with the development of the waxy epicuticular layer compared to larval stages, which are forming integuments and gut epithelium, exposing them to fungal penetration (Tanada, 1955).

The abundance, composition and virulence of entomopathogenic soil fungi vary as a function of climatic factors and land-use patterns (Barker and Barker 1998, Bidochka *et al.*, 1998; Bidochka *et al.*, 2002; Devi *et al.*, 2006; Zimmerman and Vitousek, 2012). Globally, fungal diversity declines with increasing latitude (Meiser *et al.,* 2014). At a more local scale, humidity, temperature, soil pH, light and soil content, including nitrogen and carbon mineralization, are important factors influencing community composition (Rydin *et al.*, 1997; Fiere *et al.*, 2003; Setala and McLean, 2004; Rousk, 2010; Hawkes *et al.*, 2011; Branco, 2011). Humidity and temperature are directly associated with spore germination and infection of pathogenic fungi (Tanada and Kaya, 1993); > 90% relative humidity is essential for fungal sporulation and cuticle penetration, but release of conidia is stimulated by low humidity, darkness and vibration (Tanada and Kaya, 1993). Temperature requirements vary between fungal species and ecological niches. The optimum temperature for development, survival and pathogenicity is generally between 20 to 30°C (Lipa, 1975; Samson *et al.*, 1988; McCoy, 1981; McCoy *et al.*, 1988), but species found in tropical and subtropical usually germinate in temperatures above 25°C, while species from temperate areas have a lower optimal temperature. A poor nutritional environment can also significantly reduce fungal germination (Samson *et al.,* 1988; Tanada and Kaya, 1993). Soil acidity can also affect fungal growth; growth

tends to be highest at a pH of 4.5 and can decrease growth by a factor of more than 5 toward a high pH (Rousk, 2010). The pH can also affect fungal species diversity, with a weak positive relationship to pH and diversity (Rousk *et al.*, 2010). Thus, insect species that inhabit a broad ecological range may be expected to experience pronounced spatial variation in identity and abundance of fungal pathogens.

The genus *Pogonomyrmex* is one of the most abundant genera of harvester ants in semiarid regions of North America. The closely related species *Pogonomyrmex rugosus* and *P. barbatus* are the largest harvester ants found in the southwestern United States (Whitford *et al.*, 1976; Hölldobler, 1976; Figure 2.1). *Pogonomyrmex rugosus* occurs from southern California to Southern New Mexico where habitat is more arid, with a mean annual precipitation across their range of approximately 5 cm, while *P. barbatus* is found in much more mesic habitats in northern Mexico and across the southern half of Texas that can receive an order of magnitude more precipitation annually (~50 cm, Anderson, 2006; www.nws.noaa.gov). Hybridization between these two species has given rise to two hybrid populations ("H" and "J") occurring across a wide range in an intermediate climate zone from southeastern Arizona to southwestern Texas (Helms Cahan and Keller, 2003; Anderson, 2006a,b; Schwander *et al.*, 2007a). This climate gradient from west to east suggests that the two species and their hybrids may encounter very different fungal environments in terms of overall abundance, diversity and community composition.

In this study, I characterized fungal environments and natural infection frequencies in workers and queens across the range of *P. barbatus*, *P. rugosus*, and their

hybrids. I hypothesized that the higher precipitation habitats occupied by *P. barbatus* support a higher diversity and abundance of fungi, including entomopathogens. In addition, I hypothesized that hybridization may confer increased pathogen resistance (Jackson and Tinsley, 2003), which would be manifested in lower infection incidence along with higher environmental fungal prevalence in the hybrids' geographic range relative to their parent species. To test these hypotheses, I determined abundance and diversity of fungi by culturing fungi from soil samples at multiple sites within the ranges of *P. barbatus*, *P. rugosus*, and the hybrid lineages compare them across their habitat. To evaluate natural infection frequencies, I paired the soil survey with fungal culturing of internal tissue from field-collected workers and founding queens.

Materials and Methods

Sampling procedure

Samples were collected from Arizona, New Mexico, and Texas during July 2011. Soil and worker samples were collected from 3 locations for each species and hybrid population (Figure 2.2). I also collected 50-60 newly mated queens from 1-2 sites per species (Figure 2.1). Sampling locations for each species were separated from neighboring sites by at least 100 kilometers to minimize spatial autocorrelation. At each location, colonies were haphazardly selected with a minimum distance of 10 meters between colonies. At each colony, one soil sample was taken one meter from the entrance of the colony at 360° degrees (due north). Using a hand trowel sterilized with 70% ethanol, soil samples were collected at a depth of 5 to 10 cm after removing surface litter.

Each soil sample was sealed in a sterile plastic bag (4½"W x 7"L) and refrigerated. Ten workers were also collected from each colony; when possible, I collected workers on the nest surface; on occasions where the colony was not active, the entrance was lightly excavated and exiting workers were collected. Each worker was placed in an individual 1.5 mL centrifuge tube and refrigerated. We also collected 50-60 newly mated queens from each species, this were collected during the nuptial flights. Nuptial flights occur a day after heavy monsoon rainstorms. The flight starts in the afternoon but time varies between species and lineage. For J lineage they start leaving the colony approximately 15:30 PST; H lineage flights starts approximately 16:00-17:15 PST. For parental species, *P. rugosus* mating start approximately 16-30-18:00 PST and *P. barbatus* 15:30-17:00 PST. Once they start flight, we followed them to their mating swarm. Flight last about one hour, once queens mated they where picked off the ground and place in a tube with cotton.

Isolation of Fungi from soil, ant and queen samples

To determine the fungal diversity present at each site, fungi were cultured from each of the three soil samples collected per site. To standardize the dry mass of soil analyzed, soil moisture was measured for five 1g subsamples from each colony soil sample using an OMNIMARK moisture analyzer (Sartorius Corporation) at 100°C for 10 minutes. Depending on the soil moisture content per colony sample, a soil suspension was created by adding 5-10g of soil in 20 mL of water solution with Silwet-L77 (0.02%) for 5 minutes with approximately 20 solid soda lime glass beads (3½ mm). Ten mL of

each suspension was poured through a cheesecloth filter, resuspended by vortexing, and a subsample diluted to 1/10 and 1/100 dilutions.

To isolate fungi from internal ant tissue, I used 2 ant workers per colony. To minimize external microbial contamination, individuals were washed with 75% ethanol for 5 seconds, and then rinsed in sterile distilled water. Insects were surface sterilized in sodium hypochlorite (2.5%) for 30 seconds and rinsed in three changes of sterile distilled water.

Fungal infections in queens were surveyed only for those queens that died in the laboratory during the colony-founding period. Newly mated queens were placed in individual test-tubes containing a water reservoir stoppered with cotton at one end and reared in constant darkness at 30°C and 60% humidity Sanyo incubator at 30 for 60 days. Queens do not feed during this period. Queens were censused every two days and any dead queens were removed. Queens were surface sterilized in the same manner as workers.

Three different media were used for the inoculation of soil and ant suspensions: potato dextrose agar with antibiotics to suppress bacterial growth, a selective medium for isolation of *Beauveria bassiana*, and a selective medium for *Metarhizium anisopliae*. *Beauveria bassiana* was prepared with 10 g of neopepton, 40 g of dextrose, 10 g of yeast extract, 15 g of agar, 1.1 g of syllit (fungicide), 0.1 g of rose Bengal (color dye), 1 mL of stock solution of penicillin G and 2.5 mL of stock solution of streptomycin for a total of one Liter. *Metarhizium anisopliae* medium was prepared with 72.5 g of oatmeal agar, 1 g of syllit, 4 mL of stock solution of benlate, 4 mL stock solution of crystal violet (color

dye), 1 mL of stock solution of penicillin G and 2.5 mL of stock solution of streptomycin for a total of one Liter (Goettel and Inglis, 1997). All media were solidified in 9 cm diameter petri dishes.

Soil samples were cultured in three replicates of 20 µl for each dilution level. Worker ant and queen tissue was cultured with two methods. Each individual ant was bisected, and one half was left intact and placed in the center of the medium, and the other was homogenized and streaked across the plate. Inoculated plates were incubated at 22 ± 2^0C, and after 14 days the numbers of fungal colonies (CFU) were recorded under a dissecting microscope at 40X power.

Identification of Fungi

To identify the CFUs, each fungal colony was identified to morphotype and a single representative of each fungal morphotype was inoculated onto a new plate with a sterilized needle. Identification was based on conidium development, morphology, and conidiogenesis. I prepared whole mount slides by staining a small piece of fungal hyphal tissue with methylene blue and placing it in the center of a microscope slide. All slides were observed under a microscope at 100X power. Fungi were classified as pathogenic if one or more members of the genus are known to infect insects, and non-pathogenic if they were antagonistic, plant pathogens (phytopathogenic) or saprophytic.

For CFUs that could not be unambiguously identified morphologically, I confirmed their identity genetically. DNA extractions were made using a Plant Tissue DNA extraction kit (Mini Protocol from Qiagen). After extractions were performed, a

portion of the Internal transcribed spacer (ITS) gene region (~500 bp) was amplified (Viaud *et al.*, 2000; Anderson and Cairney, 2004; Schoch *et al.*, 2012). Polymerase chain reaction (PCR) was conducted in 30-µl reactions using the fungus-specific forward primer ITS-5 F (5' GGAAGTAAAAGTCGTAACAAGG 3') and the reverse primer ITS-4 (5' TCCTCCGCTTATTGATATGC 3'), manufactured by Invitrogen Custom Primers (Carlsbad, CA). PCR was carried out using the following amplification conditions: initial denaturation at 94°C for 2 mins, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR product was examined on a 2% agarose gel and successful amplifications were sent for bi-directional Sanger sequencing. Geneious version Pro 5.6.5 was used to annotate and align the sequences. We conducted a Blast search of each sequence against the NCBI nr database to identify the best-hit fungal species; only matches with a maximum E-value threshold of 0.1 were retained.

Statistical Analyses

Analysis of Alpha-Diversity

Fungal abundance and diversity were calculated for both the complete set of fungi (pathogenic and non-pathogenic). The total abundance per soil sample was calculated by counting the total number of colonies per gram of soil between the three colonies. To determine alpha-diversity, I used generic richness and the Simpson's Index. Richness was determined by the number of genera found per site, corrected by the sample size. Generic Richness, also known as G_{total} or is the total number of genera in the community, was

calculated with the following equation:

$$G_{total} = G_{observed} + \left(\frac{a^2}{2b}\right)$$

Where $G_{observed}$ is the total number of genera identified. The variable *a* is the number of genera represented by exactly one individual ("singletons"), and *b* is the number of genera represented by exactly 2 individuals ("doubletons"). To calculate diversity, I used the Simpson's Index with the following equation:

$$D = \frac{1}{\sum p_i^2}$$

Where p_i is the proportion of individuals belonging to genus *i*.

To test whether alpha-diversity measures varied among sites and species range, I conducted nested ANOVAs with site nested within species in the program JMP Pro 10 (SAS Institute Inc., 2007).

Analysis of Beta-Diversity

To visualize and test for variation in fungal composition, I performed a multidimensional scaling (MDS) analysis using the *vegan* package in R. Differences at the species and site level were tested with a permutation ANOVA with 999 permutations.

Environmental predictors of fungal community metrics

To test whether environmental factors drive patterns of fungal diversity and infection frequency, I regressed each community metric against three principal components derived from four environmental predictor variables (elevation, observed

mean soil moisture from collected soil samples, mean annual temperature and mean annual precipitation) using a multiple regression design in JMP. Mean annual temperature and precipitation were extracted from the Bioclim database (http://worldclim.org/bioclim).

Results

Characterization of Fungal Community

A total of 17 fungal genera were found when summed over all sample types and across the entire range of the two species and their hybrids, of which two were classified as antagonistic, four were phytopathogens, five were saprophytic and six were entomopathogenic (Table 2.2).

The soil and ant tissue surveys differed significantly in both the number and identities of fungi found ($X^2 = 21.955$, df = 8, $p < 0.005$*; Figure 2.3). Soil samples had a total of 15 genera (Figure 2.3a), with the highest colony forming units (CFUs) being *Aspergillus* (35.7%), *Penicillium* (30.5%), and *Paecilomyces* (16.4%). The remaining the genera had fewer than 10 CFUs. Of the total, four genera were classified as entomopathogenic: *Paecilomyces* (16.4%), *Fusarium* (6.7%), *Metarhizium* (0.2%), and *Lecanicillium* (0.2%). Worker internal tissue contained a total of seven fungal genera, where *Beauveria* had the highest number of CFUs with 61%, followed by *Penicillium* at 18% and *Alternaria* with 10% of CFUs (Figure 2.3b). Two genera were entomopathogenic, with the majority from the genus *Beauveria* (61%) and the remainder from *Paecilomyces* (2%). Queen samples also had a total of seven fungal genera (Figure

2.3c), where *Aspergillus* was the most abundant (89.3%). Of the three entomopathogens in queens, *Beauveria* had the highest percentage (9.44%), followed by *Fusarium* (0.26%) and *Lecanicillium* (0.07%).

Geographic variation in fungal alpha-diversity

Total abundance in soil samples did not differ among the ranges of the four ant taxa (Q = 2.63, df = 3, $p = 0.4648$) (Figure 2.4). We did see differences in sites within soil samples (Q = 3.38, df = 8, $p < 0.001$*). Generic richness showed a trend toward lower richness in *P. rugosus*, while H lineage soil showed the highest richness and the J lineage and *P. barbatus* were intermediate, respectively, although this trend was marginally not statistically significant ($p = 0.064$) (Figure 2.5). Simpson's Diversity was found to be significant between soil samples ($p = 0.0083$*). *P. rugosus* soil samples had significantly lower diversity than H lineage ($p = 0.0060$*). Soils from the ranges of the J lineage and *P. barbatus* were intermediate and not significantly different from *P. rugosus* or the H lineage (Figure 2.6).

