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Sexual Conflict and Chemical Communication in Hybridizing Harvester Ants

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SEXUAL CONFLICT AND CHEMICAL COMMUNICATION IN HYBRIDIZING HARVESTER ANTS

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ABSTRACT

Sexual conflict occurs when the fitness interests of males and females do not align with one another. The resolution of sexual conflict often depends on the level of control each sex has on the behavior in conflict. In *Pogonomyrmex* harvester ants with a genetically determined caste system, two separate lineages interbreed with one another during summer mating swarms. Diploid offspring sired by a single lineage develop into reproductive queens, while offspring sired by opposite-lineage parents develop into sterile workers. This results in sexual conflict, as males which mate with opposite lineage queens will produce only workers, resulting in no fitness benefit, while queens must mate with opposite-lineage males in order to obtain workers and survive. Despite these fitness differences, males do not discriminate between lineages prior to mating. One possible reason for the lack of male discrimination is that queens "mask" their identity cues, making discrimination difficult for males. In eusocial insects, identity cues are encoded by cuticular hydrocarbons (CHC's) found on the exoskeleton of the insects. These cues contain information on the insect's reproductive status, sex, species, state, and nest membership. In addition to their communication functions, CHC's also serve as desiccation-resistance molecules, preventing water from freely passing out of the cuticle. However, molecules that are best-suited for communication functions are poor desiccation resistance molecules, and molecules that are best-suited for waterproofing lack the diversity needed for communication; therefore, a tradeoff between these two functions is expected.

In this dissertation, I explore sexual conflict in these ants and the chemical recognition cues that likely play a role in this conflict. To test for cryptic strategies in harvester ant mating swarms, I experimentally paired males and females from two interbreeding lineages of harvester ant with different fitness outcomes based on pairing, and measured the propensity to initiate copulation, pre-copulatory time, time in copula, and rate and amount of sperm transferred in each mating. Although females controlled copulation duration, males altered sperm transfer rates, resulting in no quantitative difference in total sperm transfer between lineages. To test for thermal constraints on the diversity and composition of cuticular hydrocarbon profiles, and changes in CHC profiles that occur in workers isolated from the queen, I surveyed the cuticular hydrocarbon profiles of a species complex of harvester ants. The CHC profiles of ants from more xeric environments showed evidence of constraints, while isolated workers differentiated from their queen-raised sisters, although not in queen-specific molecules. To test for queen identity masking and lack of discrimination ability in mating swarms, I tested for convergence in the CHC profiles of reproductives in two hybridizing lineages in response to the sexual conflict playing out in this species. Differences in CHC profiles were lost during the mating swarm, likely limiting male ability to discriminate between mates, limiting discrimination ability in mating swarms. To study the genetic regions that control CHC production, I created a physical linkage map of two of the interbreeding populations, and used that map to perform quantitative trait loci analysis on the cuticular hydrocarbon profile of recombinant males. One significant region associated with 13-methylnonacosane contained numerous odorant receptor genes, suggesting a link between that CHC production and the receptors that detect it, while a second region associated with n-pentacosane contained numerous genes that control expression levels. Overall, the genetic caste determination system in these ants leads to antagonistic coevolution between species. This coevolution is likely reinforced by the thermal constraints and exchange of recognition cues between species, lowering the ability of useful discrimination between lineages during mating swarms.

CITATIONS

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CHAPTER 1: LITERATURE REVIEW

The evolution of social groups, especially eusocial insect groups with sterile workers, was considered by Darwin to be one of the theory of evolution's greatest difficulties; if evolution occurs based on an organism's survival and reproduction, how has the creation of an entire class of these sterile "neuters" evolved (Ratnieks et al. 2011)? Even today biologists often disagree on which hypothesis can best account for the evolution of these social groups (Nowak et al. 2010, Abbot et al. 2011). Evolution of sociality has led to a new field of study on the biology of grouping and social behavior in living organisms, with social insects serving as the model of the highest level of social behaviors, eusociality (Wilson Edward 1975). In addition to studying the origin of social groups, eusocial insects are an ideal study system to examine other phenomena that occur within social groups. For instance, communication and conflict are ubiquitous in social groups, and communication can lead to the resolution of conflict in a less costly way, allowing for the maintenance and survival of these groups (Zahavi 1977). In this dissertation, I will focus on the role of sexual conflict and communication in *Pogonomyrmex* harvester ants, using this study system to investigate how the interactions of conflict and communication shape the evolution of social behavior.

1.1 Sexual conflict

One form of conflict that can arise is sexual conflict. Sexual conflict arises when the fitness interests of males and females are not aligned (Parker 1979, Chapman et al.

2003). This can lead to antagonistic coevolution between the sexes, since traits that benefit one sex do so at the expense of their mate (Arnqvist and Rowe 2002, 2005). One example of sexual conflict involves the optimal mating frequencies of males and females. Often, these differences initially arise from different initial levels of investment in the offspring, with females typically investing more resources into their offspring than males (Trivers 1972). This differing level of investment leads to different strategies to maximize fitness between males and females; while males can obtain greater fitness through mating as often as possible regardless of female quality, females maximize their fitness by mating with the highest quality males that they can find (Parker 1979). Therefore, conflict arises with females evolving resistance to mating with low-quality males, and males co-evolving traits to counteract female resistance (Chapman and Partridge 1996).

Two classic examples of sexual conflict leading to antagonistic coevolution are the complex genitalia of waterfowl and the clasping/anti-clasping spines of water striders (Arnqvist and Rowe 1995, Brennan and Prum 2012). In waterfowl (Aves: Anatidae), males have a complex, spiraling phallus that expands rapidly to fertilize females, with males often forcing females to mate (Brennan et al. 2010). In response, complex, labyrinth-like vaginal systems in females limit the success of forced copulations from males (Brennan et al. 2007, Brennan et al. 2010). Similar to the waterfowl, in water striders (Heteroptera; Gerridae) males have developed large claspers that are utilized to hold females during forced copulations (Arnqvist and Rowe 1995). In response to this, a

system of spikes has evolved in to prevent forceful mating, resulting in the evolution of reinforced abdominal cuticles in males (Arnqvist and Rowe 2002).

The evolution of these sexually antagonistic traits are limited by the extent that each sex can control behaviors involved in the conflict (Beekman and Ratnieks 2003). In general, each sex can only control certain aspects of reproduction. For instance, males can control the amount of sperm transferred to females after copulation commences, while females can control the degree of care an offspring receives. Sexual conflict, like all conflicts, can also resolve around the information that is available to each party in the conflict (Axelrod and Hamilton 1981). If males or females are unable to obtain the information that is needed to obtain their optimum return, then game theory predicts that a bet-hedging approach would produce the best outcome.

Although sexual conflict is generally an intraspecific process, it can also occur in an interspecific context. For example, amazon mollies (*Poecilia formosa)* are a species of all-female freshwater fish that reproduce by mating with males from three closely-related species of mollies (Schlupp 2005, Schupp and Plath 2005). However, in these matings the male's genes are not incorporated into the offspring, resulting in no fitness benefits provided to the interspecific males that mate with these amazon mollies (Heubel et al. 2009). This leads to males preferentially avoiding mating with thegynogenetic species, resulting in sexual conflict between species (Riesch et al. 2008). Another example of interspecies sexual conflict can be found in hybridization involving two species of spade-

footed toads, *Spea bombifrons* and *Spea multiplicata* (Pfennig 2007). Typically, *S. bombifrons* has a slower metamorphosis time than *S. multiplcata*, while hybrids between the two species have an intermediate metamorphosis time. In highly ephemeral ponds, *S. bombifrons* females will preferentially mate with *S. multiplicata* males, which produces hybrids with a faster development speed for *S. bombifrons*, although a slower development speed than pure-breeding *S. multiplicata* (Pfennig and Simovich 2002). Although hybridization increases the fitness *of S. bombifrons* females through the generation of faster developing offspring, it results in a loss of fitness for *S. multiplicata* males due to the lower fitness of the hybrid offspring, creating conflict between males and females of the two species (Pfennig 2007).

1.2 Sexual Conflict in Ants

Ants (Hymenoptera: Myrmecea) are eusocial insects that live in colonies with a strong reproductive skew (Wilson 1971). One or a small number of females, the queens, are responsible for all of the reproduction in the colony. Other females, the workers, perform all other tasks in the colony, such as nest maintenance, defense, brood care, and foraging for food (Hölldobler 1990). Ants, like all Hymenoptera, have a haplo-diploid sex determination system; males are haploid and produced from unfertilized eggs, while all fertilized eggs are diploid and develop into females. Therefore, males in ant colonies are produced through parthenogenesis, or the laying of unfertilized eggs.

In ants, sexual conflict can occur between males and reproductive females in species where queens mate with multiple males, known as polyandry (Arnqvist and Nilsson 2000). In singly-mating species (monandry), males and female reproductive interests are identical, and all reproductive queens are produced using the male's sperm. However, polyandrous species decouple this 1:1 fitness alignment of males and females, since reproductive daughter queens can be produced with any of the queen's male mates, limiting the fitness benefits of the male ant (Strassmann 2001). This leads to the potential for sexual conflict over sperm allocation and offspring caste in ants (Boomsma et al. 2005). In some ant species, males utilize mechanisms such as clumping and sperm biasing to increase their fitness by increasing the probability that reproductive females are produced with their sperm (Boomsma 1996, Wiernasz and Cole 2010). In ants with a lek mating system, sexual conflict during male and female interactions are greatly abbreviated, occurring only over the course of the mating event with no opportunity for males to replenish sperm between matings (Strassmann 2001). In harvester ants these mating swarms take place in under two hours (Hölldobler 1976), creating intense pressures on male fitness due to limited time available to mate and the inability of males ants to replenish their sperm reserves. These limitations greatly increase the fitness cost of mating mistakes, especially in ant species where hybridization with other species can occur (Feldhaar et al. 2008).

1.3 Harvester Ant Genetic Caste Determination (GCD) Lineages

One ant system where interspecies sexual conflict is expected is in the interbreeding lineage of *Pogonomyrmex* harvester ants (Helms Cahan and Keller 2003). In this system, two genetically distinct lineages of harvester ants from a historic hybridization event mate together in a lek mating swarm, with queens mating numerous times with males from both lineages (Hölldobler 1976, Helms Cahan and Keller 2003, Linksvayer et al. 2006). The caste of the offspring from these matings is genetically determined (Genetic Caste Determination, or GCD), with pure-lineage offspring developing into reproductive queens, while mixed-lineage offspring develop into workers (Volny and Gordon 2002, Anderson et al. 2006a).

This unusual genetic caste determination system leads to sexual conflict. In GCD harvester ants, males that successfully mate with their own lineage obtain a high fitness benefit, as their offspring develop into reproductive queens. Males who mate with opposite-lineage queens, however, receive little or no fitness benefit because those offspring become sterile workers. Queens, on the other hand, must mate with opposite-lineage males in order to produce workers and survive. This leads to interspecific sexual conflict, as queen and male reproductive interests diverge. Previous research suggests that despite this sexual conflict, no mate discrimination occurs and males and females appear to mate randomly (Suni *et al.* 2007, Suni and Eldakar 2011). One possible reason for the lack of discrimination seen in these mating swarms could be a lack of reliable

discrimination cues during initial interactions, limiting the ability of males and females to differentiate between potential mates of the two lineages. This lack of species discrimination during mating could result from a tradeoff between signals of species identity, and mate quality (Pfennig 1998).

1.4 Cuticular Hydrocarbons in Insects

In eusocial insects, the primary form of recognition cues are cuticular hydrocarbons (CHCs) (Lahav et al. 1999, Gamboa 2004, Howard and Blomquist 2005). These compounds are comprised of long chains of hydrogen and carbon molecules, and the mixture of these compounds form a waxy outer layer on insect cuticles (Blomquist and Bagnères 2010). Insects have a variety of different cuticular hydrocarbon compounds, ranging from three distinct compounds found in the American cockroach *Periplaneta americana* (Baker et al. 1963) to ant species with over 50 distinct compounds (Martin and Drijfhout 2009a). The carbon chain number of most cuticular hydrocarbons are between 20 and 50 carbons, although analysis of CHCs using MALDI-TOF mass spectrometry has found hydrocarbons with a chain length of up to 80 carbons (Cvačka et al. 2006).