The most common fungal genera across sites in soil were *Aspergillus, Paecilomyces, Cladosporium* and *Penicillium*. Seven genera were uniquely found at a single site: *Trichoderma* (J lineage: Sierra Vista, AZ), *Scedosporium* (J lineage: Willcox, AZ), *Xylohypha* (*P. rugosus*: Florence Jct, AZ), *Alternaria* (H lineage: Las Cruces, MN), *Diaporthe* (H lineage: Sierra Vista, AZ), *Metarhizium* (*P. barbatus*: Luckenbach Rd., TX), and *Lecanicillium* (*P. barbatus*: Brady-Eden, TX) (Figure 2.7). Generic composition varied significantly among sites (permutation ANOVA, $p < 0.05$*) but was marginally

not significantly different among species ($p = 0.056$; Figures 2.8, 2.9).

Soil types also differed in the functional diversity of fungi found, with the J lineage and *P. barbatus* having significantly more functional groups than *P. rugosus* and the H lineage ($X^2 = 9.348$, df = 3, $p < 0.025$*; Figure 2.10). The most abundant functional group was saprophytic for all sites except for one J site, Sierra Vista, AZ, with only half saprophytic fungi and one *P. barbatus* site, Brady-Eden, TX, where the most abundant functional group was entomopathogenic. Phytopathogenic fungi were only found in 4 sites, all in J lineage and *P. barbatus* habitat. Antagonistic fungi were only found in two sites, Sierra Vista, AZ and Willcox, AZ, again both in J lineage and *P. barbatus* habitats. Entomopathogenic fungi were found across all soil types, but were similarly most abundant in Sierra Vista and Brady-Eden (Nested ANOVA: $p = 0.060$, lineage effect; $p < 0.05$*, site effect).

The four taxa varied significantly in infection frequency in internal worker tissue ($X^2 = 14.52$, df = 3, $p = 0.003$*), with the highest frequency in *Pogonomyrmex barbatus* workers and the lowest in *P. rugosus* and the H lineage ($X^2 = 7.815$, df = 3, $p < 0.005$*) (Figure 2.11). J lineage was intermediate and did not differ from any of the other taxa. Queen fatalities associated with fungal infection were low and did not differ significantly among species, although there was a trend for higher fungal-associated mortality in *P. rugosus* queens ($X^2 = 6.66$, df = 3, $p = 0.08$; Figure 2.12).

Combining the occurrence of entomopathogenic genera across all three sources (soil, workers, queens) revealed variation among entomopathogenic fungi in their geographic occurrence, from widespread to highly species-specific (Table 2.3).

Comparing the pathogenic fungi, *Paecilomyces* was found in all regions but primarily in soil, within only one occurrence in a worker sample. *Isaria* was similarly found in soil from all ant species except the J lineage, but not in worker or queen samples. In contrast, *Metarhizium* was exceedingly rare, occurring only in a single *P. barbatus* soil sample. *Beauveria* was found in only 3 worker samples of *P. barbatus,* but at high abundance when it occurred, with 71 CFUs cultured. *Fusarium* was only found in the parental species, in both soil and workers. *Lecanicillium* was found in all species except the H lineage, and was the only entomopathogen to be widespread in queens, although absent from workers. When considered from the perspective of the hosts, *P. barbatus* overlapped with all the fungal pathogens in their habitat, while *P. rugosus* overlapped with only four. The H lineage overlapped with the least entomopathogens with only two genera, while the J lineage overlapped with three entomopathogens.

Environmental predictors of fungal community metrics

The first two principal components (PC) for environmental predictors showed that there is a positive correlation between MAT and MAP, while MAT was negatively correlated to soil moisture and elevation. Elevation was positively correlated to soil moisture and negatively correlated to MAT and MAP (Table 2.4; Figure 2.13). PC1 (56.2%) had a higher loaded value for MAT than MAP. PC2 (24.9%) also showed a higher loaded value for MAT than soil moisture, while PC3 (18.2%) showed higher loaded values for MAP and soil moisture than for MAT and elevation (Table 2.5). The multi regressions did not show any significant effect of environmental variables on any of

the diversity measurements (total number of species, abundance, richness and diversity index) (Figure 2.14).

Discussion

In this study, I tested whether and how habitat features affect the abundance and composition of fungi that *Pogonomyrmex* harvester ants encounter in their environment, and whether exposure to potential pathogens is related to infection frequency. I predicted that higher precipitation habitats support a more diverse fungal community and a higher abundance of fungal pathogens.

Our data suggest weak support for the precipitation hypothesis. I found no significant differences in total abundance or generic richness of fungi across the habitats of the different ant species. However, one diversity measure that was found to be significant was the Simpson's Index, where the driest region, the range of *P. rugosus*, was significantly less diverse than that of the other taxa. This suggests that extreme environments may inhibit successful fungal growth or transmission and reduce pathogen pressure. Within the more mesic habitats, however, the relationship did not hold, as the H lineage, which occupies a relatively dry region, was found to have a higher diversity compared to *P. barbatus*.

To test for a quantitative relationship, I tested environmental variables that should impact the level of available moisture in the soil to see if they were good predictors for abundance and diversity. Even though a recent study has shown that distance from equator and mean annual precipitation (MAP) can have a strong effect on diversity of fungi (Tedersoo *et al.*, 2014), and other studies have found that a combination of both soil

moisture and temperature were main factors which determine microbial community structure, including effects on the fungal community (Morris and Boerner, 1999; Carletti *et al.*, 2009; Brockett *et al.*, 2012), I did not see this effect. There are a number of possible explanations for this result. Our data samples were small compared to other studies looking at diversity, while I only used a few samples per habitat some studies use metagenomics analysis to obtained diversity measurements. As a result of this, it is possible I may not have been able to detect relationships with our sampling scheme. It is also possible that the effects of the environmental variables are not additive, such that linear regression would not be able to detect the true relationships. This is suggested by the fact that only *P. rugosus* soils showed significantly reduced alpha-diversity, despite the fact that precipitation increases gradually from west to east across the region. Alternatively, the relationship may exist but be obscured due to a technical problem with the fungal culturing, which delayed the processing of two of the three *P. barbatus* sites by three weeks and may have resulted in lower-than-expected spore germination. Without these two samples, PC1 explained considerably more of the observed variation in both abundance and richness than the original model (Abundance, $R^2 = 0.144$; Richness, $R^2 = 0.227$). Finally, environmental conditions in the year of collection could have had an effect on soil moisture, and therefore fungal spore production, across the study range. During 2011, the month of July precipitation was the driest ever recorded, with about only 30-40% of normal precipitation (NOAA, http://www.srh.noaa.gov/; http://www.ncdc.noaa.gov/). The extent of the drought was more sever in the east, in direct contrast to the typical precipitation trends. New Mexico (1.70" for July 2011 and

average is 2.34") and Texas (0.74" for July 2011 and average is 2.3") were the driest, while Arizona was much close to the normal precipitation (1.76" for July 2011 and average is 1.80") and California was little higher than the normal precipitation (0.22" for July 2011 and 0.18" is average). These changes in precipitation may explain why I do not see higher diversity and richness in *P. barbatus* compared to *P. rugosus* habitats.

The main difference in fungal composition was found between sites of each species/lineage. This is consistent with results from other studies; despite their capacity for air-borne dispersal, soil fungal communities show high beta-diversity and strong distance-decay relationships at both large and small spatial scales (Hibbett *et al.,* 2011; Xu *et al.*, 2012). Studies have found that plants root exudates are able to regulate soil fungal community composition (Batten *et al.*, 2006; Broeckling *et al.*, 2008). Our species' habitat varied in the amount of vegetation that surround them; where *P. rugosus* includes creosote bush and saltbush habitats while the hybrids and *P. barbatus* occur in grasslands (Johnson, 1992). Dispersal of fungal spores can be low in these areas. Most of the areas are arid and dry in these ants' habitats so dispersal of fungal spores should not be a big factor. Ant dispersal may also be reduced by spatial patchiness of appropriate habitat in canyons and valleys separated by mountain ranges; some studies have showed that J lineage queens are unlikely to disperse to other areas colonized by another population (Hölldobler, 1974; Gordon and Kulig, 1996; Suni and Gordon, 2010).

While the soil sample survey showed a high number of total genera, the distribution found to have infected the tissues of workers and queens was less diverse and more frequently pathogenic. Our data suggest that source of pathogenic fungi for our

ants is most likely soil for 4 genera (*Lecanicillium*, *Fusarium*, *Metarhizium* and *Paecilomyces*) but this is not the same for *Beauveria*, which was found in high percentages in ant workers and queens but was completely absent from soil. This may be because it is present but rare; studies have shown that using standard techniques for soil samples usually reveals only the most common fungi in the area (Jeewon and Hyde, 2007). Fungal diversity in soil was strongly biased toward a few saprophytic fungi, belonging to the genera *Penicillium* and *Aspergillus*. Alternatively, soil may not be the primary source of entomopathogenic infections, but are contracted from direct contact with infected nestmates, conspecifics, competitors, or prey. There are a few studies, which have characterized fungal composition in habitats of social insects (Schmid-Hempel, 1998; Reber and Chapuisat, 2012; Hughes *et al.*, 2004). All these studies, however, have isolated fungi from soil samples; not many studies use measures of infection to characterize fungal diversity. Soils are great samples to look at fungal diversity but the type of survey may provide us with different information about the fungal community, which social insects encounter in their habitat. Interestingly, queens and workers did not show a similar distribution of fungal infections, which may be due to different levels of exposure. Queens that were collected were newly mated, meaning they were collected after only a few hours (2-3) after leaving the parental nest in which they had matured, while foraging workers are typically older and the foraging task requires recurring exposure to the environment outside of the colony as well as potentially infected insect prey and seeds.

Our second hypothesis, that populations of hybrid origin are better able to resist

fungal infection, was partially supported. Resistance to pathogens can be affected by hybridization in different ways: hybrids might not differ from their parents (Fritz *et al.*, 1999), show additive or dominance patterns of inheritance, or show transgressive patterns of enhanced hybrid susceptibility or resistance (Fritz *et al.*, 1994). This may in turn influence the relative ability of hybrids to persist and compete with their parents in habitats with high pathogen prevalence (Fritz, 1999).

As predicted, the H lineage had the lowest proportion of spores despite the highest generic richness and diversity in soils from their collection sites. However, when only known entomopathogens were considered, the ranges of the two hybrid lineages did not correspond to regions with higher abundance or diversity, as the two non-hybrid parental species overlapped with the highest number of pathogenic taxa (Table 2.3). Similar to our studies, others have examined whether pathogen loads and the susceptibility of insects varies when the species have higher genetic diversity (Reber *et al.*, 2008). They have found that social insect colonies, which are genetically heterogenous, show a reduction of load and pathogen species richness and this was correlated with a lower infection frequency by pathogens (Liersch and Schmid-Hempel, 1998; Baer and Schmid-Hempel, 1999). An experimental approach comparing pathogen resistance between hybrid and non-hybrid populations may help to resolve this question (Chapter 3).

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Table 2.1. Location, species, elevation and mean annual precipitation (MAP) of samples collected.

Site (Species)	***Coordinates***	***Elevation (meters)***	***Mean Annual Precipitation (MAP) (inches)***
San Simon, AZ, USA (H lineage)	N 32°16.225', W 109°14.263'	1101	20.70
Columbus (BF), NM, USA (H lineage)	N 31°47.993', W 107°44.905'	1326	11.55
Las Cruces, NM, USA (H lineage)	N 32°24.385', W 106°39.317'	1394	9.74
State Line Rd., AZ, USA (J lineage)	N 31°52.286', W 109°2.883'	1254	21.13
Sierra Vista, AZ, USA (J lineage)	N 31°39.999', W 110°20.986'	1336	21.95
Willcox, AZ, USA (J lineage)	N 32°14.233', W 109°49.465'	1268	13.32
Bowie, AZ, USA (P. rugosus)	N 32°18.915', W 109°29.128'	1152	13.32
Florence Junction, AZ, USA (P. rugosus)	N 33°13.775', W 111°20.715'	561	10.31
Willow Springs Rd., AZ, USA (P. rugosus)	N 32°36.127', W 110°52.278'	1149	23.47
Brady-Eden, TX, USA (P. barbatus)	N 31°12.982', W 99°48.560'	631	28.95
Luckenbach Rd., TX, USA (P. barbatus)	N 30°13.036', W 98°44.287'	459	31.69
Ozona, TX, USA (P. barbatus)	N 30°41.098', W 101°16.243'	771	22.70

Table 2.2. Summary of fungal genera isolated from soil, queen and ant samples. Type indicates the typical functional group of each fungal genus.

Fungi Genera	***Ant Samples***	***Soil Samples***	***Queen Samples***	***Type***
Trichoderma		X		Antagonistic
Scedosporium		X		Antagonistic
Bipolaris	X	X		Phytopathogenic
Diaporthe		X		Phytopathogenic
Alternaria	X	X	X	Phytopathogenic
Colletorichum		X		Phytopathogenic
Chrysosprium or Sporotrichum	X			Saprophytic
Xylohypha		X		Saprophytic
Cladosporium	X	X	X	Saprophytic
Aspergillus		X	X	Saprophytic
Penicillium	X	X	X	Saprophytic
Fusarium		X	X	Entomopathogenic
Lecanicillium		X	X	Entomopathogenic
Beauveria	X		X	Entomopathogenic
Isaria farinosus		X		Entomopathogenic
Paecilomyces	X	X		Entomopathogenic
Metarhizium		X		Entomopathogenic

Table 2.3. Presence of entomopathogenic fungi in parental species and hybrid lineages. An X symbol indicates that the fungus was found, and a dash represents absence of the genus. Results for the three sample types (soil, workers, queens) are separated by a forward slash.

Species	*Paecilomyces*	*Beauveria bassiana*	*Isaria*	*Fusarium*	*Metarhizium*	*Lecanicillium*
P. rugosus	**X/-/-**	**-/X/-**	**X/-/-**	**-/X/-**	**-/-/-**	**-/-/X**
H lineage	**X/-/-**	**-/-/-**	**X/-/-**	**-/-/-**	**-/-/-**	**-/-/-**
J lineage	**X/-/-**	**/-/X**	**-/-/-**	**-/-/-**	**-/-/-**	**X/-/X**
P. barbatus	**X/X/-**	**-/X/X**	**X/-/-**	**X/-/X**	**X/-/-**	**X/-/X**

Table 2.4. Pairwise correlations among the four environmental predictors.

	MAT	MAP	Soil Moisture	Elevation
MAT	1.000	0.2807	-0.1121	-0.8397
MAP	0.2807	1.000	-0.3007	-0.5935
Soil Moisture	-0.1121	-0.3007	1.000	0.2275
Elevation	-0.8397	-0.5935	0.2275	1.000

Table 2.5. The amount of environmental variables loaded in each principal component.

	PC1 (56.9%)	PC2 (24.9%)	PC3 (18.2%)
MAT	0.8226	0.4371	-0.3173
MAP	0.7219	-0.3076	0.6134
Soil Moisture	-0.4127	0.8158	0.4052
Elevation	-0.9519	-0.2092	0.0155

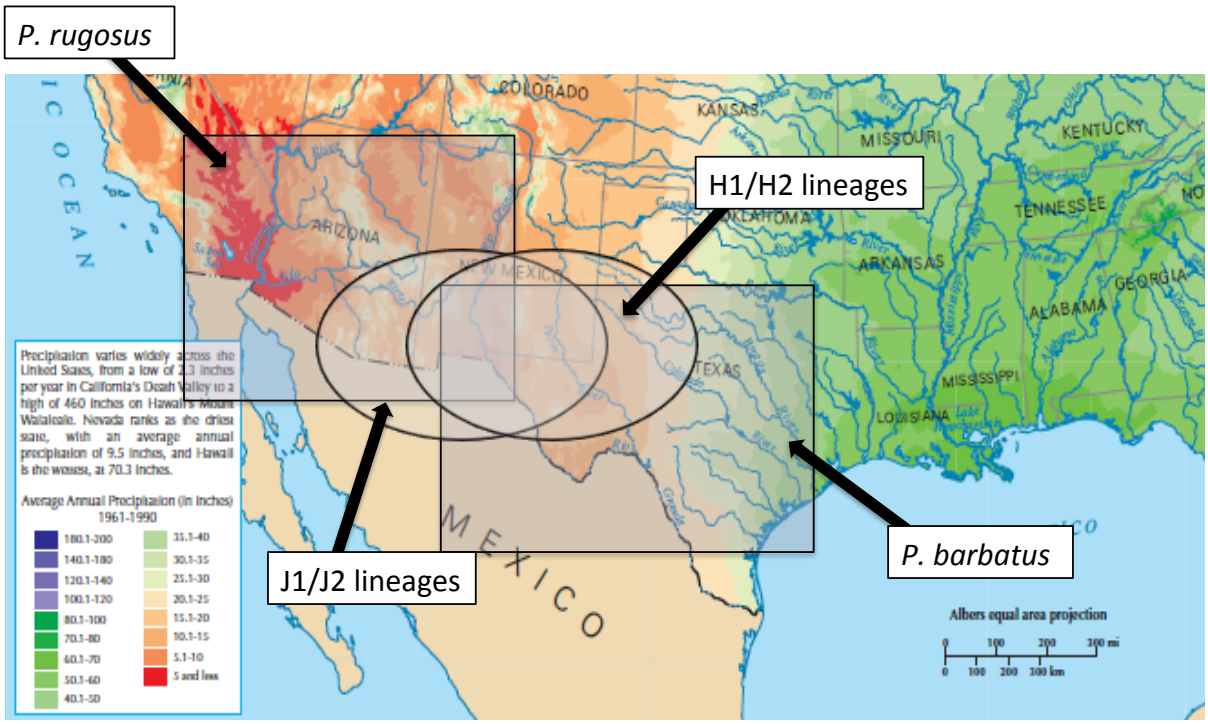

Figure 2.1. Geographical distribution of *Pogonomyrmex* barbatus, *Pogonomyrmex* rugosus and two hybrid lineages ("H" and "J") across a wider range from southeastern Arizona to southernwestern Texas.

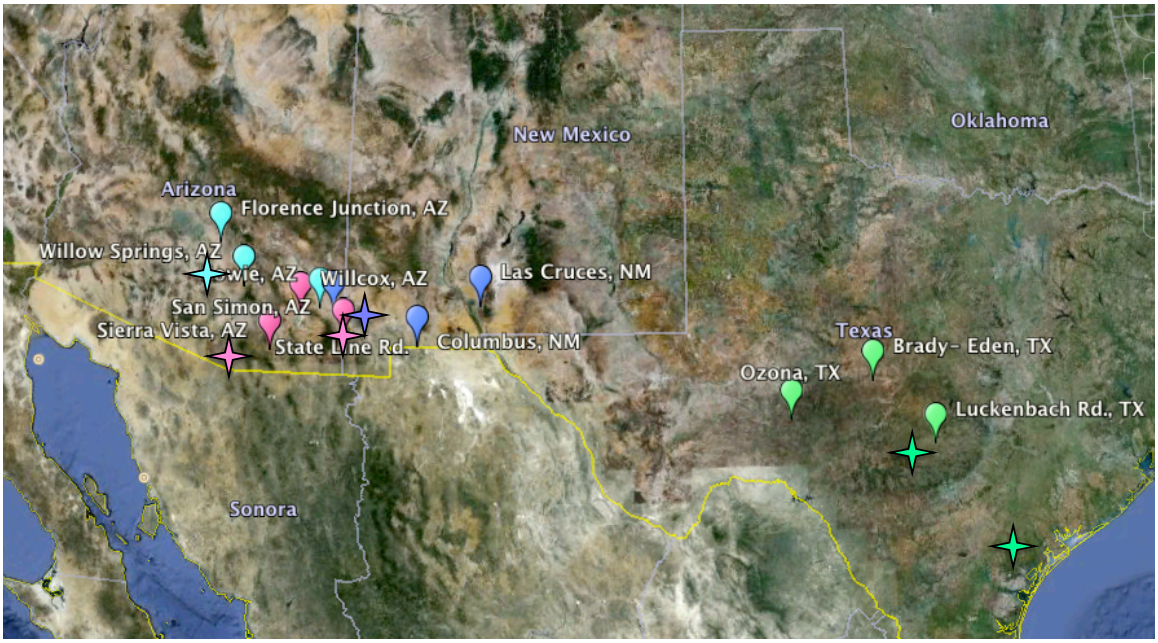

Figure 2.2. Geographic location of *Pogonomyrmex* species sites. Circles represent sites where soil and ant workers samples were collected. Stars represent where newly mated queens were sampled. Aqua color represents *P. rugosus* sites, pink represents J lineage sites, purple represents H lineage sites and green represents *P. barbatus* sites.

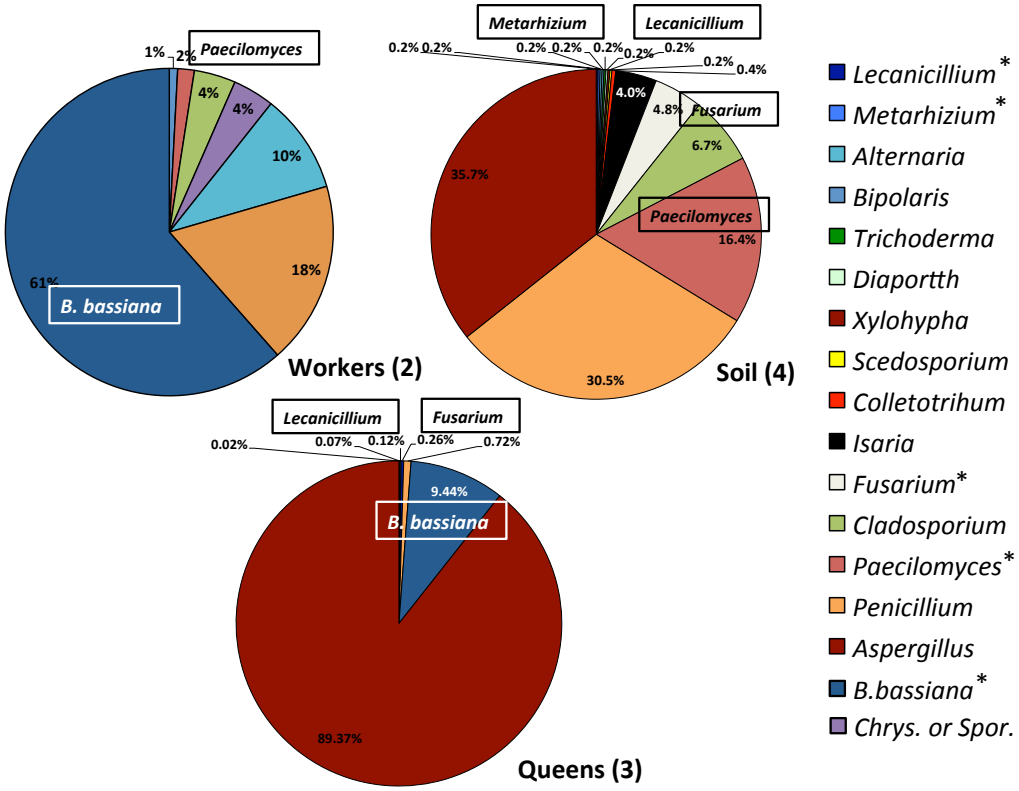

Figure 2.3. Diversity of fungi found in a. soil, b. worker, and c. queen samples. The percentages are the proportion of total CFUs. The proportion of ants infected per ant type (infected ants/total ants) for queen and ant samples. Highlighted boxes and asterisks on the legend show entomopathogenic fungi. Number of genera per sample type is in parenthesis.

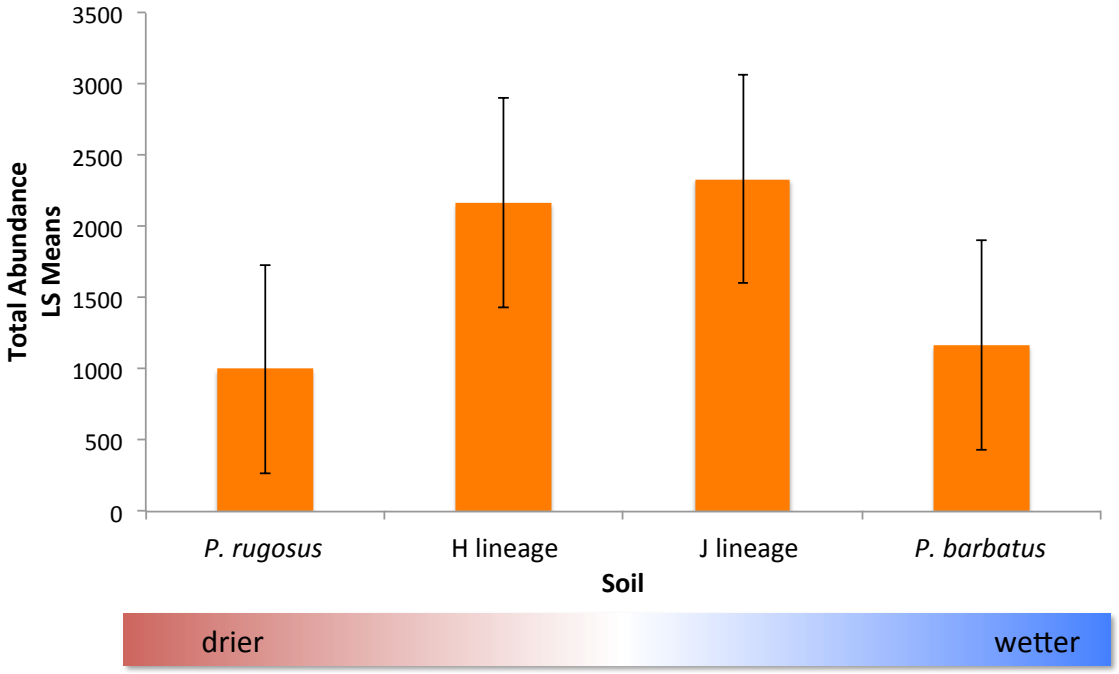

Figure 2.4. Least-squares means (+/- SE) of total abundance of CFUs found in each soil type. Bottom bar with red and blue gradient represent the precipitation gradient of the species. Q = 2.63, df = 3, p = 0.4648.

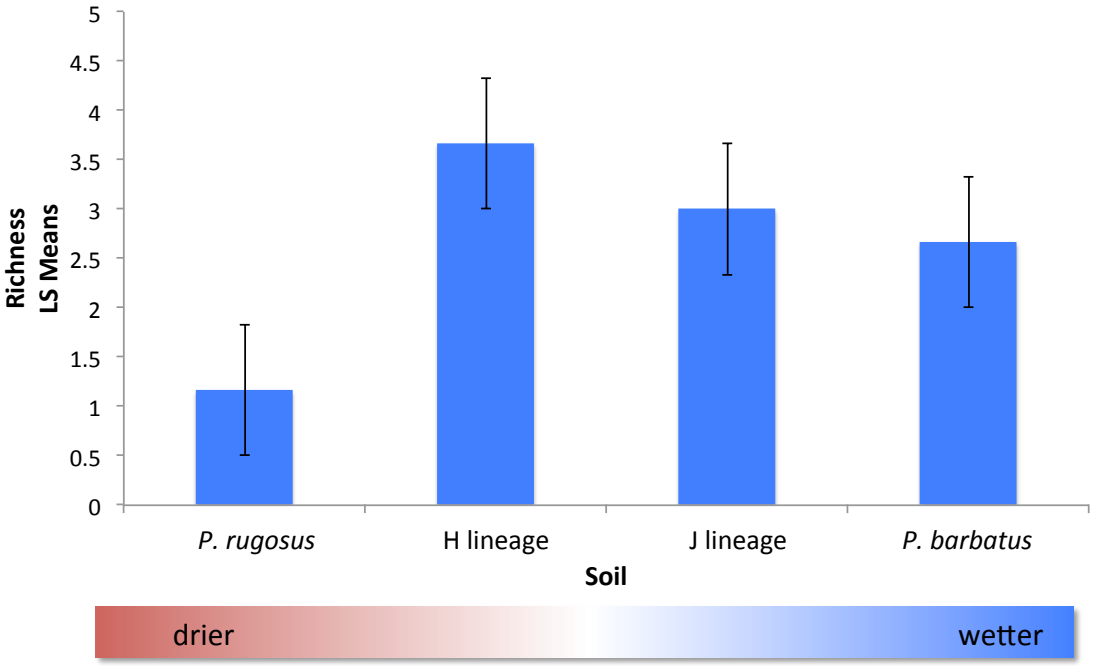

Figure 2.5. Least-squares means (+/- SE) of generic richness found for each soil type. Bottom bar with red and blue gradient represent the precipitation gradient of the species. Q = 2.63, df = 3, p = 0.064.