In insects, CHCs serve two primary roles: they serve as non-volatile chemical communication molecules, and also help water-proof the cuticles of insects, preventing desiccation (Gibbs 1998). In general, straight-chain n-alkane molecules with a lower density, no functional groups, and a higher melting point tend to provide a more waterproof layer, resulting in better protection from desiccation in insects (Gibbs and Pomonis 1995). However, these molecules are poor communicators, since they can only vary by chain length and lack the diversity needed to code for meaningful communication cues. Methyl-alkanes and alkenes, on the other hand, have a high level of variation and physical differentiation, making them excellent communication molecules (Dani et al. 2005, Greene and Gordon 2007). These molecules have virtually unlimited molecule diversity, with varying number and type of functional group, chain length, and position of functional groups within the main hydrocarbon chain. However, these additional functional groups reduce the density and melting point of these compounds, limiting the effective desiccation resistance of these compounds (Gibbs and Pomonis 1995).

The two different functions of these CHC molecules are predicted to lead to a trade-off between hydrocarbons that are effective communication resistance molecules, and hydrocarbons that act as informative communication cues; hydrocarbons that increase desiccation resistance are too simplistic to transfer meaningful communication, while hydrocarbons that are complex can easily code for information at the cost of poor desiccation resistance function. Therefore, in hot xeric environments, one might expect

positive selection for the proportion of desiccation resistance hydrocarbons, limiting the number of communication hydrocarbons in favor of higher proportion of n-alkanes.

CHCs are produced *de novo* in insects through a well-studied pathway of enzymes (Blomquist 2010a). They are heritable in insects (Thomas and Simmons 2008, van Zweden et al. 2010), and serve as honest kin recognition cues in eusocial insects (Nehring et al. 2011). The evolution of kin-informative recognition cues leads to several questions: first, how is the diversity of these recognition cues maintained, especially if the cues are linked to beneficial social behavior (Holman et al. 2013)? Second, how much of an ant's CHC profile is produced de novo, and how much is altered by contributions from their social environment? And finally, are the genetic regions that control the production of these compounds linked to genes that are linked to a specific social behavior or trait?

1.5 Dissertation Questions Overview

In this dissertation, I explore the manifestation of sexual conflict and the evolutionary and ecological dynamics of chemical recognition cues that likely play a role in its resolution. The four research chapters in this dissertation examine the expected sexual conflict and the role of chemical communication in these conflicts using *Pogonomyrmex* harvester ants as a model system. In Chapter 2, I utilized natural GCD populations to look for the manifestation of sexual conflict between species, examining the reproductive behaviors of males and females that would allow each sex to exert their interests on interspecific mating partners. In Chapter 3, I tested for evidence of thermal

constraints on hydrocarbon composition by characterizing the cuticular hydrocarbon profiles of queens and workers in two ECD specie and four GCD lineages of *Pogonomyrmex* ants. In chapter 4, I tested for a mechanism to prevent assortative mating through identity masking in GCD *Pogonomyrmex* males and females during mating. In chapter 5, I tested for the genetic basis of CHC production by searching for genomic regions associated with the production of several cuticular hydrocarbons.

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CHAPTER 2: INTER-GENOMIC SEXUAL CONFLICT DRIVES ANTAGONISTIC COEVOLUTION IN HARVESTER ANT

2.1 Abstract

The reproductive interests of males and females are not always aligned, leading to sexual conflict over parental investment, rate of reproduction, and mate choice (Parker 1979). Traits that increase the genetic interests of one sex often occur at the expense of the other, selecting for counter-adaptations leading to antagonistic coevolution (Rice 1996, Chapman et al. 2003). Reproductive conflict is not limited to intra-specific interactions; inter-specific hybridization can produce pronounced sexual conflict between males and females of different species, but it is unclear whether such conflict can drive sexually antagonistic coevolution between reproductively isolated genomes (Pfennig 1998, Helms Cahan and Keller 2003, Schupp and Plath 2005). We tested for hybridization-driven sexually antagonistic adaptations in queens and males of the socially hybridogenetic "J" lineages of *Pogonomyrmex* harvester ants, whose mating system promotes hybridization in queens but selects against it in males. We conducted no-choice mating assays to compare patterns of mating behavior and sperm transfer between inter- and intra-lineage pairings. There was no evidence for mate discrimination on the basis of pair type, and the total quantity of sperm transferred did not differ between intra- and inter-lineage pairs; however, further dissection of the sperm transfer process into distinct mechanistic components revealed significant, and opposing, cryptic manipulation of copulatory investment by both sexes. Males of both lineages increased their rate of sperm transfer to high-fitness intra-lineage mates, with a stronger response in the rarer lineage for whom mating mistakes are the most likely. In contrast, the total duration of copulation for intra-lineage mating pairs was significantly shorter than for inter-lineage crosses, suggesting that queens respond to prevent excessive sperm loading by prematurely terminating copulation. These findings demonstrate that sexual conflict can lead to antagonistic coevolution in both intra-genomic and inter-genomic contexts. Indeed, the resolution of sexual conflict may be a key determinant of the long-term evolutionary potential of host-dependent reproductive strategies, counteracting the inherent instabilities arising from such systems.

2.2 Introduction

Although males and females in sexually reproducing species interact to produce offspring, the fitness interests of the two sexes are not identical, often leading to divergent optima for mating frequency, mate identity, and parental care distribution (Chapman and Partridge 1996). Such sexual conflict is expected to result in antagonistic coevolution between the sexes, as adaptations that shift trait values toward the optimum of one sex necessarily move them farther away for the other, increasing selection for counteradaptation (Parker 1979, Arnqvist and Rowe 2002). Sexual conflict can be manifested throughout the process of mating and reproduction, from mate choice and receptivity, to sperm transfer, storage, and utilization, to post-copulatory offspring provisioning and care (Parker 2006, Brennan and Prum 2012).

Sexually antagonistic coevolution is typically thought of as an intraspecific process, but mating interactions between species can also generate interspecific conflict between the sexes that in some cases may impose even stronger selective pressures than those acting intraspecifically (Schartl et al. 1995, Pfennig 2007). The costs and benefits of hybridization can vary in magnitude and even direction between species and sexes (Landmann et al. 1999, Engeler and Reyer 2001, Pfennig and Simovich 2002), with the same hybrid progeny representing a fitness benefit for one parent but a cost to the other. This is most evident in gynogenetic and hybridogenetic species complexes, where

obligately hybridizing females parasitize the sperm of host species males to initiate parthenogenetic offspring development (gynogenesis) or to produce progeny that discard the paternal genome during reproduction (hybridogenesis) (Beukeboom and Vrijenhoek 1998). Whether the strong selective pressures in such systems can drive sexually antagonistic coevolution, however, is not yet clear; experimental studies of inter-genomic conflict over mate choice have yielded contradictory results (Engeler and Reyer 2001, Riesch et al. 2008).

One case where strong inter-genomic sexual conflict is predicted is social hybridogenesis in the harvester ant genus *Pogonomyrmex* (Helms Cahan and Vinson 2003). Ants have a eusocial colony structure, with sterile worker and reproductive queen castes. In the two socially hybridogenetic populations described (the "H" and "J" populations), reproductive caste is mediated by genetic ancestry. Each population is composed of two genetically distinct but interbreeding lineages (lineages "1" and "2") derived from historical inter-specific hybridization between two extant species, *P. barbatus* and *P. rugosus* (Helms Cahan and Keller 2003). Although the two lineages are derived from hybridization, lineage "1" is more closely related to *P. rugosus*, while lineage "2" is more closely related to *P. barbatus*; interbreeding lineages are as genetically distinct from one another as are their parent species, with no evidence of inter-lineage gene flow (Helms Cahan and Keller 2003). Queens mate multiply during a single mating swarm (Hölldobler 1976), and the female progeny of intra-lineage matings develop only into reproductive females, whereas inter-lineage hybrids result primarily in

worker offspring (Julian et al. 2002, Volny and Gordon 2002, Helms Cahan and Keller 2003)(Fig. 2.1). This leads to a conflict of interests, as queens must mate with both lineages to produce both daughter queens and the workforce to care for them (Helms Cahan et al. 2004), but males gain fitness returns only by mating with queens of their own lineage. The cost of mating mistakes for males is potentially quite high, as mating occurs within swarms on a single day over a period of only 1-2 hours, with intense male-male competition (Hölldobler 1976); thus, males that hybridize may lose the opportunity to mate with a female of their own lineage, especially if, as is often the case, one lineage is relatively rare in the population (Anderson et al. 2006b). An adaptive male preference for intra-lineage copulations, however, would be in direct conflict with the interest of queens, who are largely unable to produce workers in the absence of hybridization and suffer direct productivity costs as the proportion of inter-lineage matings declines (Schwander et al. 2006). Importantly, the selective pressures acting on males and females over hybridization run counter to how intraspecific sexual selection typically acts in harvester ants, leading to clear alternative predictions for how the sexes should behave under these two forms of sexual selection; rather than mate indiscriminately, the high costs of mating mistakes in this case would select for male selectivity, while queens normally expected to be selective may instead benefit from higher mating frequency and diversity.

In this study, we investigated the effects of inter-genomic sexual conflict on males and females of the "J"-lineage socially hybridogenetic population. We generated

controlled crosses in the field and compared the mating behaviors of intra- and inter-lineage pairs to test whether either or both sexes show evidence of adaptation to hybridization-driven sexual conflict. We considered pre-copulatory mate choice as well as more cryptic control mechanisms over investment once copulation is initiated, including copulation duration, rate of sperm delivery, and total sperm transfer. Because males receive no direct benefits from hybridization, we predicted that if mating patterns reflected male interests, inter-lineage matings (J1/J2 and J2/J1 pairs) should be less likely to occur, result in lower sperm transfer, and/or be shorter in duration than intra-lineage matings. In contrast, females require sperm from both male types, and thus under female control we expected to see either preference for an equal mating investment or compensatory bias by females toward inter-lineage males in response to male biasing strategies.

2.3 Methods

2.3.1 Field Experiments

Field experiments were performed during natural mating flights in July 2010 (2 flights) and July 2011 (2 flights) near Portal, Arizona on the Arizona-New Mexico border in the southwestern United States. Mating flights occur 1-2 days after a heavy monsoon rain storm at approximately 15:30 PST (Hölldobler 1976). Adult colony lineage frequencies are predictably biased in J-lineage populations, with the J2 lineage comprising approximately 67% of mature colonies (Anderson et al. 2006b, Schwander et

al. 2007). Once a mating swarm site was located, incoming virgin males and females were captured in the air as they approached the swarm using insect nets and isolated by sex in plastic boxes. Mating observations were conducted continuously by one to four observers per swarm until natural copulations ceased at approximately 17:00 PST. For each mating observation, a single male and female were selected at random and placed into a clean 20 mL PET clear plastic tube for observation; the two lineages cannot be distinguished by eye, so observers were blind to the lineage identities of interacting pairs. Initiation of successful copulation was defined operationally by male release of the female's thorax following genital contact, such that the pair remained attached only by the copulatory organs. If copulation was not initiated after five minutes, the mating was considered failed and the male was replaced, for a maximum of two pairings per queen. If the pair successfully mated during this period, willingness to mate was quantified as copulation latency, the length of time between introduction and the initiation of copulation.

For pairs in which copulation was initiated, copulation duration was measured to the nearest second and specimens were placed on ice immediately after separating. A total of 87 experimental pairings were used. Two of these mating pairs were discarded from the analysis; in one pair the male escaped following copulation, and in the second the pair failed to detach successfully after eight minutes in copula. All individuals were shipped on ice overnight to the University of Vermont for genetic analysis and sperm quantification.