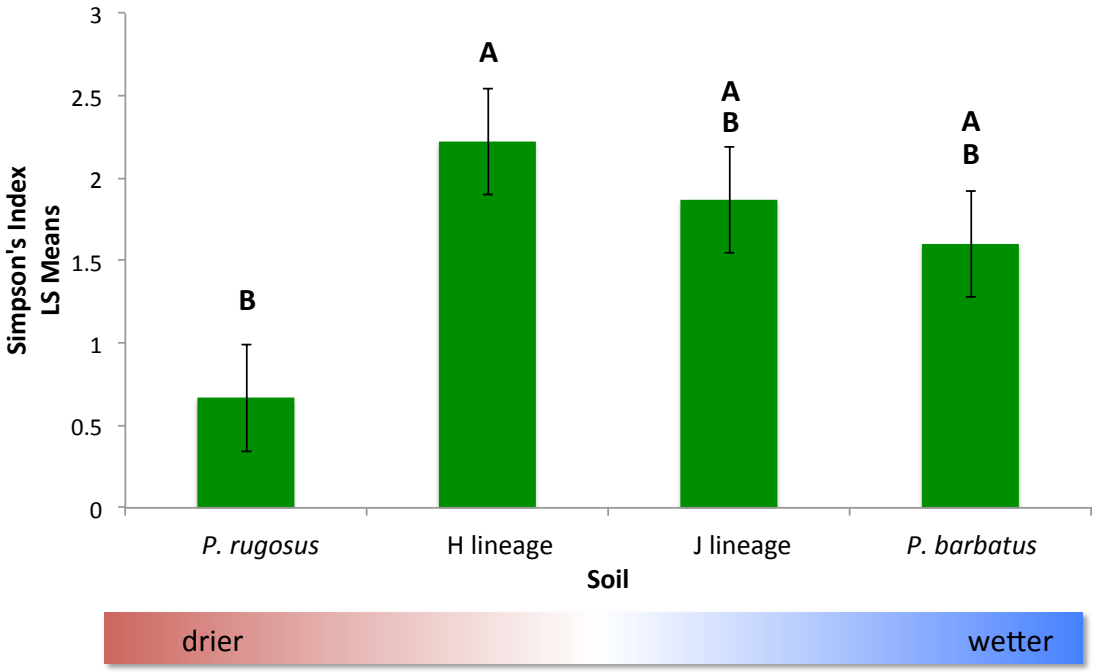

Figure 2.6. Least-squares means (+/- SE) of Simpson's Index diversity index found in each soil type. Bar represented by different letters are significantly different. Bottom bar with red and blue gradient represent the precipitation gradient of the species. Q = 2.63, df = 3, p = 0.0083*.

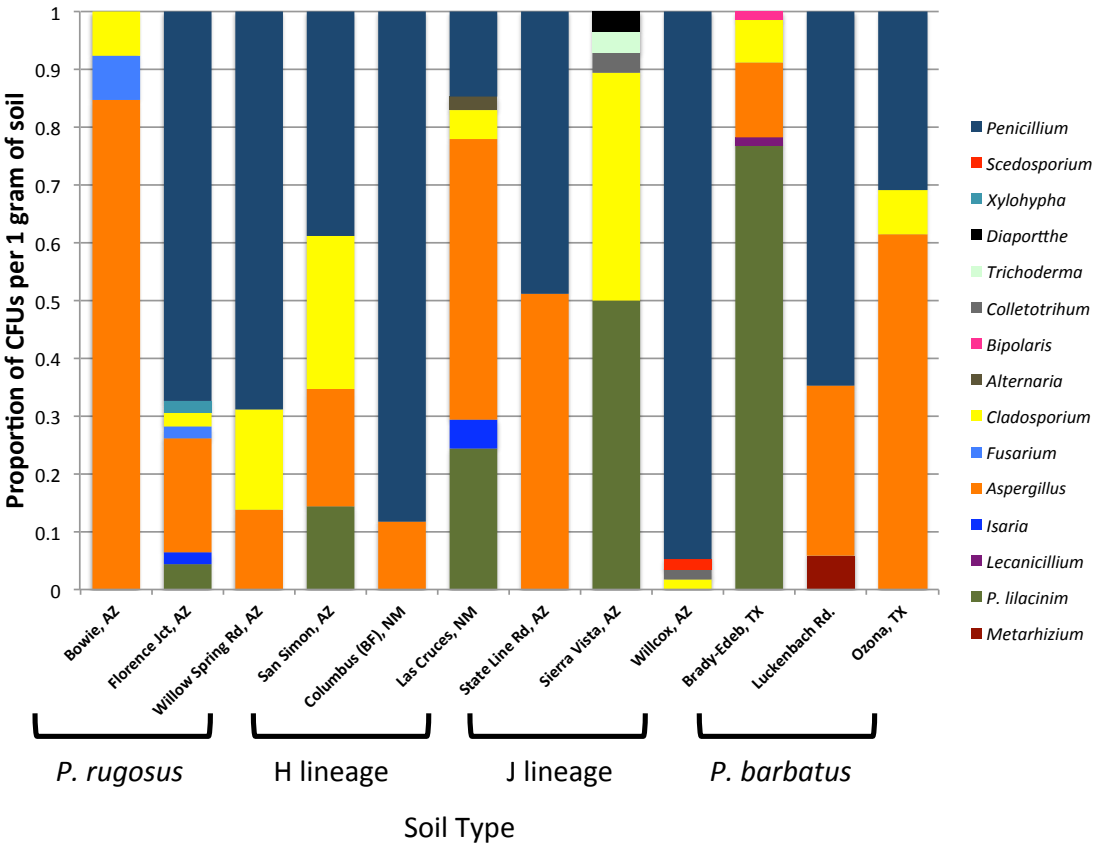

Figure 2.7: Proportion of CFUs of genera across collection sites, grouped by species range. Each bar represents the proportion of each genus found in a specific site. Permutation test: $p = 0.056$, lineage effect; $p < 0.05$*, site effect.

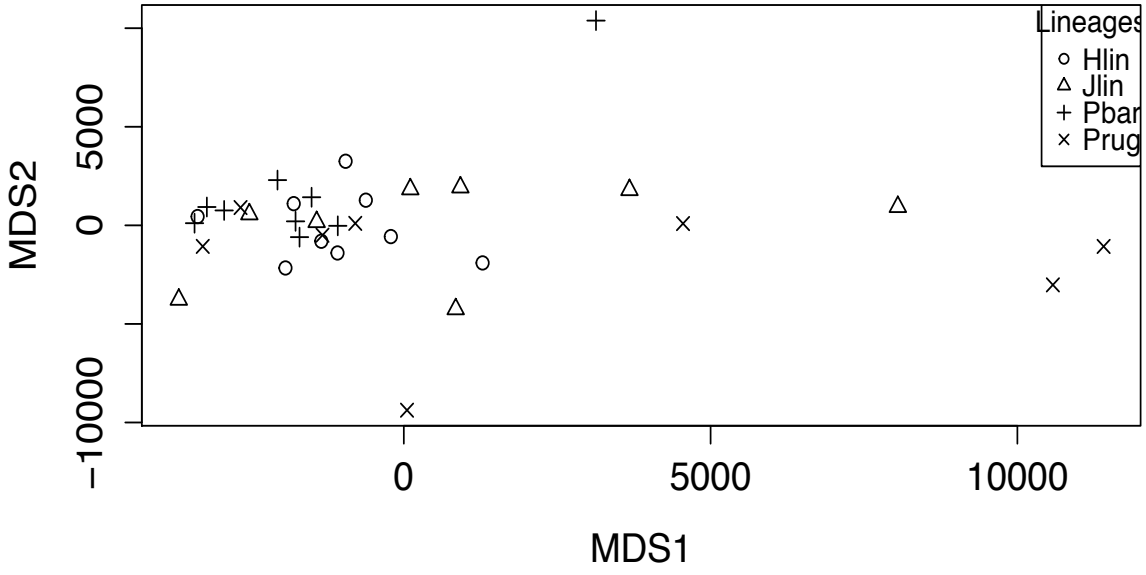

Figure 2.8. Multidimensional scaling analysis showing variation in fungal community composition among lineages. Symbols represent the different species, circles represented by H lineage, triangles represent J lineages, plus signs represent *P. barbatus* species and time sign represents *P. rugosus*. $p = 0.056$.

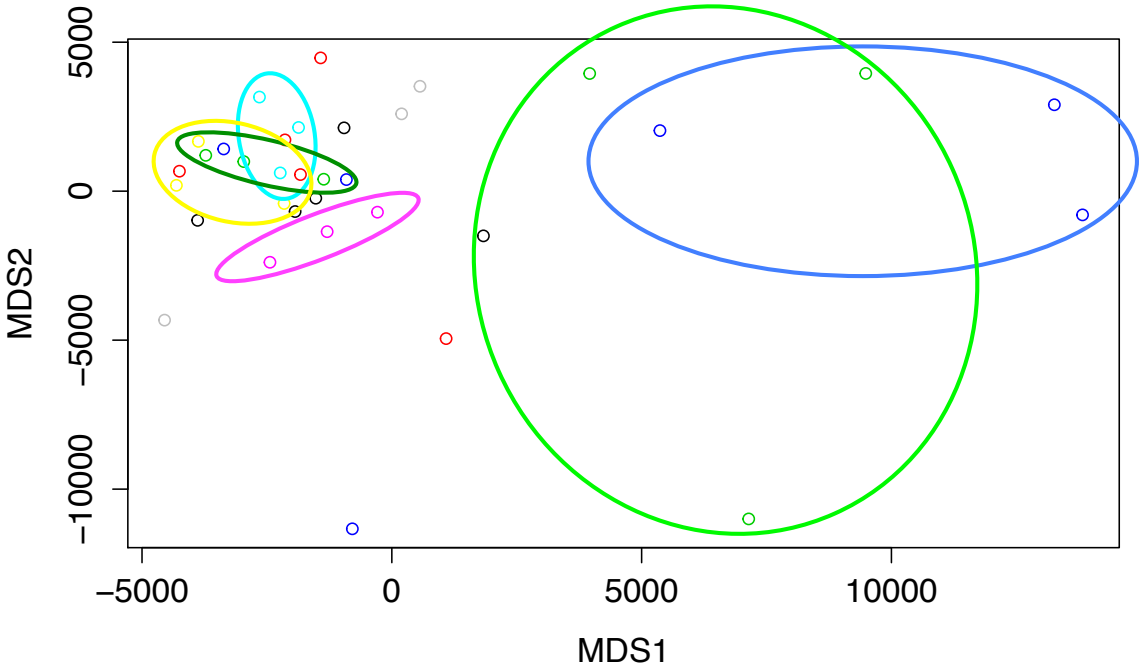

Figure 2.9. Multidimensional scaling analysis showing variation in fungal community composition across sites. Different colored ellipses indicate six representative sites; each ellipse shows where the samples from the same site occur. p <0.05*.

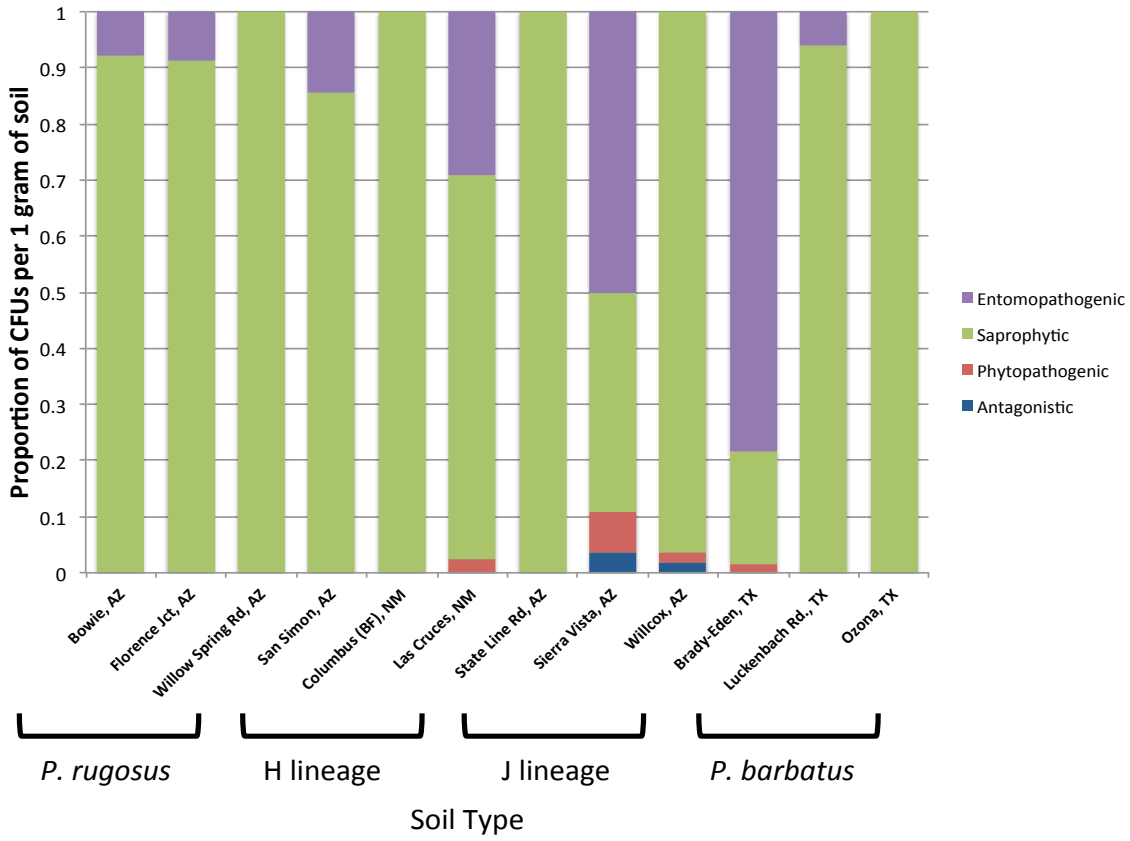

Figure 2.10. Proportion of CFUs of each functional type per site, grouped by species range. Each bar represent the proportion of each fungi type found in a specific site. Significant differences between functional groups with in sites are mark with an asterisk. Nested ANOVA: $p = 0.060$, lineage effect; $p < 0.05$*, site effect.

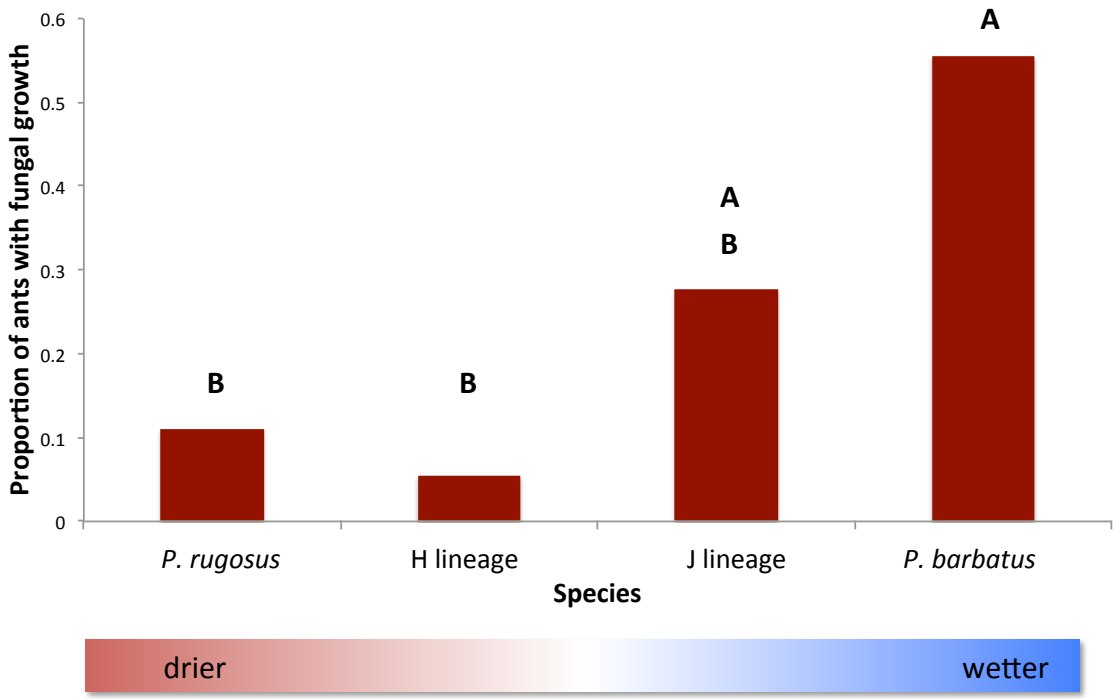

Figure 2.11. Proportion of ants infected with one or more fungi in the four ant taxa. Bars represented by different letters are significantly different. Bottom bar with red and blue gradient represent the precipitation gradient of the species. $X^2 = 14.52$, df = 3, p = 0.003*.

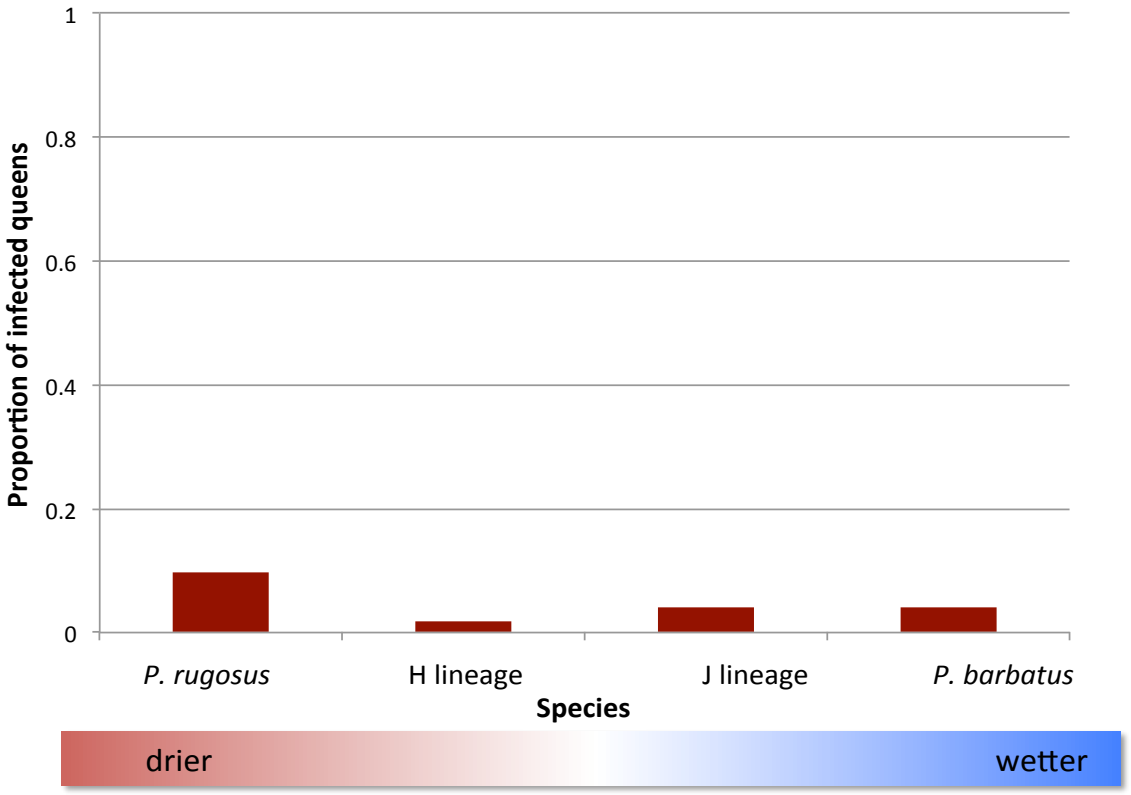

Figure 2.12. Proportion of queens that died during colony founding and were found to be infected with fungus. Bottom bar with red and blue gradient represent the precipitation gradient of the species. $X^2 = 6.658$, df = 3, $p = 0.0836$.

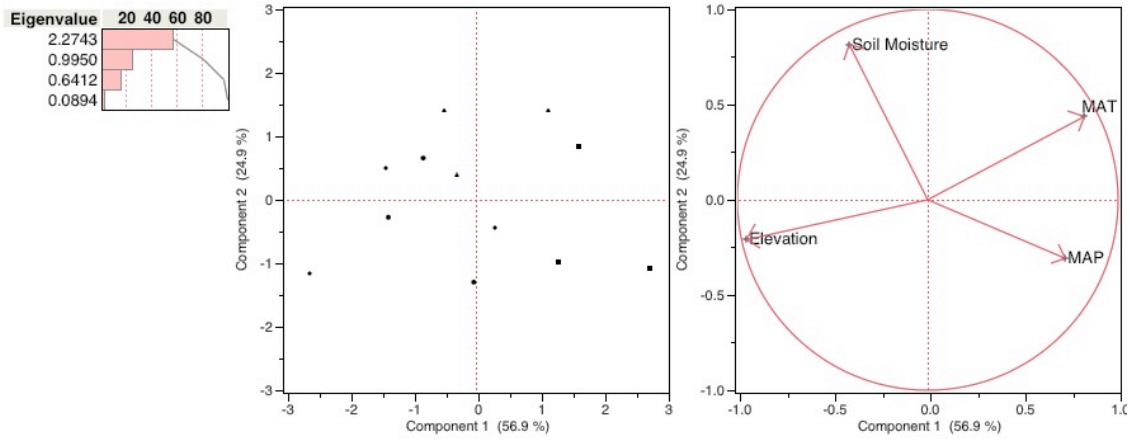

Figure 2.13. Principal component plot showing correlation of the environmental predictors.

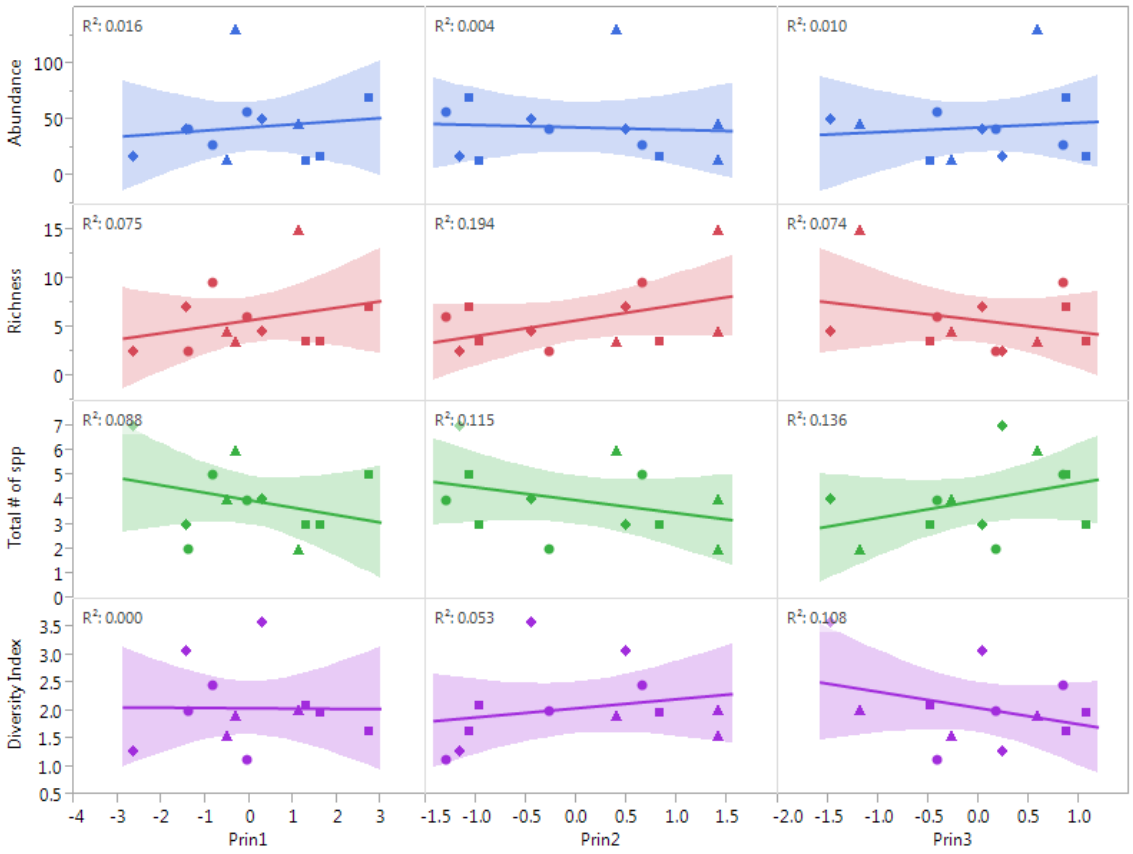

Figure 2.14. Scatter plots of the relationship between the first three Principal Components and diversity measurements. Different shapes in the graph represent species and lineages. Diamonds represent H lineage, circles represent J lineages, squares represent *P. barbatus* species and triangles represent *P. rugosus*. (N=12, sites)

CHAPTER 3

DO HYBRIDIZATION AND/OR HABITAT DIFFERENCES PLAY A ROLE IN PATHOGEN RESISTANCE?

Abstract

Understanding how hosts adapt to pathogens can help elucidate mechanisms of coevolution of pathogen and host. I generated four different hypotheses that could explain why different populations or species of host might differ in resistance to pathogens: pathogen load (high pathogen environments will have higher resistance), genetic diversity (outbreeding and hybridization increase genetic diversity, reducing probability of disease), local adaptation (individuals in their local environment will have higher resistance than individuals in foreign environments), and life history tradeoffs (individuals in high-stress environments will allocate resources to defense compared to individuals in other environments). The harvester ant species *Pogonomyrmex barbatus* and *P. rugosus* live along a precipitation gradient in southwestern North America, along with two populations of hybrid origin. The combination of both environmental and genetic variation across the group makes this an ideal system to experimentally discriminate among these hypotheses. A fully factorial experiment was conducted using soil from their natural environment to test the effects of species identity and soil type on the rate of infection of isolated pupae. *Pogonomyrmex rugosus* was found to have the highest resistance to fungal infection, while *P. barbatus* had the lowest. Our results supported the life history tradeoff hypothesis: *P. rugosus* lives in a harsher environment and has a lower growth rate, suggesting they invest more energy in protection from pathogens than reproduction because of the stressful environments they live in.