2.3.2 Lineage Identification and Body Size Measurement

To determine lineage identity, all samples were typed with lineage-diagnostic restriction assays of the *cox1* mitochondrial gene (bp = 433) (Helms Cahan and Keller 2003). DNA was extracted from each sample with a rapid Chelex extraction protocol (Schwander et al. 2007) on tissue from two legs. Lineage identification was performed by PCR amplification as in Helms Cahan and Keller (2003), followed by enzymatic digest using the enzymes MfeI and BsaI. These enzymatic digests can be used for diagnostic identification of lineage, since MfeI matches a restriction site unique to the J1 lineage haplotype, while BsaI matches a site unique to the J2 lineage haplotype(Helms Cahan and Keller 2003). Positive J1 and J2 controls were included in each digest to confirm enzyme activity. The resulting fragments were run on a 1.5% agarose gel at 120 mV until separation could be resolved. Several individuals who could not be identified with this method due to DNA degradation were genotyped for the microsatellite locus *Myrt-3* following Helms Cahan& Keller (2003), which shows fixed differences between the two lineages (Volny and Gordon 2002).

Head width was used as a proxy for body size of males and females (Ferster and Traniello 1995). The distance between the eyes (above each eye for males and below each eye for females) was measured to the nearest 0.05mm using an optical micrometer. Sex-specific standardized z-scores (standard deviation units from the mean) were used instead of raw head width values in all statistical analyses.

2.3.3 Sperm quantification

Sperm quantification was conducted using an insect sperm fluorescent quantification method (Reichardt and Wheeler 1995), modified by substituting SYBR Green I dye for Hoechst dye and using manual cell counting rather than automated fluorometry. 51 of the 53 mated queens were successfully dissected in 1X PBS solution, and the spermatheca and reproductive tract of each individual were removed intact (Fig. 2.2A). Sperm was extracted from the spermatheca and reproductive tract by opening each of these structures in 200 µL PBS/1% BSA. 200 µL of DMSO was added, and the sample was centrifuged at 15,000 g for 20 minutes. 200 µL of solution was removed, and the sample was sonicated 2X for 30 seconds. 2.5 µL of 10X SYBR Green I was added to 7.5 µL of each sample, and 10 ul mixed with an equal volume of Vectashield (Vector Labs) was placed on a slide for quantification. Once prepared, each slide was counted under 10X magnification on a fluorescent microscope using Stereo Investigator software (MBF Bioscience, Williston VT). The program photographed and randomly partitioned each slide into 9-12 subsets, then the number of cells in each subset were manually

counted, allowing the program to estimate the total cell count on the slide (Fig. 2.2B). In order to compare the quantity of sperm transferred per mating to the total sperm reserves of males, five virgin "J1" and five "J2" lineage males collected immediately before a mating flight were dissected and the *vas deferens* and testes were removed. Sperm was extracted and quantified using the same method used for the spermathecae. Total sperm contents of J1 and J2 males did not differ significantly (Mean Sperm Count, J1: 26.1 x $10^6 \pm 2.5$ x 10^6; J2: 29.99 x $10^6 \pm$ 14.0 x 10^6; Student's t-test, $t = 0.59$, $p = 0.58$).

2.3.4 Statistical Analysis

All statistical analyses were performed in JMP 9.0. Male and female headwidths were compared between lineages using a student's T-test. All other variables were analyzed using a stepwise GLM, with either a binomial (willingness to mate) or a normal (copulation latency, total and rate of sperm transfer, and copulation duration) distribution. Lineage identity of males and females and their interaction were locked into the model to ensure that they were always included; AICc-based model selection was used to select additional covariates for the final model for each variable. Covariates included the number of minutes after commencement of the mating swarm at which the pairing began (time after swarm, or TAS), male size, queen size and their interaction, and the date of the swarm.

2.4 Results

2.4.1 Mating swarm dynamics

As expected from adult colony frequencies, both males and queens collected in the mating swarm were biased toward the J2 lineage (males: 60% J2, queens: 66% J2). J1 and J2 queens did not differ significantly in size (t_{76} = 0.3 p= 0.8); J1 males, however were significantly smaller than J2 males (Mean head width, J1: 2.05± 0.1 mm; J2: 2.10 ± 0.11mm; Student's T-test,t_{85} = 1.98, p< 0.03). Despite this size difference, there was no significant effect of male size, queen size, or the interaction of male and female size on mating success, copulation latency, or copulation duration.

2.4.2 Mate choice

The proportion of successful copulations declined significantly over the course of the swarm (χ^2_1=12.48, p< 0.001), and copulation latency of successful matings increased (χ^2_1= 5.37, p<0.03). Contrary to expectations, intra- and inter-lineage pairings were equally likely to lead to copulation (χ^2_1=0.52, p= 0.47), although queens from the less common J1 lineage were significantly less likely to mate overall than the more common J2 lineage (χ^2_1= 12.48, p<0.001;Fig.2.3). Copulation latency for successful mating pairs showed a similar pattern, with both J1 queens and males taking significantly longer to initiate copulation than J2 queens and males (Males:χ^2_1 = 4.14, p< 0.0417;Queens χ^2_1= 6.84, p< 0.01); however, there was no interaction effect between lineages, suggesting that mating success was not biased toward a specific type of cross (χ^2_1 = 1.04, p= 0.31).

In contrast, the duration of successful copulations showed significant differences between intra- and inter-lineage pairings ($\chi^2_1 = 4.33$, $p < 0.04$). When controlling for time of day ($\chi^2_1 = 11.85$, $p < 0.001$), intra-lineage copulations were 13.1% shorter than inter-lineage copulations (Least Squared Means, intra-lineage pairs: 217.1 ± 15.1 sec; inter-lineage pairs: 249.8 ± 16.2 sec). Both types of intra-lineage pairings (J1/J1, J2/J2) showed a similar reduction in copulation duration (Fig. 2.4A).

2.4.3 Sperm transfer

Males transferred an average of $19.164 \times 10^6 \pm 1.081 \times 10^6$ sperm when mating with a virgin female, which represents 68.5% of their entire sperm reserves (total in virgin males: $27.991 \times 10^6 \pm 3.067 \times 10^6$, n = 10). As expected, total sperm transfer was positively correlated with copulation duration ($F_1 = 7.07$, $p<0.02$), but total sperm transferred to queens did not differ between intra- and inter-lineage pairings ($\chi^2_3=0.74$, $p= 0.38$; Fig 2.2C).

When considered per unit time in copula, however, the rate of sperm transfer was significantly higher in intra-lineage than in inter-lineage pairs (male x female lineage interaction effect, $\chi^2_1= 11.74$, $p< 0.001$). As predicted from their relative rarity in the

population, the sperm transfer rate of J1 males responded more strongly to queen lineage identity, with transfer rates to high-fitness J1queens nearly double the rate of low-fitness matings with J2 queens (Dunnett's test, p=0.03); sperm transfer by J2 males to intra-lineage J2 queens was also significantly higher , but to a smaller degree (Fig. 2.4B, Dunnett's test p=0.01). The rate of sperm transfer was also significantly higher overall for J1 males than J2 males (Least squared means ± SE: J1 = 122,080 ± 12,275 sperm/sec, J2 = 86,200 ± 7556 sperm/sec; $\chi^2_1 = 6.65$, $p < 0.01$), and increased over the course of the swarm ($\chi^2_1 = 7.31$, $p < 0.01$).

2.5 Discussion

Hybridogenetic mating systems generate divergent selective pressures on males and females over investment into conspecific and heterospecific matings. Previous work on the socially hybridogenetic harvester ant lineages found no deviation from random mating in these populations despite strong interspecific sexual conflict, suggesting that they may have lacked the capacity or time to evolve adaptive responses (Helms Cahan et al. 2004, Anderson et al. 2006b, Schwander et al. 2006). The results of this study, however, suggest that this overall pattern is not indicative of an absence of adaptation, but instead reflects the net result of counteracting effects of sexually antagonistic coevolution over copulatory investment. Males of both lineages strategically adjusted

sperm transfer rate based on the lineage identity of their mate, increasing transfer rate with same-lineage females, with the strongest response occurring in the rarer lineage whose males were most likely to make mating errors. In isolation, the effect of such selective sperm transfer would be to improve male fitness at the expense of female procurement of sperm for worker production, with costs to colony foundation and growth and potential destabilization of the hybridogenetic system as a whole (Anderson et al. 2006b, Schwander et al. 2006). Such an effect is not realized, however, because increased sperm transfer rate toward same-lineage females is balanced by a significant reduction in the duration of such copulations. The overall result is a similar total quantity of sperm transferred per mating despite the fact that both males and females manipulate aspects of copulation to maximize their fitness.

One of the most common and obvious manifestations of sexual conflict occurs during pre-copulatory mate choice, with one sex (typically males) pursuing matings that are actively resisted by the other sex (Holland and Rice 1998); however, our results suggest that pre-copulatory mate choice is not the primary mechanism by which males and females impose their genetic interests in this system. We found no evidence of pre-mating discrimination between lineages in either the likelihood or latency of mating initiation. This is consistent with earlier studies demonstrating a significant correlation between the relative frequencies of the two lineages in the population at large and the relative mating success of males inferred from patriline distributions in adult colonies (Helms Cahan and Julian 2010, Suni and Eldakar 2011). It important to note, however,

that males and/or females may still display preferences when both lineages are available for direct comparison; such simultaneous mate choice experiments could not be conducted here due to the time constraints caused by the limited duration of mating swarms and the difficulty of identifying lineage in the field.

Both discrimination costs and informational constraints may contribute to the absence of mate preference. As in other sperm parasites in which mate discrimination is low (Landmann et al. 1999) or absent (Engeler and Reyer 2001), *Pogonomyrmex rugosus* mating swarms have a strongly male-biased operational sex ratio, with an average of four males per female physically competing in a characteristic "mating ball" (Hölldobler 1976). Intense contest competition between males can make the delay associated with mate assessment more costly than accepting some proportion of low-fitness matings, even if inter-lineage females can be identified (Pfennig 1998, Engeler and Reyer 2001). Alternatively, individuals may be unable to discriminate lineage identity before initiating copulation, preventing mate selection from occurring. Notably, males and queens of the two J lineages are visually indistinguishable (Schwander et al. 2007), despite being originally derived from differently-colored parental species (Helms Cahan and Keller 2003). This same pattern of convergence in queen, but not male, coloration occurs in the "H" lineage pair (Helms Cahan et al. 2002). Similarly, the volatile mating pheromone is suggested to be highly conserved within the *P. barbatus* complex (Hölldobler 1976), making it unlikely that it contains information on individual lineage. Correct assessment of cuticular hydrocarbon cues, a common method for

reproductive isolation among many insects (Howard and Blomquist 2005) might be unreliable or time-intensive in mating swarms; although CHC assessment is generally very fast and reliable, recognition in mating swarms can become more difficult when individuals exchange hydrocarbons with other reproductives as they come into physical contact, homogenizing their profiles and reducing their ability to use lineage-specific CHC differences (Volny et al. 2006) to accurately identify a potential mate's lineage before copulation.

In the absence of a pre-copulatory cue, males may instead benefit from maximizing mating frequency and thus limit the fitness consequences of any individual copulation (Suni and Eldakar 2011). For queens, the primary risk of a suboptimal mating distribution is failure to procure inter-lineage sperm required for worker production; the converse problem, failure to produce queen-destined progeny, is less costly because queens can recoup some fitness returns through parthenogenetically-produced haploid male progeny (Helms Cahan and Julian 2010). This suggests that mate identity costs increase for queens as a lineage becomes more common; in the absence of mate preference, queens of the more common lineage appear to respond to local lineage skew by increasing mating frequency (Suni et al. 2007), which could explain why the more common J2 lineage was significantly more willing to mate and initiated copulations sooner than the J1 lineage in this study.