Introduction

Organisms with opposing fitness interests, such as predators and prey, resource competitors, or pathogens and their hosts, are expected to continually evolve adaptations to one another, leading to an evolutionary arms race (Van Valen, 1973; Dawkins and Kerbs, 1979). In the case of pathogens, interaction with their hosts plays an important role in their evolutionary trajectory (Van Valen, 1973; Clay and Kover, 1996; Bert and Hamilton, 1996). This co-evolutionary process can lead to strong differences in pathogen impact among individuals and populations, as pathogens adapted to infect one genotype

in a particular may not be able to infect other genotypes (Halden, 1949; Lively, 1996; Hughes and Boomsma, 2004). Similarly, hosts may respond to pathogens by evolving specific or generalized resistance to infection via adaptive changes in hygienic behavior, morphological defenses and immune system function (Evans *et al.*, 2006; Fernández-Marín *et al.*, 2006; Ugelvig and Cremer, 2007; Cremer and Sixt, 2009; Schlüns and Crozier, 2009; Hamilton *et al.*, 2011; Konrad *et al.*, 2012).

A number of different ecological and genetic factors may influence the extent to which hosts can defend themselves against potential pathogens. One key environmental factor, which can influence host resistance, is pathogen pressure (Horrocks *et al.*, 2011), the rate of exposure to pathogens in the environment. Pathogen growth is favored by high humidity, moisture and warm temperatures, increasing pathogen pressure in such environments (Tanada and Kaya, 1993). Since resistance is costly, organisms should match their investment toward defense traits with their expected fitness impact; organisms that encounter high pathogen pressure are expected to experience stronger selection for resistance to avoid disease despite the costs, shifting their allocation toward increased immune function. Studies have showed that when species face high numbers of pathogens they increase their level of defense; for example, ants performed more hygienic behavior when exposed to more pathogens (Reber *et al.*, 2008).

Pathogen resistance may also be influenced by life-history tradeoffs. When organisms have limited resources, allocating more energy to one function comes at the expense of another (Stearns, 1989; Zera and Harshman, 2001). Investing in reproduction, for example, may reduce energy available for immune function, increasing susceptibility

to pathogens (Perlman, 2008; Reavey *et al.*, 2014). Trade-offs between pathogen defense and growth and reproduction are likely to be affected by the environment. In a rich environment, the possibility of rapid growth and reproduction may reduce the relative benefit of investing in costly defense. However, in harsh environments, the increase in extrinsic mortality risk selects more strongly for survival mechanisms (including pathogen defense) at the expense of growth and reproduction. For example, snails have been shown to vary in life history traits expressed in response to varying degrees in the ability of a trematode parasite to infect the snails (Minchella, 1985).

Host-pathogen arms races can also lead to adaptations that are specific to their habitats, through the process of local adaptation. As long as dispersal between populations is not high, this process should lead to differentiation in host and pathogen traits even if the habitat is identical (Schulte, 2011). Local adaptation can be detected by differences in fitness between populations when placed in different habitats compared to their own habitat (Lively, 1996: Kawecki and Ebert, 2004). Pathogen resistance that evolved in a particular habitat may be ineffective in a different one. A population from a specific location has adapted to particular pathogens from that habitat, but when an individual is introduced to a different habitat, the probability of getting infected is higher because they have never encountered pathogens from that area (Kaltz and Shykoff, 1998; Ebert and Hamilton, 1996; Lively and Dybdhl, 2000).

Genetic factors can also play an important role in disease resistance. Outbreeding introduces unrelated genetic material into a breeding line. This increases genetic diversity and reduces the probability of infection of an individual (Thronhill, 1993). An extreme

version of outbreeding is interspecific hybridization, which introduces more heterozygosity than expected under random mating in a single population (Harrison, 1993; Dowling and Secor, 1997), and generates mixtures of genes in novel combinations to which pathogens may be poorly adapted, leading to lower infection rates. Empirical evidence for enhanced hybrid resistance, however, has been mixed (Fritz *et al.*, 1994; Fritz, 1999; Carlsson-Granér *et al.*, 1999, Krebs *et al.*, 2011). Some studies have not observed any differences between parents and hybrids (Fritz *et al.*, 1994; Fritz *et al.*, 1998; Jackson and Tinsley, 2003). In a bird hybrid zone system, resistance in hybrids was intermediate compared to their parental species but interestingly the prevalence of pathogen infection is less in younger individuals and older individuals was found to be intermediate prevalence of infection (Wiley *et al.*, 2009). A study of interspecific crosses between two mouse species, however, found that hybrid mice had lower parasite loads than their parental species (Moulia *et al.*, 1995).

Harvester ants (genus *Pogonomyrmex*) are an ideal group with which to test both environmental and genetic hypotheses for the evolution of host resistance. Ant colonies are made up of highly related individuals that live in close proximity, which can facilitate the spread of infectious diseases and parasites (Hölldobler and Wilson, 2009). As soil-dwelling-insects, ants are exposed to a diverse community of entomopathogens, including mites, helminth worms, fungi, bacteria, viruses and protozoa (Cremer *et al.*, 2007). The *P. barbatus* species complex includes two wide-ranging, ecologically dominant species that together span a large environmental gradient across the southwestern US; the rough harvester ant, *P. rugosus*, occurs in the Mojave, Sonoran and Chihuahuan deserts from

California to New Mexico, where it overlaps with the red harvester ant, *P. barbatus*, which inhabits more mesic habitats from higher-elevation Sky Island mountain ranges in southern New Mexico to low coastal plains in southeast Texas (Whitford *et al.*, 1975; Hölldobler, 1976). Comparisons of fungal communities in soil as well as worker infection rates in the field suggest that *P. rugosus* encounters lower fungal pathogen pressure (Chapter 1). In addition to environmental variation, historical interspecific hybridization between these two species has produced at least two obligately hybridizing species pairs, the "H" and "J" lineages, that occur between the ranges of the two parental species (Helms Cahan and Keller, 2003; Anderson, 2006a,b; Schwander *et al.*, 2007), whose worker progeny are exceedingly heterozygous due to their F1 ancestry (Helms Cahan *et al.*, 2002; Helms Cahan and Keller, 2003).

These characteristics allowed us to generate four contrasting predictions of host resistance under the pathogen pressure, life history tradeoff, local adaptation, and genetic diversity hypotheses. If pathogen pressure determines investment in resistance, we expect a negative relationship between environmental harshness and resistance, reflecting the lower pathogen pressure expected in such environments. In contrast, resistance driven by life history tradeoffs would produce the opposite pattern, as ants in harsh environments should lower investment into growth and reproduction in favor of allocating more energy toward pathogen defenses. If resistance to disease is primarily based on local adaptation, we predict that for each species, resistance will higher in their local habitat than in a foreign habitat. Finally, if genetic diversity is the primary factor which influences resistance to disease, then I predict that hybrids should be more resistant to disease than

their parental species because of their novel, highly heterozygous genotypes.

To test these hypotheses, we experimentally tested individual resistance to soil-borne fungal pathogens sourced from sites across the ranges of the two species and their hybrids. We set up a fully factorial experiment, in which we exposed each of the ant types (*P. barbatus*, *P. rugosus*, H lineage and J lineage) to each of the different soil types (*P. barbatus*, *P. rugosus*, H lineage and J lineage).

Materials and Methods:

Sample collection

Soil samples were collected from Arizona, New Mexico, and Texas during July 2012 (Figure 3.1). Soil samples were collected from 10 locations for each species and hybrid population. Sampling locations for each species were separated from neighboring sites by at least 100 kilometers to minimize spatial autocorrelation. At each location, three colonies were haphazardly selected with a minimum distance of 10 meters between colonies. At each colony, one soil sample was taken one meter from the entrance of the colony at 360° degrees (due north). Using a hand trowel sterilized with 70% ethanol, soil samples were collected at a depth of 5 to 10 cm after removing surface litter. Each soil sample was sealed in a sterile plastic bag (4½"W x 7"L) and refrigerated. Pupae were obtained from laboratory colonies derived from colony-founding queens collected in Summer 2011 during nuptial flights (Sites: Animas, NM (H lineage); Welder, TX (*P. barbatus*); Fredericksburg, TX (*P. barbatus*); McCartney Rd., AZ (*P. rugosus*); State Line Rd, AZ (J lineage); Santa Cruz, AZ (J lineage)). Nuptial flights occur a day after heavy monsoon rainstorms. The flight starts in the afternoon but time varies by species and

lineage. Flying virgin queens and males were followed to the mating swarm, and fully mated queens that had removed their wings were picked off the ground and placed in a tube with moist cotton. Colonies were maintained in a temperature control room at 28°C with two 16 x 150 mm test tubes with a water reservoir stopped with cotton provided as a nest. A mix of seeds (wheat germ, cornmeal and oats) and one mealworm was provided weekly.

Soil preparation

Soil samples from each location were passed through a 2.0 mm sieve and ground with a mortar and pestle. Three soil samples from colonies of ten sites collected for each species were pooled and homogenized. The soil was moistened by spraying with sterile water until the soil was damp but did not readily clump when compressed. Approximately seven grams of moistened soil sample was placed in a 35 x 10mm petri dish.

Bait Experiment

For each petri dish, one *Pogonomyrmex* pupa was partially embedded in the soil, the petri dish lid was replaced and partially sealed with parafilm, and the dish was incubated at 30°C for 15 days. For each soil type and ant type there were 30 replicates, having a total of 480 plates. As a control, 30 pupae of each species were maintained in individual petri dishes with moistened filter paper. Soil samples were examined and watered to keep soil moist every two days. When signs of fungal growth were visible or

the pupa was dead, identifiable when they turned dark to black, the pupa was removed from the petri dish and surface-sterilized by washing with 10% Bleach for 3 minutes under a fume hood. After washing with bleach, they were rinsed three times with distilled water. To determine whether the ant internal tissue was infected, and to grow sufficient fungal tissue for genetic analysis, the pupa was placed in a small petri dish plate with a moist filter paper, sealed with parafilm, and incubated at 30°C for an additional 7-15 days. Samples were examined every 2 days. When signs of fungal growth were visible, the fungal tissue was scraped off the surface of the ant pupae and placed in a microcentrifuge tube for DNA isolation.

Identification of Fungi

DNA from the isolated fungal tissue was extracted using the Plant Tissue kit (Mini Protocol from Qiagen) according to the manufacturer's specifications. To identify fungal species identity, the Internal transcribed Spacer (ITS) gene was amplified. This gene was used because it has the highest probability of identification of a broad range of fungi (Viaud *et al.*, 2000; Anderson and Cairney, 2004; Schoch *et al.*, 2012). Polymerase chain reaction (PCR) was conducted in 30-µl reactions, containing 16.35 µl of dH2O, 3.0 µl of 10x Std. Taq Reaction Buffer, 1.5 µl ofdNTP, 1.5 µl of forward primer, 1.5 µl of reverse primer, 0.15 µl of Taq and 6 µl of DNA. The primers used were the fungus-specific forward primer ITS-5 F (5' GGAAGTAAAAGTCGTAACAAGG 3') and the reverse primer ITS-4 R (5' TCCTCCGCTTATTGATATGC 3'), manufactured by Invitrogen Custom Primers (Carlsbad, CA). PCR was carried out using the following

amplification conditions: initial denaturation at 94°C for 2 mins, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 10 min. Successful amplification was verified using 2% agarose gel electrophoresis. Samples were Sanger sequenced in both forward and reverse directions and Geneious version Pro 5.6.5 was used to annotate and align the sequences (http://www.geneious.com/). We used NCBI's BLAST nr database to identify the fungi to species; the top match was retained if it returned an E-value less than 0.1. All sequences were aligned with Geneious and a neighbor-joining tree was constructed; this allowed inference of species or generic identity of samples with no significant match when nested within identified clades.

Statistical Analysis

To test the differences between control and treated samples a chi-square test of independence was performed. Logistic regression was used to test the effect of ant species identity, soil source, and their interaction on the probability that a pupa would become infected. Two logistic regressions were run: in the first, all four ant taxa (*P. barbatus*, H, J, *P. rugosus*) were treated as separate categories. In the second, I explicitly tested the genetic diversity hypothesis by combining the H and J lineage samples into a single "Hybrid" category; because the first analysis indicated a difference between the two parent species, these were left as separate taxa. All statistical analyses were carried out in JMP version Pro 10 (SAS Institute Inc., 2007).

Results

Exposure to fresh soil significantly elevated pupal mortality relative to filter paper controls ($X^2 = 65.42$, df = 1, $p < 0.0001$*; Figure 3.2). Fungi isolated from the few pupae that had died in the controls were identified as *Aspergillus,* a cosmopolitan saprophytic fungus, and *Candida,* an opportunistic vertebrate pathogen. In contrast, a total of 16 fungal genera were reared from pupae in the fresh soil treatment (Figure 3.3).

Both soil origin and ant species identity significantly affected the likelihood of fungal infection, but there was no significant interaction between ant and soil type (Soil origin: $F_{3,468} = 3.2241$; $p = 0.0225$*; Ant species: $F_{2,468} = 12.3223$; $p < 0.001$*). H lineage soils had the lowest infection rate, while J lineages soil had the highest (Q = 2.57, df = 3, $p = 0.0395$*) (Figure 3.4). *Pogonomyrmex rugosus* soil resulted in infections from a total of 12 species from 11 genera, while H lineage soil yielded 9 species from 8 genera, J lineage soil yielded 13 species from 9 genera, and P. *barbatus* produced infections of 8 species from 5 genera (Figure 3.3). When the distribution of fungal genera across soil types for each site was compared to the grand mean across all soil types, J lineage soil was the only site that differed significantly in fungal distribution, with an excess of *Mortierella*, *Modicella* and *Lecanicillium* and a deficit of *Candida* infections (Table 3.1).

Among species, *P. rugosus* ants were significantly less likely than all other taxa to be infected by fungi regardless of soil type ($p < 0.0001$*, *P. barbatus*; $p < 0.0001$*, H lineage; $p = 0.042$*, J lineage). Hybrid lineages ants were significantly different from each other ($p = 0.0253$*). The H and J lineages were generally intermediate and did not differ significantly from *P. barbatus* (p = 0.0610). To test the hybrid resistance

hypothesis, we pooled the H and J lineages as hybrids and reanalyzed the infection data, with identical results (Figure 3.5). Infection types across the four ant types were not equally distributed (Table 3.2); the H lineage was more infected than expected by *Modicella,* but had fewer than expected *Fusarium* infections.

Discussion

Pathogen resistance is expected to be shaped by costs and benefits of investment in resistance, the extent of local antagonistic coevolution, and the genetic diversity of individuals and populations. We tested these hypotheses with a factorial design that exposed two species of ants and their hybrids to soil-borne fungi from across the range of the species complex. Our results provide support for the Life-History Tradeoff hypothesis, as the slow-growing, desert-adapted species *P. rugosus* was significantly more resistant to fungal infection than the other taxa. In contrast, we found no evidence for an effect of pathogen pressure, genetic diversity, or local adaptation on host resistance.

The pathogen load hypothesis predicted that ants living in more productive, mesic habitats would experience a higher pathogen load in their environment and therefore invest more heavily into pathogen resistance, reducing their infection probability. Mean annual precipitation increases from west to east, and overall infection rates from soil showed a similar positive correlation with precipitation level even under uniform laboratory conditions, suggesting that the bank of fungal spores present increases as conditions became more favorable for fungal growth and reproduction. However,

comparison of ant susceptibility to infection showed the opposite pattern from that predicted by the pathogen pressure hypothesis: *P. rugosus*, which comes from the most arid environment of the four taxa, was the most resistant to fungal infection, while the most mesic-habitat species, *P. barbatus*, was among the least resistant. This matches the results of a field survey, which found higher prevalence of fungal infection in internal tissue of adult *P. barbatus* workers than in *P. rugosus* or the hybrid lineages (Chapter 2).

The genetic diversity hypothesis predicted higher resistance to disease in hybrid populations compared to parental species. This hypothesis was not supported because it predicted that hybrid would have a higher number of uninfected ants than parental species. Previous studies have shown that hybrid species, especially in plants, are not less susceptible to pathogens, although but they do show benefits against herbivores (Fritz *et al.*, 1994; Fritz *et al.*, 1999). However in a study of mice results showed that hybrid mice had less parasite loads than parental species suggesting hybrids vigor (Moulina *et al.*, 1995). Although other studies of social insects show increased pathogen resistance related to genetic diversity (Liersch and Schmid-Hempel, 1998; Tarpy, 2003; Reber *et al.*, 2008; Ugelvig *et al.*, 2010; Calleri *et al.*, 2006), our data suggest that hybridization does not play a role in pathogen resistance in *Pogonomyrmex* ants.

The local adaptation hypothesis stated that ants should have a higher resistance to disease in their local habitat than in a foreign habitat. This hypothesis was not supported, as the ant species were no more resistant to fungi when exposed to their own soil than when exposed to foreign soil. Similar to our study, Zhan and colleagues (2002) did not find any global evidence of local adaptation in two species of wheat that had different

levels of resistance to one fungal pathogen, *Mycosphaerella graminicola,* although such an effect could be detect for a subset of specific fungal strains tested. There have also been studies that have found maladaptation of fungi to their host when a sexually-reproducing host is infected by a selfing pathogen, limiting the evolutionary potential of the pathogen (Kaltz *et al.*, 1999; Gandon and Michalakis, 2002). In contrast, other studies have found evidence of coevolution produced by local adaptation in different hosts and parasites (Imhoof and Schmid-Hempel, 1998; Thrall *et al.*, 2002; Schulte *et al.*, 2011). No evidence for local adaptation was found in this study system, but it should be noted that this study looked at all the fungal genera together, and therefore had little power to detect species-specific effects. Testing a single species may provide more insight into whether there are patterns of local adaptation.

The life history tradeoffs hypothesis stated that ants that have a higher investment in reproduction would have a lower resistance to disease. Studies looking at life history tradeoff and host-pathogen interactions have found that species vary in their allocation of resources towards resistance versus other tasks such as reproductive effort (Minchell, 1985; Richner, 1998; Gwynn *et al.*, 2005). In pea aphid, this shift to invest more in immunity comes at a cost for fecundity, because individuals carry a secondary symbiont associated with parasite resistance leading to fewer offspring (Gwynn *et al.*, 2005). Once infected, however, individuals may shift to allocate more towards growth and reproduction in order to recoup some fitness in the face of increased mortality risk (Pagán *et al.*, 2008). Our results showed that *P. rugosus* had the highest resistance in all soil types. Helms Cahan and colleagues (2009) looked at the growth rate for both parental

species and hybrid lineages and found that *P. rugosus* has a lower growth rate. They suggest that this difference of growth rate it is an adaptation to stressful environments. Our results suggest that there is a difference in life history between our species that lead to differences in pathogen resistance.

Identification of fungi for this experiment showed a greater number of non-pathogenic fungi than expected in our samples. We saw a number of plant pathogens in our data as well as endophytic fungi (endosymbionts of plants). *Pogonomyrmex* are granivores and bring a lot of seeds to their colonies; it is possible that spores of these fungi were dormant in the soil of the ant colonies. As for *Candida*, human pathogen, they are known to live in endosymbiosis in insect host tissue (Nguyen *et al.*, 2007; Suh *et al.*, 2008), which could explain the high concentration of these fungi in our sample.

Alternatively, the types of fungi in the soil environment that can effectively infect and invade *Pogonomyrmex* pupae may be greater than the set of taxa commonly considered to be entomopathogens. Overall low resistance to pathogens in immature stages may be expected in eusocial insects such as ants, which are typically cared for by adults throughout development. This study only measured inherent resistance, and did not take into consideration any social immunity effects. Having this in mind we might see differences in the response to pathogens in our ants. Social insects have a series of mechanism at the individual and colony level that helps with resistance towards pathogen. Social immunity mechanisms include allogrooming, where one individual ant grooms another one removing spores from their bodies (Fernández-Marín *et al.*, 2006; Reber *et al.*, 2011). In bumblebees, brood can receive trans-generational immunity via the

mother's egg (Sadd *et al.*, 2005; Sadd and Schmid-Hempel, 2007). By not testing this we might have missed some information regarding mechanism of resistance.

Table 3.1. Number of fungi genera found between the ant types. Fungi were identified by functional groups. A chi-square test of goodness-of-fit was performed to determine whether the ant types were equally preferred ($X^2_{(0.050,\ df=4)} = 9.488$).

Fungi Genera	Type	*P. rugosus*	H	J	*P. barbatus*	*Total*
Cunninghamella	Antagonistic	1	2	1	1	5
Candida	Human pathogen	1	2	1	8	12
Kodamaea	Human pathogen	0	1	0	0	1
Mortierella	Saprophytic	2	4	2	2	10
Modicella	Endophytic	0	7	2	3	12
Ceratobasidium	Saprophytic	0	0	0	1	1
Aspergillus	Saprophytic	0	2	1	2	5
Preussia	Endophytic	0	0	1	0	1
Cochliobolus	Phytopathogenic	0	0	3	0	3
Curvularia	Phytopathogenic	0	2	0	0	2
Alternaria	Phytopathogenic	0	1	0	1	2
Chaetomium	Endophytic	1	0	1	0	2
Xylariaceae	Phytopathogenic	1	0	0	0	1
Lecanicillium	Entomopathogenic	0	0	1	2	3
Fusarium	Entomopathogenic	4	1	9	8	22
Rhizopus	Phytopathogenic	1	0	0	1	2
Chi-square		2.6060	9.8135*	3.9526	4.6659	

Table 3.2. Fungal genera found across the soil types. Fungi were identified by functional groups. A chi-square test of goodness-of-fit was performed to determine whether the distribution of fungal taxa was similar across soil types ($X^2_{(0.025,\ df=4)} = 11.143$).

Fungi Genera	Type	*P. rugosus*	H	J	*P. barbatus*	*Total*
Cunninghamella	Antagonistic	1	2	1	0	4
Candida	Human pathogen	3	7	1	1	12
Kodamaea	Human pathogen	0	0	1	0	1
Mortierella	Saprophytic	1	2	6	1	10
Modicella	Endophytic	1	2	9	0	12
Ceratobasidium	Saprophytic	1	0	0	0	1
Aspergillus	Saprophytic	1	4	0	0	5
Preussia	Endophytic	0	0	0	1	1
Cochliobolus	Phytopathogenic	3	0	0	0	3
Curvularia	Phytopathogenic	0	2	0	0	2
Alternaria	Phytopathogenic	1	0	1	0	2
Chaetomium	Endophytic	0	0	1	1	2
Xylariaceae	Phytopathogenic	1	0	0	0	1
Lecanicillium	Entomopathogenic	0	3	0	0	3
Fusarium	Entomopathogenic	2	4	9	7	22
Rhizopus	Phytopathogenic	2	0	0	0	2
Chi-square		6.5270	6.0042	13.0946**	8.3070	

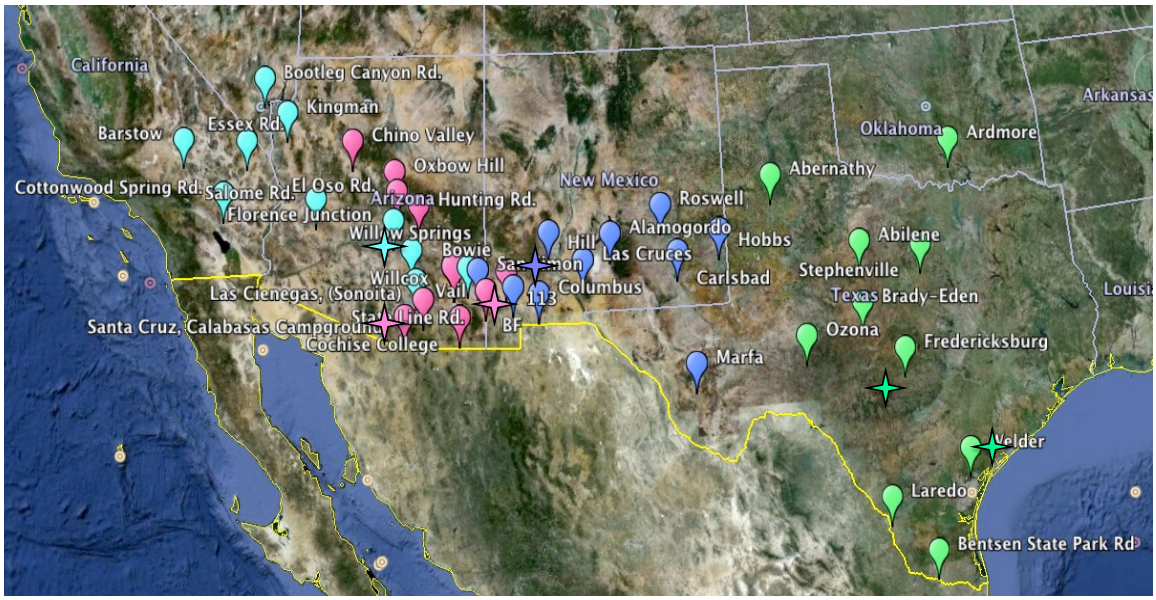

Figure 3.1. Geographic location of *Pogonomyrmex* species sites. Circles represent sites where soil and ant workers samples were collected. Stars represent where newly mated queens were sampled. Aqua color represents *P. rugosus* sites, pink represents J lineage sites, purple represents H lineage sites and green represents *P. barbatus* sites.

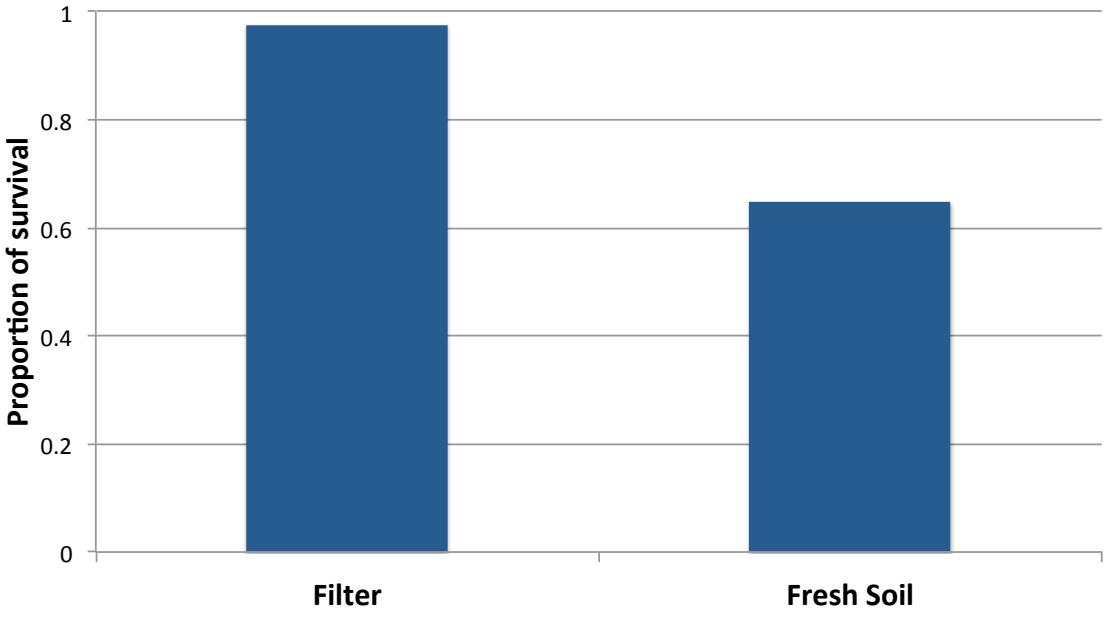

Figure 3.2. Proportion of pupae surviving to adult eclosion in filter paper control and fresh soil. Chi-squared test: $X^2 = 65.42$, df = 1, $p < 0.0001$*