In contrast to events prior to copulation, the dynamics of copulation itself showed striking differences between inter- and intra-lineage pairs that reflected the fitness interests of males and females. As suggested in earlier work (Baer 2011), the first copulation by a male constitutes nearly 70% of his total lifetime sperm transfer, imposing selection on males to bias copulatory investment toward high-fitness mates. Because males actively pump sperm into the female reproductive tract during copulation, sperm transfer rate is expected to be primarily under male control; consistent with male interests, the rate of sperm transfer was significantly higher for both J1/J1 and J2/J2 intra-lineage matings than for either inter-lineage cross. This suggests that some cues indicating lineage identity are available to males, although they may not be detectable or acted upon when mate choices are being made. It is possible that after copulation begins, males have a better opportunity to ascertain differences in cuticular hydrocarbon (CHC) profiles(Volny et al. 2006) to identify female lineage in a way that would be too costly or time consuming before copulation begins.

Unlike queens, for whom costs increase as a lineage becomes more common, male costs increase as a lineage becomes more rare, increasing the likelihood that the first mating will be with a low-fitness inter-lineage female. Across J-lineage populations, the J1 lineage is consistently under-represented, making up approximately one-third of the adult colony population (Schwander et al. 2005, Anderson et al. 2006b, Gordon et al. 2013), with similar proportions in the mating swarms investigated in this study. Consistent with their relative rarity, the difference in sperm transfer rate between intra-

and inter-lineage copulations was particularly high for J1 males, whose intra-lineage sperm transfer rate was the highest of all the pair types and nearly double that observed when J1 males were paired with low-fitness J2 queens (Fig. 2.4B).

The difference in sperm transfer rate between intra- and inter-lineage matings did not translate into a difference in overall sperm transfer due to a second difference between these pair types that acted in the opposite direction: same-lineage copulations were significantly shorter in duration than those occurring between lineages (Fig. 2.4A). Although males are typically thought to also control copulation duration, female control via a genital locking mechanism is known to occur in some insects with a polyandrous mating system (Davidson 1982) to regulate the sperm contributions of individual males and prevent male mate-guarding (Brown and Baer 2005).Polyandry is nearly universal in the genus *Pogonomyrmex* (Hölldobler 1990); the existence of such a mechanism may have enabled queens to respond to selection in hybridogenetic systems, terminating insemination by same-lineage males earlier to reduce their relative sperm contribution.

Because male and female biasing strategies were of similar magnitude but differed in direction of effect, the net result of conflict over copulation was equal sperm transfer (Fig. 2.2C), the same outcome as would be expected in the absence of conflict. These results show the importance of fully characterizing different mechanisms when investigating sexually antagonistic coevolution. Mate choice tends to be the most commonly studied form of sexual conflict, but both males and females may also impose

their genetic interests via cryptic mechanisms that are more difficult to assess (Arnqvist and Rowe 2002). Such mechanisms may play a particularly important role in species for which mate choice is costly or constrained. Moreover, the counteracting effects of adaptive changes in complementary control mechanisms may mask the existence of conflict when only a summary measure of reproductive success is observed. By decomposing the mating process into its component parts, the conflict over control of each aspect became apparent and revealed a dynamic coevolutionary process.

Within species, sexually antagonistic coevolution has the potential to lower the mean fitness of an entire population if enough costly adaptations are implemented (Rice 1996). Paradoxically, however, the interaction between males and females in hybridogenetic populations may play an important role in maintaining such systems over evolutionary time (Schupp and Plath 2005). If males were fully successful in discriminating and selectively transferring sperm to their own lineage, queens would be unable to produce workers (Gordon et al. 2013) and populations would quickly collapse. The tight ecological linkage between hybridogenetic taxa and their hosts, imposed by obligate hybridization, appears to provide the evolutionary opportunity for both sexes to evolve in response to inter-genomic sexual selection. This suggests that strong antagonistic sexual conflict may facilitate evolutionary persistence of interdependence at higher taxonomic levels such as occurs in harvester ants, despite the intrinsic disadvantages such complex mating systems entail.

2.6 Figures

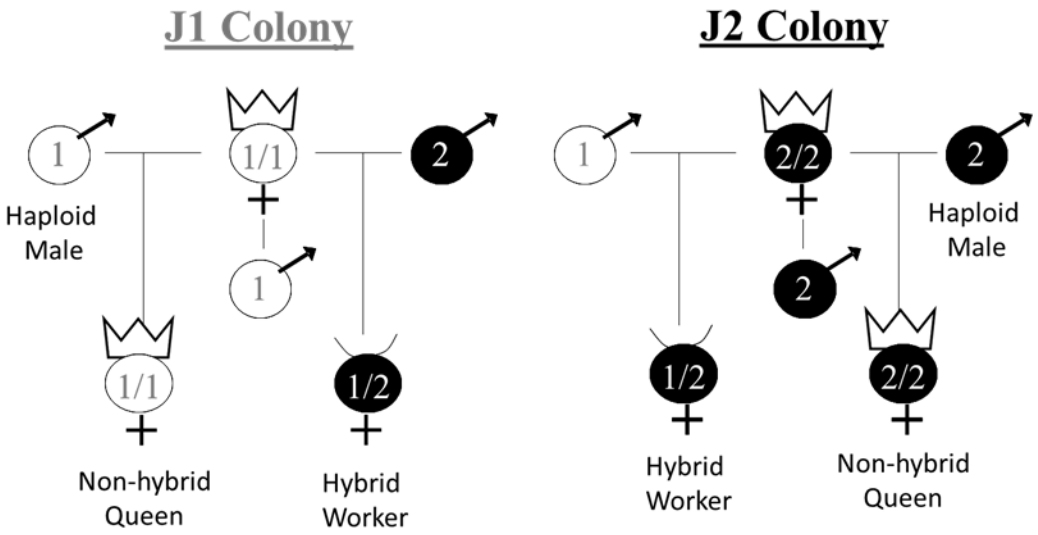

Figure 2.1: Pedigree illustrating the hybridogenetic reproductive system of Pogonomyrmex harvester ants. Female symbols with crowns are reproductive queens, andfemale symbols with crescents are non-reproductive workers. Numbers refer to the identity (lineage '1' or '2') and number of genome copies (one digit ¼ haploid,two digits ¼ diploid) of the genomes of each individual type.

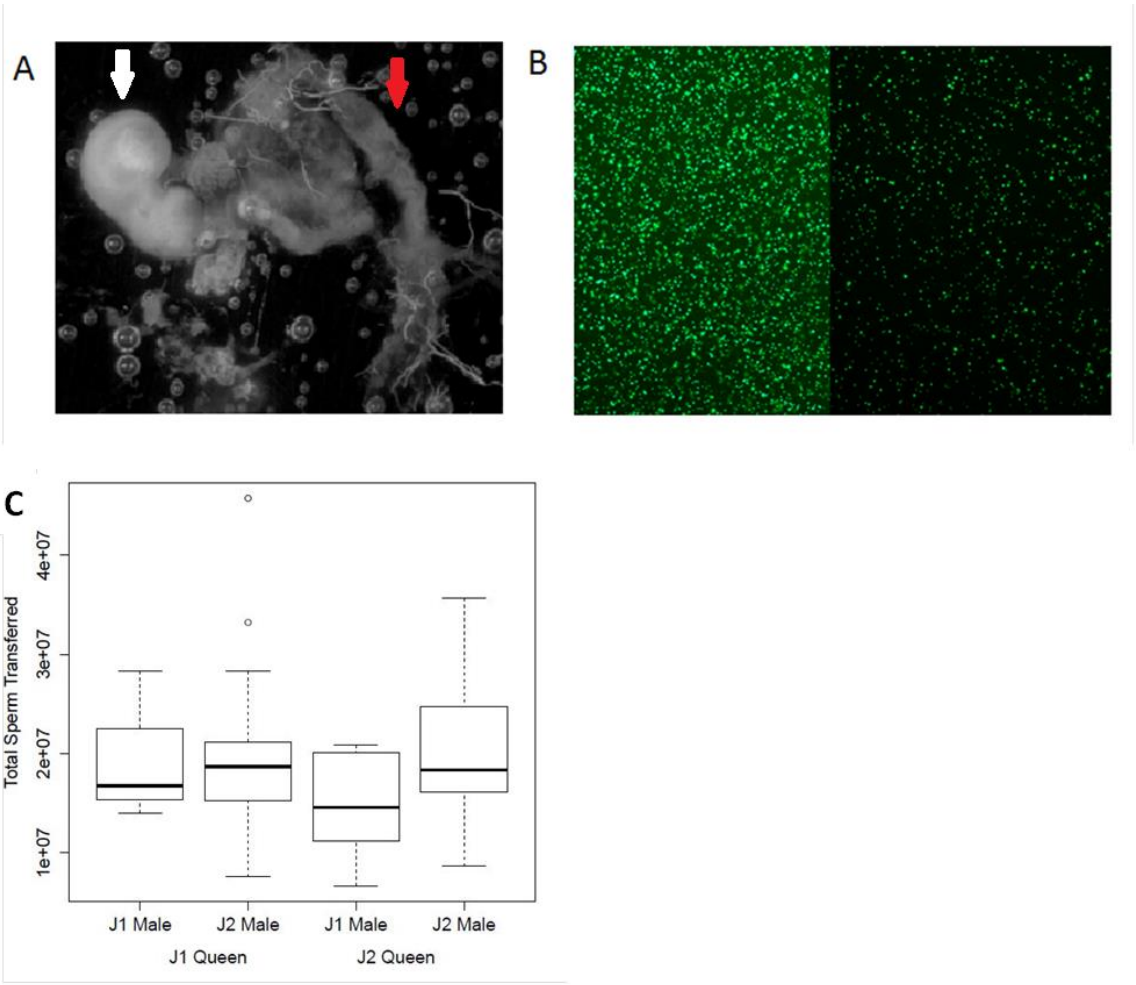

Figure 2.2: Images of (a) extracted spermatheca (white arrow) and reproductive tract (white-outlined arrow; red online), (b) fluorescent sperm under 10_ magnification for individuals with high sperm transfer (left) and low sperm transfer (right) and (c) boxplot of total sperm transferred by mating pair type. Error bars show the furthest datapoint within 1.5 quartiles of the box.