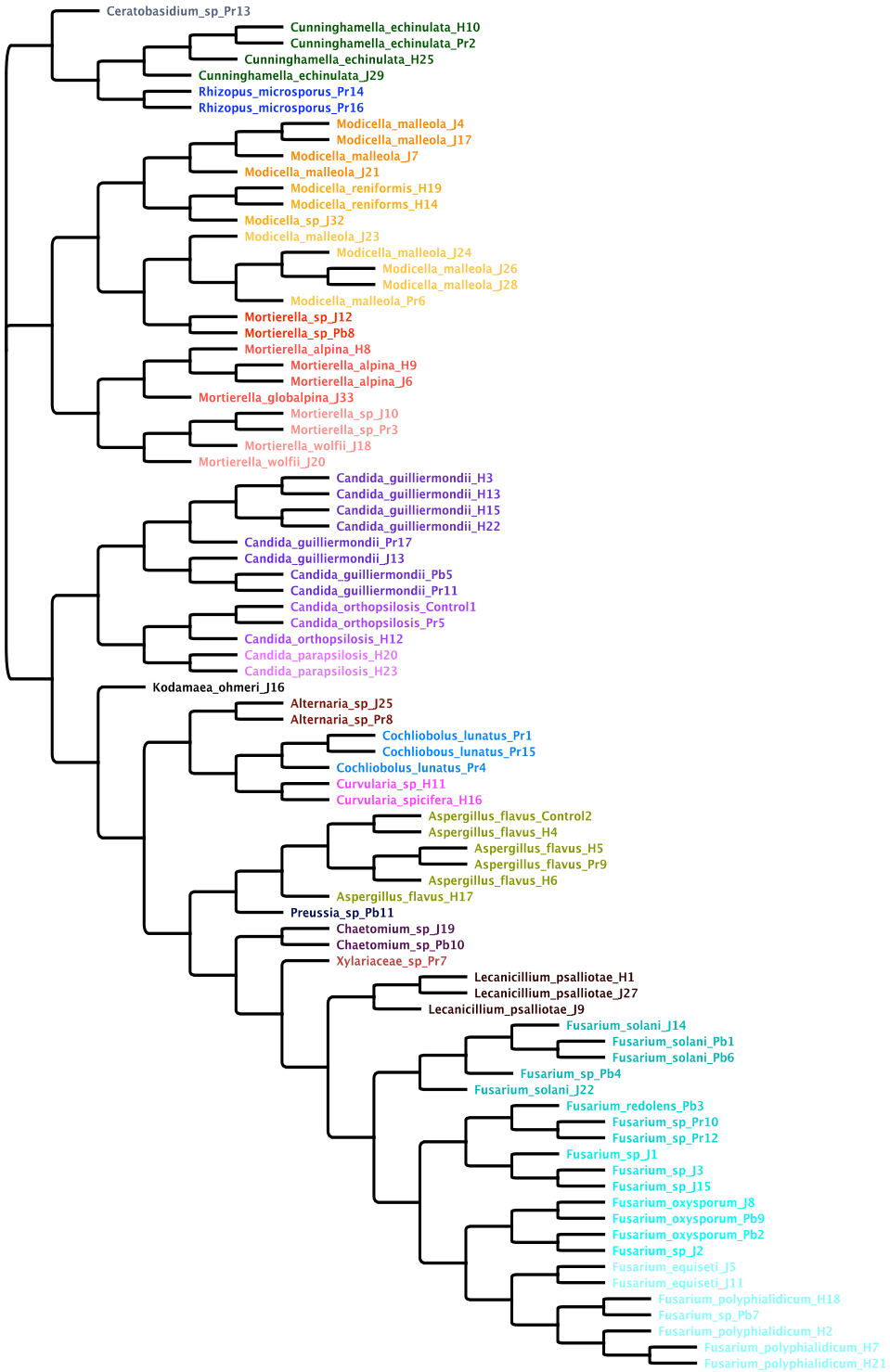

Figure 3.3. Neighbor Joining Tree of the ITS gene amplified from fungi recovered in the bait experiment. Each branch represents one sample; the name is the fungi species followed by the soil sample it was extracted. Different color represents each fungal genus and the shades indicate different species within a genus.

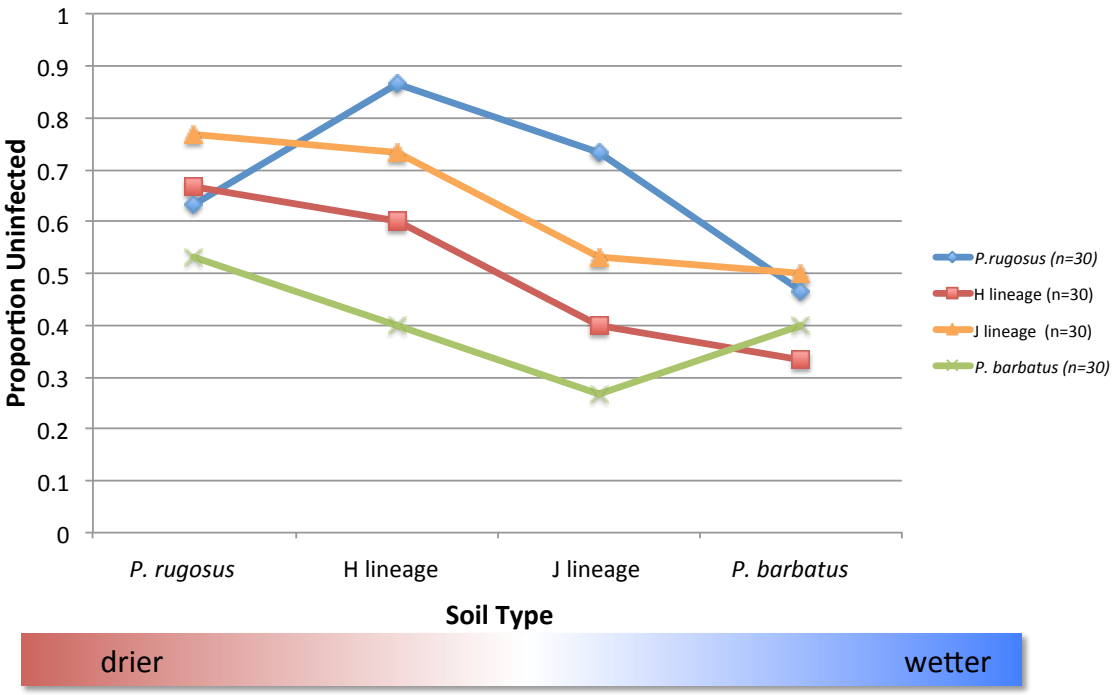

Figure 3.4. Proportion of uninfected pupae in soil types. Ant Species: $F_{2,468}$; $p < 0.001$*. Soil Samples: $F_{3,468}$; $p < 0.0225$*

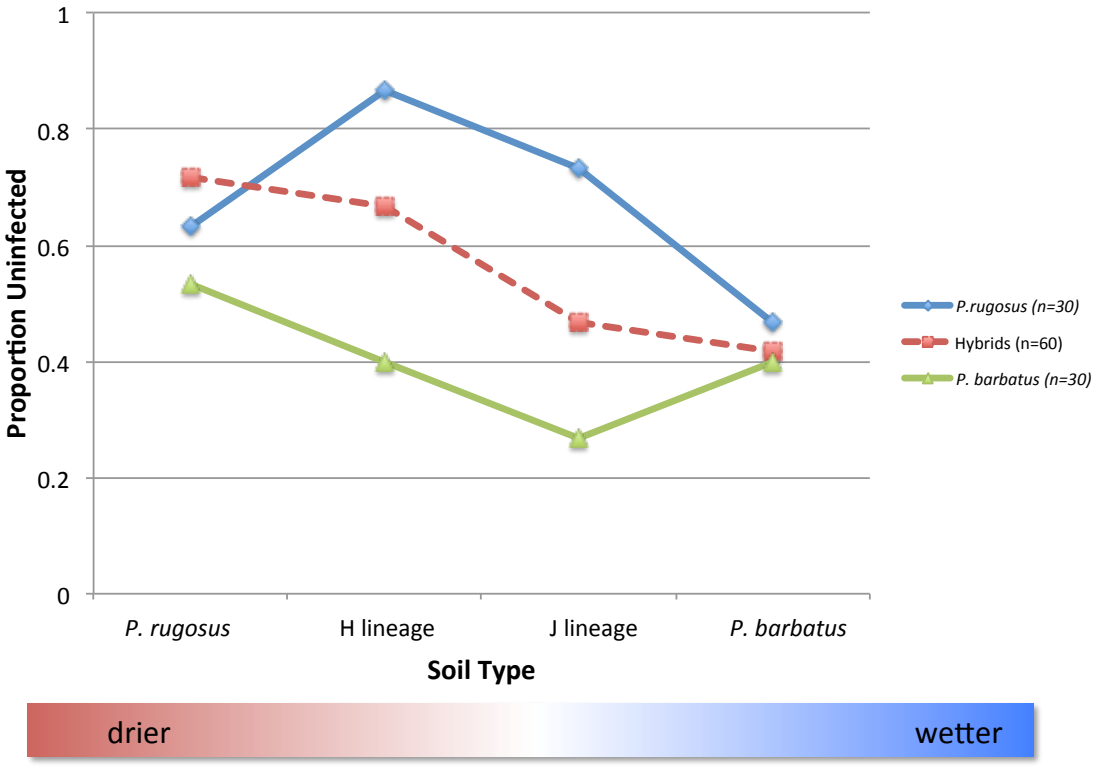

Figure 3.5. Proportion of uninfected pupae in soil types with hybrids together. Ant Species: $F_{2,468}$; $p < 0.001$*. Soil Samples: $F_{3,468}$; $p < 0.0225$*

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CHAPTER 4

FUTURE DIRECTIONS

Future projects on fungal diversity and pathogenicity on harvester ants would benefit from more detailed sampling. To gain a better understanding or predictor for abundance and diversity of fungi, I would recommend going out in the field and collect more samples from the entire range and collect environmental data (pH, temperature and soil moisture). Although the use of standard microbiology techniques was useful to characterize the fungal community that are present in these ant habitats, these techniques tend to overestimate abundance of some fungal species and identify only the most common species (Jeewon and Hyde, 2007). In the future, it would be useful to utilize different methods to gain a better understanding of fungal species composition and pathogen load in our habitats. Large-scale meta-barcoding using DNA extraction of soil samples would allow us to obtain more information on fungal diversity in the soil environment. Using this method, we can extract ribosomal DNA and functional genes from genomic DNA from the soil samples, sequence it, then identify fungal community in the soil samples based on these sequences (Elsas and Boersma, 2011; Prosser, 2002). This approach will allow us to obtain more accurate relative abundances of the fungi found in the soil. Also, would allow us to capture more diversity and is less biased.

A clear understanding of pathogen-host dynamics emerges from matching the fungal diversity that ants experience in their environment and probability of infection. Results from the first chapter showed that there are distinct differences in entomopathogenic fungi present in our species, but the source of this variation has not

been determined through this work (Table 1.3). Some were found in all of our ants while other pathogens were only found in one of the ant species. For example, *Beauveria bassiana* was found in both parental species and one of the hybrid lineages, but not in the other lineage tested. Chapter two showed differences in infection frequency among species, with *P. rugosus* ants having the lowest infection frequency. It is possible that there are differences in virulence between strains of entomopathogenic fungi among the ant species. *Beauveria bassiana* has been found to have high genetic differentiation between isolates. Some studies have shown that genetic differentiation is related to differences in virulence against certain insect taxon (Lai *et al.,* 1982; Jones *et al.*, 1996; Bidochka *et al.*, 2002; Cruz *et al.*, 2006; Rao *et al.*, 2006). To determine the level of virulence in ant hosts, we could experimentally infect ants with the different pathogens and measure virulence. *Fusarium* seems to have the higher abundance in our second experiment, suggesting it is an effective pathogen in our ants. We could verify if *Fusarium* is a pathogenic fungi for *Pogonomyrmex* species. Very little is known about the prevalence and diversity of fungal pathogens actually infecting social insects in natural conditions (Briano *et al.*, 1995; Pereira, 2004; Rodrigues *et al.*, 2010; Evans *et al.*, 2011).

One fascinating problem that still remains is how ants defend against fungal pathogens. It would also be beneficial to look at what mechanism ants use to defend against pathogens. There were no differences between hybrids and parental species in terms of resistance towards pathogens. We have observed differences between parental species that were unexpected. *P. rugosus* was found to have the lowest infection frequency compared to the other species. It is known that *P. rugosus* has a lower growth

rate and live in stressful environments (Helms Cahan *et al.*, 2009). One possible explanation for these differences is that *P. rugosus* invested more energy in protection than in reproduction to cope with disease risk. Specialized social sanitary behaviors, such as allogrooming decreases the number of fungal spores on the surface of contaminated workers and lower the rates of transmission, supporting the idea that behavioral traits can decrease prevalence of fungal infection within a colony (Fernández-Marín *et al.*, 2006; Reber *et al.*, 2011). *P. rugosus* might be investing a significant amount of time in allogrooming to avoid infections, therefore lowering their susceptibility to the pathogen.

Overall this will helps get a better understanding of the fungal composition, diversity and abundance that harvester ants experience in their habitats. Especially the different species of entomopathogenic fungi that they encounter that can help us look at susceptibility of pathogens for these ants. Determining relative pathogenicity and virulence can help understand a little more about how these pathogens might affect the host and at what level of virulence. Understanding mechanisms that generate virulence can give insight on how virulence evolves.

CHAPTER 5

COMPREHENSIVE BIBLIOGRAPHY

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