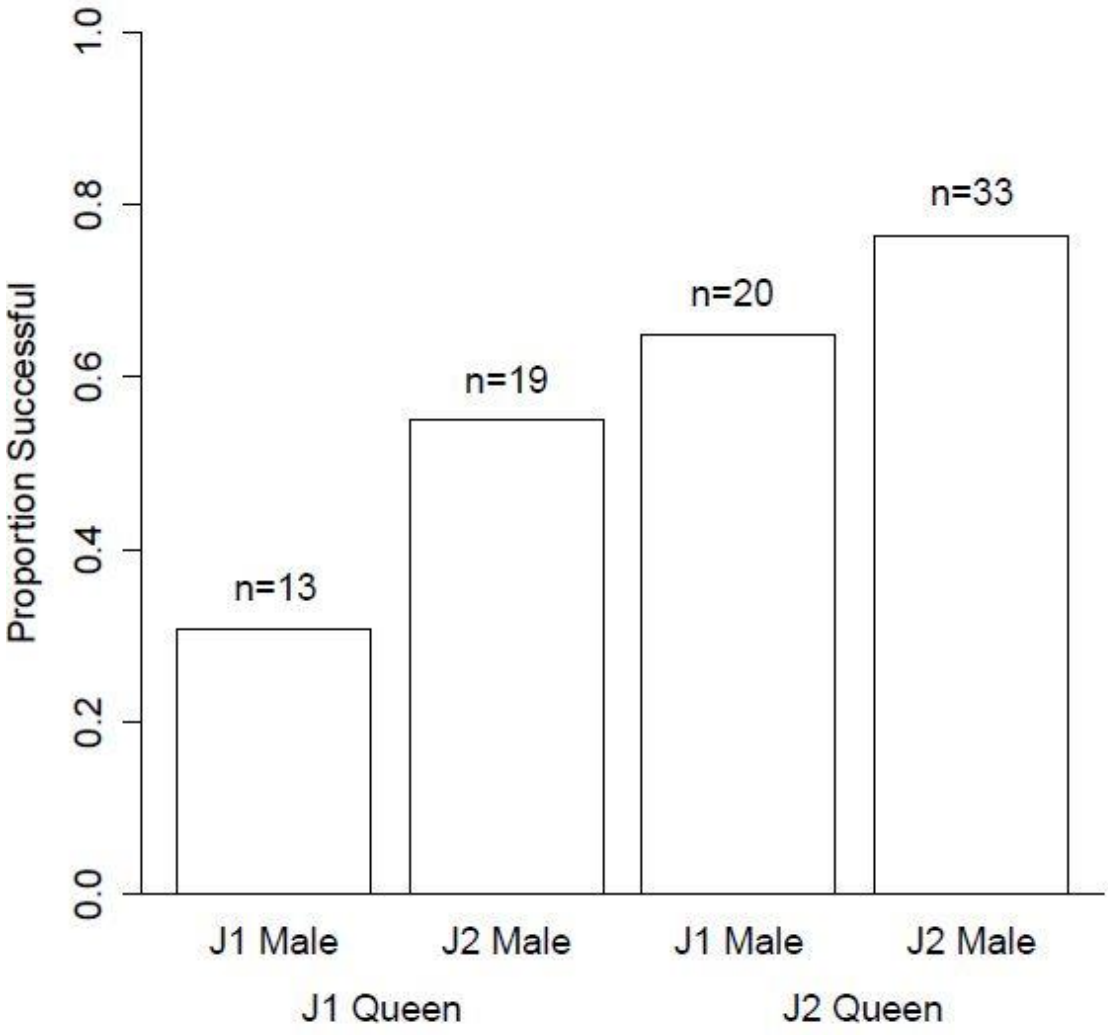

Figure 2.3 : Proportion successful matings for the four mating pair types. The outer two pairings (J1/J1 and J2/J2) are the pure-lineage matings that produce new queens, whereas the inner two pairings are inter-lineage matings that produce workers.

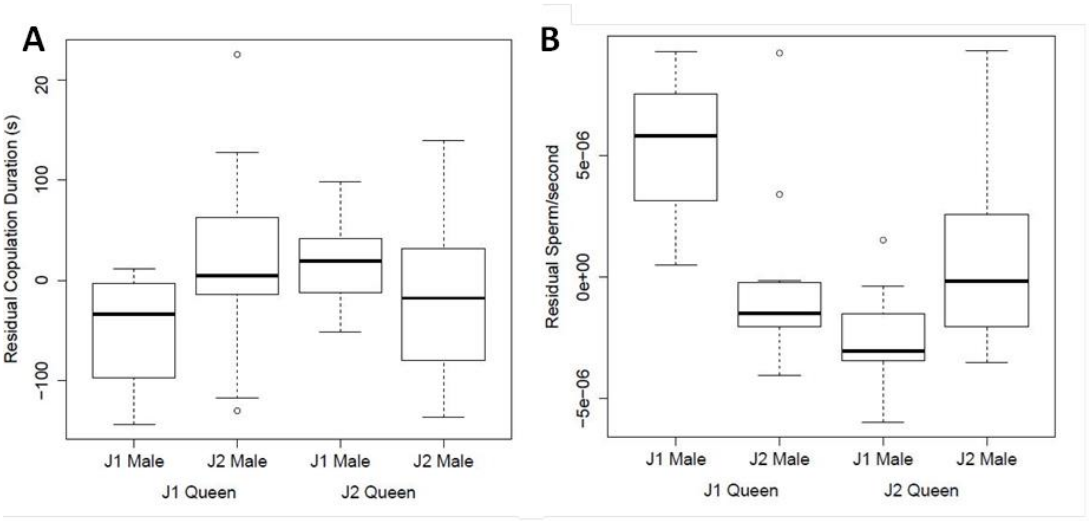

Figure 2.4 : Boxplot of (a) copulation duration and (b) sperm transfer rate by mating pair type. Error bars show the furthest datapoint within 1.5 quartiles of the box.

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CHAPTER 3: EVIDENCE FOR A DESICCATION-COMMUNICATION TRADEOFF IN THE CUTICULAR HYDROCARBON PROFILES OF *POGONOMYRMEX* HARVESTER ANTS.

3.1 Abstract

Cuticular hydrocarbons (CHCs) are multipurpose compounds, used for both desiccation resistance and communication. Hydrocarbons that are optimized for desiccation resistance (n-alkanes) have limited variation, reducing their usefulness for communication, while communication-specific hydrocarbons (methyl-alkanes and alkenes) which are more variable, perform poorly in providing resistance to desiccation, leading to a predicted trade-off between these two functions in hydrocarbon composition. We characterized the hydrocarbon profiles of queens and workers from a species complex of *Pogonomyrmex* harvester ants that occur across a range of environments, from mesic coastal plains to xeric desert habitats. Interspecific hybridization between the mesic *P. barbatus* and the xeric *P. rugosus* has resulted in at least two relic hybrid zone populations, where lineages derived from the two species obligately interbreed to produce F1 workers while reproductive females are produced from pure-lineage progeny. We hypothesized that thermal constraints would lead to less complex CHC profile in more xeric species. In addition, we hypothesized that a subset of signaling compounds would differentiate between species and caste, allowing for discrimination. Our results suggest that thermal constraints play a role in limiting the hydrocarbon profile, with the more xeric species having the lowest percentage of information-rich methyl-alkanes and alkenes, as well as the highest melting points and a less diverse CHC profile. Consistent with the predicted thermal constraints of these desert-adapted ants, statistical discrimination of species identity based on CHC composition was relatively poor, particularly for taxa occupying similar habitat types. Under low-precision detection conditions such as mating swarms, quantitative differences in low-quantity compounds may be difficult to distinguish, facilitating mis-identification of mates and potentially leading to the forms of hybridization found in these species. Queens and workers, however, differed significantly in CHC composition; worker profiles were biased toward desiccation-resistant alkanes, while queens contained greater quantities of signaling molecules, including nonacosene, a highly conserved fertility signal in the eusocial Hymenoptera. Workers experimentally isolated from the queen shifted away from the CHC profile of their non-isolated sisters, suggesting that queen-mediated signals affect overall colony CHC profiles. This work highlights the complexity of function and information-coding of CHC profiles in harvester ants, and reinforces the role of thermal constraints on the diversification of communication molecules.

3.2 Introduction:

Cuticular hydrocarbons (CHCs) are multi-purpose molecules covering the cuticle of arthropods (Blomquist and Bagnères 2010). These hydrophobic waxes are used by terrestrial insects to prevent excess evaporation across the cuticle, especially in dry, hot environments (Rouault et al. 2004, Frentiu and Chenoweth 2010), and have been evolutionarily co-opted for use in communication (Dietemann et al. 2003, Martin and Drijfhout 2009a, Greene 2010). CHCs are often species- and sex-specific, facilitating correct mate selection (Ferveur 2005, Peterson et al. 2007, Zhang et al. 2014). Quantitative variation in CHC composition, particularly the abundance of specific metabolically expensive molecules, can convey information about mate quality (Howard and Blomquist 2005, Ingleby et al. 2013).

The communication function of cuticular hydrocarbons in the eusocial insects has expanded to communicate information across a variety of hierarchical levels, resulting in complex hydrocarbon profiles in these insects(Blomquist and Bagnères 2010). In the eusocial Hymenoptera as well as in termites, cuticular hydrocarbons are the primary nestmate recognition cues (Breed 1983, Breed 1998, Gamboa 2004, Howard and

Blomquist 2005, Nunes et al. 2011). Although individuals produce their own unique CHC signatures, hydrocarbons are transferred among individuals within the colony through allogrooming, creating a more uniform colony "gestalt" odor that allows for colony identification (van Zweden et al. 2010). Accurate nestmate discrimination relies upon the presence of colony-level variation in CHC production, which has a significant genetic component (Thomas and Simmons 2008, van Zweden et al. 2009); in some invasive species, for example, the loss of genetically-based variation in CHC composition during initial bottlenecks is thought to be one factor underlying the loss of inter-colony aggression (Giraud et al. 2002, Brandt et al. 2009). Within colonies, social insects also use cuticular hydrocarbons to signal queen fecundity and status (Meunier et al. 2010), worker task (Howard and Blomquist 1982, Wagner et al. 1998, Greene and Gordon 2003), and to distinguish social parasites from the natal brood (Solazzo et al. 2013).

Although CHC molecules serve both desiccation resistance and communication roles, the relative value of individual molecules for these two functions tends to be negatively correlated, generating a trade-off between optimizing informational content of the CHC profile and maximizing desiccation resistance (Gibbs and Pomonis 1995). The hydrocarbon profile can include alkanes, alkenes, methyl-alkanes, as well as a smaller quantity of alkynes and esters. Straight-chain alkanes generate a high density water-resistant wax layer, limiting water loss across the cuticle (Gibbs 1998); however, straight-chain alkanes vary only in overall chain length, limiting the molecular diversity possible and thus the amount of information that can be used for recognition. More complex CHC

molecules show the opposite pattern: the addition of one or more methyl groups or unsaturated bonds increases the number of possible molecular variants because these functional groups can occur at any of the carbons across the hydrocarbon chain, with different physical properties at each location (Gibbs 1998). However, the addition of each of these functional groups decreases the density of the CHC wax layer, limiting their effectiveness in desiccation resistance (Gibbs and Pomonis 1995).

This trade-off between communication and desiccation molecules may be especially important in insects that live in extreme xeric environments, where selective pressures have the potential to limit the proportion of the CHC profile that can be dedicated to information-rich desaturated and methylated hydrocarbons and thus constrain information transfer. A pattern consistent with this trade-off has been shown in *Drosophila*, with warmer climates associated with longer, straight-chain hydrocarbons (Frentiu and Chenoweth 2010). In ants, the impact of thermal and desiccation stress on the production of signaling molecules has been difficult to test, in part due to environmental factors that can also influence the composition of hydrocarbons (Wagner et al. 2001, Martin and Drijfhout 2009a). If signaling is constrained in extreme environments, however, it could reduce the effectiveness of discrimination in desert species, potentially resulting in reduced intercolony aggression and increasing the likelihood of mating errors and hybridization.

Ants from the genus *Pogonomyrmex*, which use CHCs as the primary form of nestmate recognition (Hölldobler and Lumsden 1980, Wagner et al. 1998, Wagner et al. 2000), provide an ideal system to study the evolutionary dynamics of cuticular hydrocarbon profiles. This New World genus originated in South America and has radiated throughout the Americas, extending in North America into the Chihuahuan, Sonoran, and Mojave deserts in the United States (Anderson et al. 2006a, Schwander et al. 2007). Many species form large, long-lived colonies with extensive group foraging trail systems; previous work on the Red harvester ant, *P. barbatus*, has shown a significant influence of worker task on individual hydrocarbon profile, with foragers that are exposed to the hottest surface environments having the greatest number of straight-chain alkanes (Greene and Gordon 2003). This species is a member of a larger clade, the *P. barbatus* complex, which spans a range of thermal and humidity conditions that may favor CHC divergence; *P. barbatus* is found in a wider range of temperatures, but generally in the higher precipitation mesic habitats of central and eastern Texas, New Mexico, and central and eastern Mexico (Fig. 3.1A). The closely-related congener, *P. rugosus*, is found further west in drier, more xeric habitats in Arizona, Nevada, southern California, and western Mexico (Anderson et al. 2006a, Schwander et al. 2007). In addition, *P. barbatus* and *P. rugosus* have historically hybridized in the Chihuahuan grassland region where their ranges overlap, leading to at least four genetically-depauperate remnant lineages (J1, J2, H1, H2) that are now reproductively isolated from allopatric populations (Helms Cahan and Keller 2003, Anderson et al. 2006a, Cahan et al. 2006, Schwander et al. 2007). Although separated from their respective parent species,

these lineages obligately hybridize with one another to produce F1 hybrid workers, while rearing daughter queens from pure-lineage progeny (Julian et al. 2002, Volny and Gordon 2002, Helms Cahan and Keller 2003). This unusual system, referred to as Genetic Caste Determination, or GCD, provides a unique opportunity to investigate the effects of ancestry, population bottlenecks and hybridization on CHC composition and diversity.

In this study, we analyzed the cuticular hydrocarbon profiles of queens and workers of *P. barbatus*, *P. rugosus*, and the four GCD lineages reared under common-garden conditions in order to investigate the roles of habitat-mediated selection, genetic composition, and reproductive caste on the composition of CHC profiles. We predicted the more xeric species, *P. rugosus*, would possess a simpler CHC profile, composed of a higher proportion of straight-chain desiccation-resistant hydrocarbons and a higher overall average melting point, when compared to the mesic species *P. barbatus*. Because each GCD lineage is derived genetically primarily from a single parent species (Helms Cahan and Vinson 2003), we expected that the profiles of queens of the four GCD lineages would reflect their genetic ancestry, with significantly less heterogeneity in CHC profile among individuals of these lineages due to their reduced genetic diversity. We also tested whether the reciprocal F1 hybrid workers produced by each pair of interbreeding lineages showed CHC profiles intermediate to their parents or displayed phenotypes consistent with dominance or maternal effects. Last, we tested whether queen-specific components of the CHC profile, expected to signal reproductive status and fertility, were conserved across taxa as predicted by comparative studies across the

eusocial Hymenoptera (Van Oystaeyen et al. 2014). To further investigate the level of control that queens exert on worker profiles, we compared sibling workers reared in experimental colony fragments established with and without a queen.

3.3 Methods

3.3.1 Samples

Experimental colonies were raised from newly-mated queens collected from the field following mating flights in Texas, Arizona and New Mexico (Table 3.1). To determine the lineage of the GCD queens, which are not distinguishable by eye, the lower portion of a single middle leg was surgically removed from each queen following collection, from which DNA was extracted by homogenizing the leg and heating it with 250 µl of Chelex 100 chelating resin (Bio-Rad Laboratories, Hercules, CA) at 90°C for 20 minutes. The DNA was genotyped at four microsatellite loci (Pb5, Pb7, Pb8, and *Myrt*3) showing diagnostic or significantly different allele frequencies between interbreeding lineages as in Helms Cahan & Keller (2003). Colonies were raised in common-garden conditions in a steady temperature environment at 28°C with a 12-hour light/dark cycle. Individual colonies were housed inside 13x17.5x7 cm plastic boxes with two 16x155mm culture tubes filled approximately 1/3 full of water held in place by a small cotton ball to provide a humid nesting area. All colonies were fed an identical diet

of seed mixture (wheat germ, cornmeal, and oat bran) and two mealworms per week. Five queens of each species or lineage were sampled within 8 weeks of the emergence of the first workers; a single worker was sampled from an additional five colonies when the colonies were 3-4 years old, with the exception of *P. rugosus*, for which too few colonies of this age were available, so older colonies collected from the same site were also sampled. Worker samples used for CHC analysis were removed from the foraging area of each nest box to ensure that only mature workers were collected. Each sample was collected live, placed into a 1.5 mL centrifuge tube and immediately frozen at -20° C until CHC extraction.

To test for queen-mediated effects on the CHC profile, we created worker-only colony fragments by isolating worker pupae from full colonies during development and transferring them to a separate worker-only colony box where they were allowed to develop. By isolating workers as pupae, we ensured that the adult workers in these colony fragments only possessed worker-created hydrocarbon profiles, with no queen-derived compounds passed on from the colony gestalt odor. Five colonies from each of the four GCD lineages, for a total of 20 colonies, were used in this experiment. From these twenty colonies, an additional twenty colony fragments were created by transferring worker pupae found in the queen-right colonies over a 42-day period to each colony's respective worker fragment. At the end of this period, five workers from queen-right colonies and five workers from isolated worker fragment colonies were collected for each lineage and frozen for CHC analysis.

3.3.2 CHC Analysis

Cuticular hydrocarbons were extracted from individual ants in 500 µl of pentane in a 5 ml glass vial. The vial was agitated for two minutes to ensure that the solvent had contact with the entire cuticle, and incubated for an additional 8 minutes. The ant was removed and the pentane evaporated.

To analyze the hydrocarbon profile, each sample was reconstituted in 50 µl of hexane, placed in a 1.5 ml autosampler vial with a 300ul glass conical insert, and run through an Agilent/HP 5973 gas chromatography-mass spetrometer, using electron impact ionization (EI) and a quadrapole mass analyzer with a Zebron ZB-1 column. We used a protocol modified from Wagner (1998, 2000), with an inlet temperature of 300° C. A 5:1 split/splitless injection was used, and the carrier gas was helium at 1 cm^3/s .The oven was started at 100°C, increased at 25°C/min until 240° C, held for 15 minutes, then ramped to 320 at 25° C/min. A five-minute solvent delay was used to ensure the hexane solvent did not affect the gas chromatography.

Compounds were identified by comparing the mass spectrum of each component in each sample against published spectra from the NIST Chemistry Webbook database, then checked against the published CHC profiles of Pogonomyrmex ants in Wagner (1998, 2000) for verification. The elution profile area of each component was integrated from the total ion count for each sample using Chemstation software (Agilent co.); each component was then divided by the total ion count to obtain proportional amounts of each

component. The resulting values were arcsine-transformed to normalize for statistical analysis. Composite melting points for each sample were estimated using melting temperatures obtained through the NIST Chemistry Webbook when available, and using extrapolation from the methylation and unsaturation measurements in Gibbs 2002 for compounds without a measured melting point, to create a weighted melting point average.

3.3.3 Statistical Analysis

Data analysis was performed in RStudio Version 0.98.1091. To test whether different classes of compounds differ in the extent of variability across samples, we compared the coefficients of variation of the three main classes of hydrocarbons: alkanes, alkenes, and methyl-alkanes. The mean average temperature and average precipitation for the ECG and GCD lineages were obtained by using coordiantes from published collection data (Anderson et al. 2006a, Schwander et al. 2007) and obtaining environmental variables for these coordinates through the Worldclim climate database (Hijmans et al. 2005).To compare the desiccation-resistance properties of CHC mixtures among taxa, two measures of desiccation resistance were calculated: the average melting temperature, with the melting temperature of each compound weighted by its relative abundance, and the total proportion of straight-chain n-alkanes. Mixture diversity was quantified using Hurlbert's Probability of Interspecific Encounter (PIE) statistic (Hurlbert, 1978). Compounds that comprised less than 0.1% of a sample's CHC profile were set at zero for diversity measurements. Compound variability, environmental precipitation and

temperatures, percent alkane, estimated melting points, and PIE measurements were compared across taxa or compound class with an ANOVA, with post-hoc pairwise comparisons using a Tukey's HSD test.

Overall individual CHC profiles were compared without any prior groupings using principal component analysis. Because PCA minimizes the effects of collinearity through combining variables, all compounds were included in this analysis. K-means analysis was then used as an unsupervised method to obtain cross-validation scores to test placement in the PCA. To test whether elements of the overall profile reflected species, ancestry, or caste affiliations, cuticular hydrocarbon compositions of specific groupings were also analyzed using linear discriminant analysis (LDA) on relevant sample subsets for each hypothesis. Variables used in the LDA were checked for collinearity; any compounds that had a correlation coefficient higher than 0.9 were defined as collinear, and one of the variables removed. In all the subsets, the two ester compounds were collinear (Correlation coefficient = 0.97), so the second ester compound was removed from all analyses. Additional collinear compounds that were excluded in specific analyses are listed in Supplemental Table 3.1. Within-group variation was calculated for each sample by calculating the variation from the group mean from the first two discriminant axes. Accuracy of LDA groupings was determined using leave-one-out cross validation to test whether each sample could be accurately placed in the proper cluster. To test for potential fertility signaling compounds, queens were tested against workers, for the entire sample set as well as in a separate analysis for each taxon. To

investigate species-level differentiation and the relationship between CHC similarity and genetic ancestry of the GCD lineages, only queens of the two parent species and the four GCD lineages were compared (5 queens from each species/lineage, 30 total). To investigate inheritance patterns of CHC profiles in the F1 hybrid workers of the GCD lineages, for each pair of interbreeding lineages (H1-H2 and J1-J2) we separated queens of the two lineages with LDA and evaluated the placement of workers as "unknowns" relative to the two lineage references. To test for queen-mediated effects on worker compounds, an additional LDA analysis was performed on 38 samples from the isolated worker fragments and their parental colonies, separated by the environment that the workers were reared in.

3.4 Results

A total of 42 compounds were identified across the 98 CHC profiles; differences were quantitative in nature, and no compounds were unique to a single lineage or species (Table 3.2, Fig. 3.2, 3.3). The smallest compound was n-icosane ($C_{20}H_{42}$), with a molecular weight of 282.55 g/mol, while the largest compound identified was n-dotriacontane ($C_{32}H_{64}$), with a molecular weight of 450.9 g/mol. The compounds were comprised of 13 unbranched n-alkanes, 11 methyl-branch alkanes, 11 unsaturated alkenes, two esters, and one double-methylated alkane. Compound types differed significantly in the extent of variation across samples (ANOVA on coefficients of variation; $F_{5,54}$= 3.8, p = 0.03): methyl-branched alkanes (coefficient of variation (CV) = 1.25 ± 0.37) were significantly more variable than the straight-chain alkanes (CV = 0.91±

0.28, p = 0.025), but the straight-chain alkanes and alkenes (CV = 1.15 ± 0.18), as well as the alkenes and methylated alkanes did not differ significantly from one another (n-alkane vs. alkene p = 0.15, n-alkene vs. methyl-alkane p=0.7).

Compound diversity, measured by Hurlbert's Probability of Interspecific Encounter (PIE), showed significant differences among taxa ($F_{5, 54}$=2.66, p=0.03, Fig. 3.4). The PIE values for *P. rugosus* showed the lowest diversity, while J2 lineages were the highest, although no other pairwise comparisons were significant (Tukey HSD J2-Pr: p = 0.02).

3.4.1 Desiccation resistance

Species differed significantly in average melting temperature (Fig. 3.5A, $F_{5,54}$ = 3.26, p = 0.012), with the highest values in *P. rugosus*, while the GCD lineages and *P. barbatus* tended to be lower (Fig. 3.5A); in pairwise tests, only H1 and *P. rugosus* were significantly different (Tukey HSD p=0.02). Species identity also had a significant overall effect on the percentage of alkanes, again driven by elevated alkane proportions in *P. rugosus* (Fig. 3.5B, $F_{5,54}$ = 2.52, p=0.039). As with average melting temperature, in pairwise comparisons *P. rugosus* (75.4 ± 8.7%) was significantly higher than H1 (54.2 ± 16%), while there were no other significant differences between groups. The proportion of alkenes did not show a significant effect of species ($F_{54,5}$ = 2.05, p=0.08), but the proportion of methylalkanes did ($F_{54,5}$ = 5.4, p < 0.001, Fig. 3.6). P. rugosus had

significantly lower amounts of methylalkanes than H1 (Tukey's HSD : $p < 0.01$), H2(Tukey's HSD : $p < 0.01$), and J1 ants (Tukey's HSD : $p < 0.01$).

3.4.2 Caste-specific Hydrocarbons

Queens and workers were able to be separated with high accuracy from a combined dataset of all species and lineages using linear discriminant analysis (cross-validation=100%). The top 5 queen-loading compounds were all unsaturated alkenes, while 3 of the 5 top worker-loading compounds were n-alkanes (Table 3.3). However, when comparing each species independently, very few compounds were repeatedly found to be I overrepresented in queens or in workers; the one exception to this was the compound nonacosene ($C_{29}H_{58}$), which was highly loaded in queen samples from all species.

When separated from the queen prior to eclosion, individuals in isolated colonies developed a different overall profile than sibling workers that remained in queen-right colonies (cross-validation=100%). Workers raised in a queen-less environment differed from the workers in a standard colony, converging with the queenless workers in their sister lineage. However, these differences were not comprised of compounds that were identified as queen-specific or worker-specific in the caste LDA with all species; the workers that were separated and raised in queenless environments did not show any significant changes in the top five queen-specific and work-specific

compounds from workers that remained with the queens. Discriminant analysis using queen-right workers as a training set that creates variable loadings based on caste differences, and queenless workers as an unknown test set showed a slight loss of discrimination ability between the two lineages (Fig, 3.6, cross validation J-lineages: queen-right = 67%, isolated = 60%, H-lineages : queen-right 80%, isolated 70%). Two queen-specific hydrocarbons, 9-triacontene and nonacosene, appear more variable in the isolated workers, although a Levene's test does not find significant differences in variation between these groups (Fig. 3.8)

3.4.3 Interspecific CHC profile differences

When all samples were combined, without any prior grouping, principal component analysis did not reveal distinct clusters among species (Fig. 3.9, k-means cross-validation: 18.3%). Although species did not separate, the queens clustered together with higher scores on first two principal component axes while workers had lower scores on these axes (Fig. 3.9, k-means cross-validation: 65%).

Unlike in the principal component analysis, queen CHC profiles show separation between species when analyzed with discriminant analysis. Queen CHC profiles showed *P. barbatus* queens with a different CHC composition than *P. rugosus* and the GCD lineage queens. When only queens from each species/lineage were included in the LDA analysis and separated by species, the GCD lineages and *P. rugosus* clustered closely on the first discriminant axis (Fig. 3.10), which accounted for 75% of the total

variation. Queens had low cross-validation between lineages that is only slightly higher than a cross-validation in a random model (16.7%), primarily arising from the *P. rugosus* and GCD lineage clustering tightly together, preventing lineage discrimination (Fig. 3.10, cross validation=20%,). There was no significant difference among the species groups in the extent of variation across samples (ANOVA of variances for each sample, $F_{5,24}$ = 2.23, p = 0.08). Of the 5 compounds that were more heavily loaded towards *P. barbatus*, two were methyl-alkanes, two were unsaturated alkenes, and one was an alkane (Table 3.3). Of the five most *P. rugosus*-biased loading compounds, 2 were methyl-alkanes, 1 was an unsaturated alkene, and 2 were alkanes.

3.4.4 Worker CHC Profiles

Analysis of the workers from both GCD and ECD lineages showed that although GCD queens of all four lineages clustered closely with *P. rugosus*, GCD workers did not cluster in this pattern. J2 workers were clustered with and had a confidence interval overlapping with *P. barbatus* and *P. rugosus* workers on the first discriminant axis, while the other three lineages were negative on the first discriminant axis; the resulting cross-validation is not much greater than the null model (12.5%), due in part to the large overlapping clusters of workers (Fig. 3.11, cross-validation = 17%, Table 3.4). On the second discriminant axis, J-lineage workers clustered with *P. barbatus* workers, and H-lineage workers clustered with *P. rugosus*. In addition, while the J-lineage workers clustered on one axis, the H-lineage workers clustered together on both axes (Fig.3.10). When comparing the loadings on the first discriminant axis, eight of the top 10

compounds were more complex molecules (alkenes and methyl-alkanes), while two were alkanes (Supplemental Table 3.2). The extent of variation across samples differed significantly among taxa ($F=6.15$, $p<0.001$), although post-hoc pairwise comparisons did not reveal any significant pairwise differences. J2 ants tended to have the highest variation, while H1 ants had the lowest.

Although GCD workers are genetically F1 hybrids between interbreeding lineages, when evaluated along the axis of variation that differentiated queens of the two lineages, CHC profiles of each reciprocal cross resembled their maternal parent. All J and H-lineage workers were correctly classified by their maternal lineage, while all J-lineage and nine of the 10 H-queens workers were correctly classified (Fig. 3.12).

3.5 Discussion

Cuticular hydrocarbons in insects are used for both desiccation resistance and numerous communication purposes, with these multiple uses potentially leading to tradeoffs based on CHC function. Our results suggest that environmental constraints may play a role in shaping ant cuticular hydrocarbon profiles that are produced in each species. Comparison of the CHC profiles of *Pogonomyrmex* species spanning a range of precipitation regimes supported the hypothesis that harsh environments shift CHC composition toward desiccation-resistant, information-poor compounds at the expense of

reliably communicating species identity. Despite being found in lower abundances, communication compounds varied more than desiccation-resistance compounds, suggesting different selection on the different classes of compounds. In addition, caste differences appeared to be conserved across taxa, with queen-specific communication compounds driving the caste differences. Although the overall CHC profiles of the 6 species studied were similar, differences in the small amounts of complex molecules allowed for species identification based on hydrocarbon profiles.

The samples used in this study were collected from lab-reared colonies that were housed in identical conditions and fed the same diet; this allowed us to minimize confounding effects of environment that affect the cuticular hydrocarbon profile (Liang and Silverman 2000, van Zweden et al. 2009), and instead focus on differences arising from genetic effects and intracolony interactions alone. The composition of cuticular hydrocarbons closely resemble previous published work on field-collected populations of *Pogonomyrmex* ants (Wagner et al. 1998, Wagner et al. 2000, Wagner et al. 2001, Volny et al. 2006), suggesting that the hydrocarbon profiles of these ants is largely genetically controlled, and environmental variation has limited effects on each insect's CHC profile.

Compared to previous studies, our method detected two additional smaller alkanes, n-eicosane and n-docosane (Fig. 3.2, 3.3); both of these compounds were found in very small quantities and have a melting point of less than 40°C, which would put the melting-point of these compounds below the ambient temperatures that these ants

experience during foraging in desert environments. Although these compounds had the lowest melting point of the alkanes studied, the addition of functional groups in the methyl alkanes and alkenes lowered melting points to a greater extent than even these smallest alkanes. Therefore, a small portion of the cuticular hydrocarbon profile would be in liquid at typical foraging temperatures. Based on the lipid-melting theory of cuticular hydrocarbons, these compounds may melt to a degree within the cuticular matrix, but the water retention ability of the cuticle can remain intact as long as the majority of other compounds in this mixture do not melt (Gibbs 2002). Because of the loss of desiccation-resistance function found in communication molecules, desert species such as the *Pogonomyrmex* ants in this study would need to limit the number of these compounds present in their CHC profiles, which could lead to convergence in overall CHC profiles.

Both the average percent alkane and estimated CHC melting point showed trends that support environmental constraints on the cuticular hydrocarbon profiles. *Pogonomyrmex rugosus*, which lives in the most xeric environment (Fig. 3.1), had both the highest mean estimated melting point and the highest percentage of n-alkanes. The GCD lineages have melting points and percent alkanes closer to *P. barbatus*, possibly due to living in areas with more rainfall than *P. rugosus*. In addition to higher melting points and percent alkanes, *P. rugosus* had the least diverse profile, suggesting that the increase in n-alkanes comes at the expense of communication signaling diversity. The main climate difference between *P. rugosus* and the other species is primarily annual precipitation, and not mean average temperature (Fig. 3.1B, C). This would suggest that

low levels of precipitation, not high temperatures, are the main driver for selection of desiccation resistance. Precipitation and water availability have been shown to be an important predictor of species ranges in insects, again suggesting that water availability is a driving selective pressure on insect hydrocarbon composition (Chown et al. 2011, Roura-Pascual et al. 2011).

Both measures of desiccation resistance, however, have limitations. First, the exact process of how an insect's cuticular hydrocarbons change physical state from a solid to a liquid are not known, particularly when insects encounter temperatures that are high enough to cause a phase shift in lower melting point hydrocarbons within a mixed profile (Gibbs 2002). The average melting point is based on the phase transition model, which relates the temperature at which hydrocarbon melting (Critical temperature, or T_c) causes the loss of the cuticle's ability to retain water (Gibbs and Pomonis 1995). However, this model loses its ability to predict water loss through the CHC layer in species that have especially high T_c values, possibly due to other thermal damage to the cuticle at extreme temperatures. Finally, alternative mass spectrometry techniques such as MALDI-TOF have suggested that some insects may have hydrocarbons as large as 70 carbons in length (Cvačka et al. 2006). These compounds would remain undetected using our methods, altering both the percent alkane and mean melting point analyses. However, what role these larger compounds play and what proportion of the cuticular hydrocarbon profile is made up of these extremely large hydrocarbons is not clear.

In *Pogonomyrmex* mating swarm involving more than one species, reproductives mate assertively, suggesting that discrimination can occur in these species. Mating flights that contained both H-lineage and *P. rugosus* reproductives display positive assortative mating when they co-occur in sympatry, preventing mating between these species (Schwander et al. 2008), and male mate choice experiments between four *Pogonomyrmex* species showed that males preferred to mate with female of their own species over others, although the preference was not as strong between *P. rugosus* and *P. barbatus* (Hölldobler 1976). However, in GCD lineages males and females mate randomly, regardless of the lineage pairing (Chapter 2). The lack of assortative mating observed in these populations could be reinforced by the limited diversity of compounds and higher level of alkanes in the more xeric *P. rugosus* limiting the communication ability of the hydrocarbon profiles in these ants, which in turn can lead to identification and communication mistakes, especially during mating. Therefore, the role that the limits in the CHC differentiation plays on mate choice only affects the interbreeding lineages, suggesting that the thermal constraints on communication signaling may simply lower the threshold for interspecies mating, rather than cause it outright.

The different functions of the compound classes that comprise the CHC profile are expected to lead to different selective forces affecting the abundance and diversity of each class. In order to increase desiccation-resistance, we expect all of the species to exhibit a high level of n-alkanes, with selection forcing these compounds to remain high across ant species and castes in that live in extreme desiccation-risk environments (Gibbs

1998). In the desert-adapted *Pogonomyrmex* ants used in this study, the percentage of n-alkanes ranged from an average of 46% to 75%, which is much higher than the levels of alkanes found in non-desert species such as *Formica exsecta* and *Lasius niger* that contained less than 30% alkanes in their hydrocarbon profiles (Martin and Drijfhout 2009b). As the specific length of n-alkanes minimally alter the desiccation-resistance function when compared to the addition of functional groups, n-alkanes are interchangeable in the desiccation-resistance role, leading to numerous variety of different alkane compounds in the profiles of desert-adapted ants despite stabilizing selection on overall quantity (Gibbs and Rajpurohit 2010). Despite the numerous alkanes found, the variation of these alkanes between species remains low. The communication compound classes are expected to show the opposite pattern of alkanes, with higher levels of variation between species due to different selection pressures based on the communication function of each compound; nest mate compounds would be under disruptive selection, while species-identification compounds would be under stabilizing selection, especially in reproductive individuals (Greene and Gordon 2007, Martin and Drijfhout 2009b). In addition to the identity information, the higher variation in communication compounds can also be attributed to the numerous aspects of an ant's state that are communicated by these hydrocarbons such as task (Wagner et al. 1998, Greene and Gordon 2003), age (Haverty et al. 1988), and reproductive status (Smith et al. 2011a, Holman 2012, Van Oystaeyen et al. 2014). As workers have temporal polyethism, their tasks within a colony change over time (Beshers and Fewell 2001), and since ants were randomly selected within colonies these state cues will vary depending on which

ants were selected. The need for colony specificity, as well as the numerous other information types encoded into these communication molecules, would lead to higher levels of variation in these compounds due to the informational load that they carry. This is supported by the significantly lower variation found within n-alkanes between species (primarily desiccation-resistance compounds) compared to one class of communication compounds (alkenes), and marginally lower coefficients of variation than a second class of communication compounds (methyl-alkanes).

Although caste differences were resolved in an unsupervised principal component analysis, no species differences were seen (Fig. 3.9). This suggests that while caste differences can be detected in the unsupervised principal component analysis of CHC profiles, the high variation in communication molecules and overall similarity in the more numerous alkanes between species prevent species-level differences from resolving. Despite the overall similarities in these recognition cues, however, *Pogonomyrmex* harvester ants have been shown to be able to accurately identify caste, task, species and nest-membership in field and in the lab, echoing the results of our discriminant analysis (Hölldobler and Lumsden 1980, Wagner *et al.* 2000, Cahan *et al.* 2006, Volny *et al.* 2006). Unlike principal component analysis, discriminant analysis allows us to detect differences between groups by more heavily weighting compounds that differ between groups, allowing the separation between species as well as caste (Fig. 3.10).

In the discriminant analysis separating caste, the top five queen-loading compounds were unsaturated alkenes, while worker-loading compounds showed three alkanes in the five top loadings. The queen loadings in particular suggest that, in these *Pogonomyrmex* ants, unsaturated alkenes serve as the primary queen-signaling compounds (Supplemental table 3.2). This result contrasts with recent work that found methyl-alkane compounds as the primary conserved queen signaling molecules (Van Oystaeyen et al. 2014). One alkene of particular interest is nonacosene; this compound was the fourth highest queen-loading compound, and was loaded higher in queens than workers in all six species examined. This compound is a known fecundity signal in beetles (Ginzel et al. 2006), suggesting that it could serve as an honest fecundity signal in ants as well. The relationship between this nonacosene and methylnonacosane that appears to be a conserved queen signal in ants is not known, although both molecules appear to have the functional group in the same configuration.

The caste-specific differences in CHC profiles could play a role in the differentiation of the CHC profiles of isolated workers compared to their queen-right siblings. When GCD worker fragments were isolated from their queens, we predicted that some of the queen-loading compounds would decrease due to the workers being isolated from the queen, while others could increase due to worker ovary activation to produce male offspring which would trigger fecundity signaling. Isolated workers did show a change in the overall hydrocarbon profile, separating out from workers that remained with queens in linear discriminant analysis. The top-loading queen-specific compounds,

as well as the top-loading worker-specific compounds from the previous analysis, surprisingly, did not show a significant difference between isolated and non-isolated workers (Fig. 3.8). The differentiation observed between isolated and queen-right workers seen in the discriminant analysis with environment as the factor could possibly be a result of some of the isolated workers activating their ovaries, which in turn would activate any queen-specific compound that serves as an honest fecundity signal. This would explain the rise in variation in several of the queen-specific compounds, as only a fraction of some of the isolated colonies would activate their ovaries. Due to the low relatedness between workers in *Pogonomyrmex* colonies, worker fitness interests would support raising queen-laid males rather than worker-laid males (Moore and Liebig 2010, Suni and Eldakar 2011). Therefore, the detection of honest fecundity signals would provide a fitness benefit to workers by allowing them detect and remove workers who begin to produce male eggs when a healthy queen is present. Alternatively, since the queen-loading compounds were found at higher amounts on queens, the queen-specific compounds on workers are low proportions begin with, making comparisons between worker groups difficult since even slight differences will be amplified.

The historic origin of the hybridizing lineages show that H1 and J1 lineages are derived from *P. rugosus*, while H2 and J2 lineages are derived from *P. barbatus* (Helms Cahan and Keller 2003, Sirvio et al. 2011). However, the GCD lineage queens did not cluster with their closest ancestor species; instead, the GCD lineages cluster with *P.*

rugosus, while *P. barbatus* was isolated by the first discriminant axis (Fig. 3.10). This suggests that, rather than conserving the CHC profile of their closest ECD ancestor, the 4 ECD lineages shifted their CHC profile to be more similar to their interbreeding lineage, and all of the GCD lineages become more similar to *P. rugosus* as a result. One possible hypothesis for why this pattern occurs could relate to the environmental variables of each species' habitat; while the mean annual temperatures of all the species were similar, the annual precipitation of P. *barbatus* habitats was higher than the rest of the species (Fig. 1). However, the results of our desiccation resistance measurements do not support this idea; the GCD lineages were actually closer to *P. barbatus* in melting point and percent alkanes than they were to *P. rugosus*. Loadings for this discriminant analysis suggest that on the first axis, the top 5 negative loading compounds (most like *P. barbatus*) were all alkenes and methyl-alkanes, suggesting *P. barbatus* had a greater amount of specific signaling hydrocarbons that were not seen in the other species groups, possibly separating them from all other species. This difference could be in response to less severe selective pressures against signaling molecules in *P. barbatus*, allowing them to differentiate more than *P. rugosus* and the GCD lineages.

Due to the GCD workers in interbreeding lineages having a hybrid genome, we expected the hybrid workers to appear more similar to their interbreeding lineage's hybrid workers than to other taxa. When workers remained with the queen, both J-lineage and H-lineage worker hydrocarbons did not show convergence between sister lineages and instead clustered with the queen lineage they were raised with. This could be a result

of epigenetic effects from the queen, or from an interaction between epigenetic effects and nuclear genes. Alternatively, the CHC profiles of these workers could also be modulated by the social environment they are raised in (Fig. 3.12). These workers maintained the lineage differences seen between queens, likely as a result of sharing CHC compounds within the colony and creating a colony-specific gestalt odor (van Zweden et al. 2010). When these workers were isolated, however, the differentiation between interbreeding lineages decreased slightly (Fig. 3.7). However, this does raise questions as to how these queen-mediated CHC profiles are spread in larger colonies, where the number of workers stabilizes around 12,000 workers, and many workers never have direct contact with the queen (Gordon 1995). As colonies members share CHCs throughout the colony and form a "gestalt" odor (van Zweden et al. 2010), it is possible that queen-mediated compounds are simply passed between workers, with queen signals appearing in a more diluted form in foraging workers. This could explain why aggression between interbreeding workers appears to be lower than between GCD and ECD workers; in natural colonies H-lineage workers react aggressively towards other H-lineage workers, but are much more aggressive to *P. rugosus* workers (Julian and Cahan 2006).

Despite the species differences found in workers, the F1 worker offspring of interbreeding lineages were more similar than the other species when only workers were used. The H1 and H2 workers cluster next to each other on both discriminant axes , and

the J-lineages separate on discriminant axis 1, while clustering on discriminant axis 2 (Fig. 3.11). This could be an artifact of the loadings favoring compounds that cluster the H-lineages and ECD workers on that axis, loading compounds that do not differ between H-lineages.

Overall, the hybridizing GCD lineages are intermediate to both parental species, but also have traits that differ from either parent. The hybrid origin of the GCD lineages can explain some of the intermediate profiles that are seen here, although drift or selection pressures have led to hybridizing species being distinct from either parental group as well. This results in an interesting split between the parental species and the hybridizing species; while the lineages share many cuticular hydrocarbon traits from both parental species, they also show unique CHC traits that are absent in *P. rugosus* and *P. barbatus*. These differences could have accumulated in the bottleneck event that is thought to have created these hybridizing populations (Sirvio et al. 2011), which would have limited the genetic diversity of the hybridizing populations, allowing rare hydrocarbon signatures to become common.

In conclusion¸ this work highlights the complex nature of cuticular hydrocarbon profiles. Comparisons among the CHC profiles of 6 desert ant species showed evidence of environmental constraints on the amount of hydrocarbons that can be utilized for communication, which would limit the communication potential of these molecules in high desiccation risk environments, especially drier environments with low precipitation.

Although the overall CHC profiles of the ants in this complex were similar overall, differences were seen between species, caste, and worker environment. The differences between caste and species were primarily concentrated in complex molecules used for communication, likely a result of diversifying selection on these molecules compared to desiccation-resistance molecules. The levels of selection based on molecule type could help maintain the genetic diversity needed to allow for genetic kin recognition in these ants.

3.6 Figures and Tables

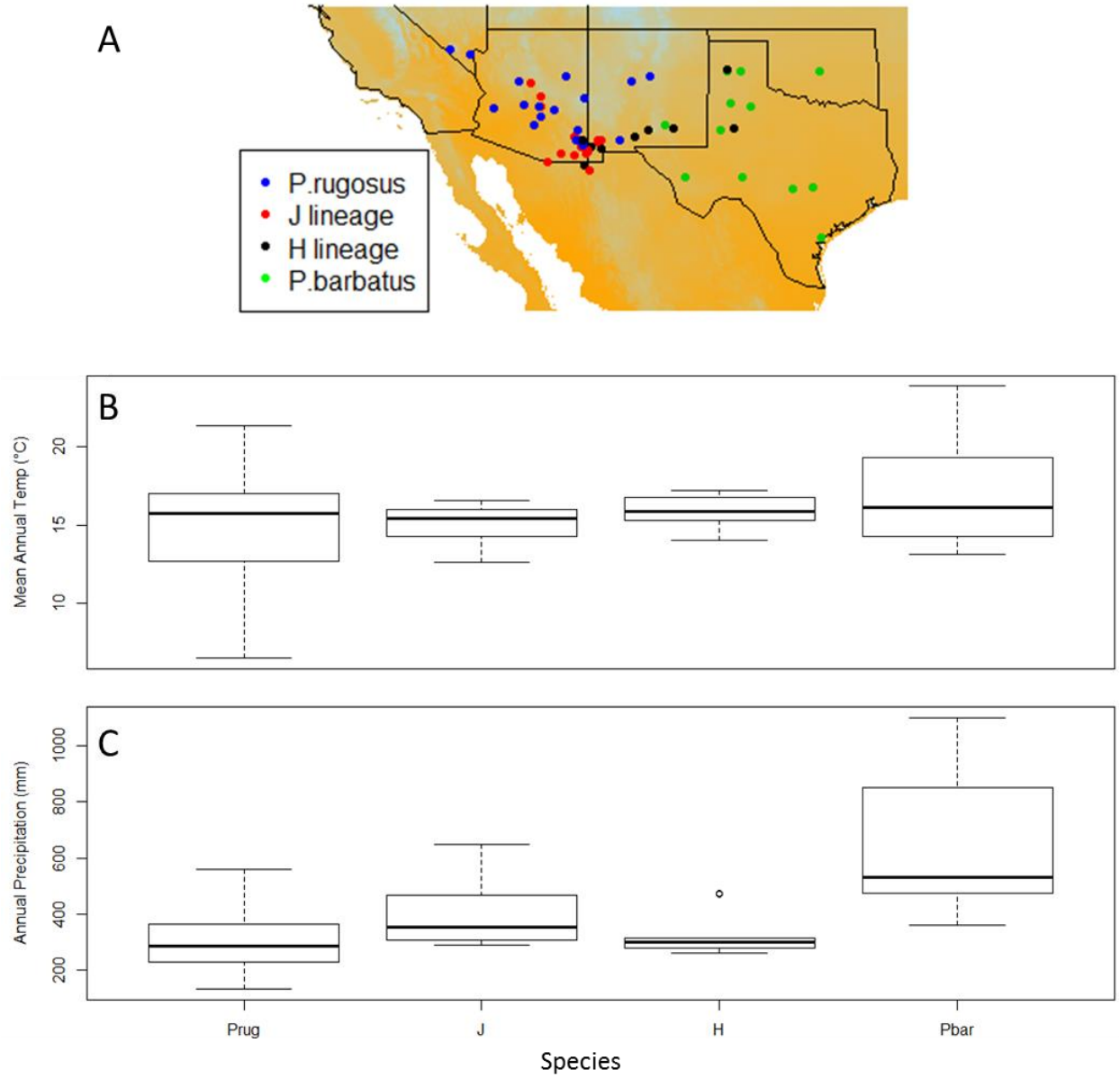

Figure 3.1 : Range and environmental conditions of species used in this analysis, obtained from published works. A. The range of each species/lineage of harvester ants used in this study. All species extend into Mexico, but only samples collected in the US are shown. Map gradient correspond to mean annual temperature in each area, obtained from the worldclim dataset. B. Boxplot of mean annual temperature by species. C; Boxplot of the annual precipitation by species.

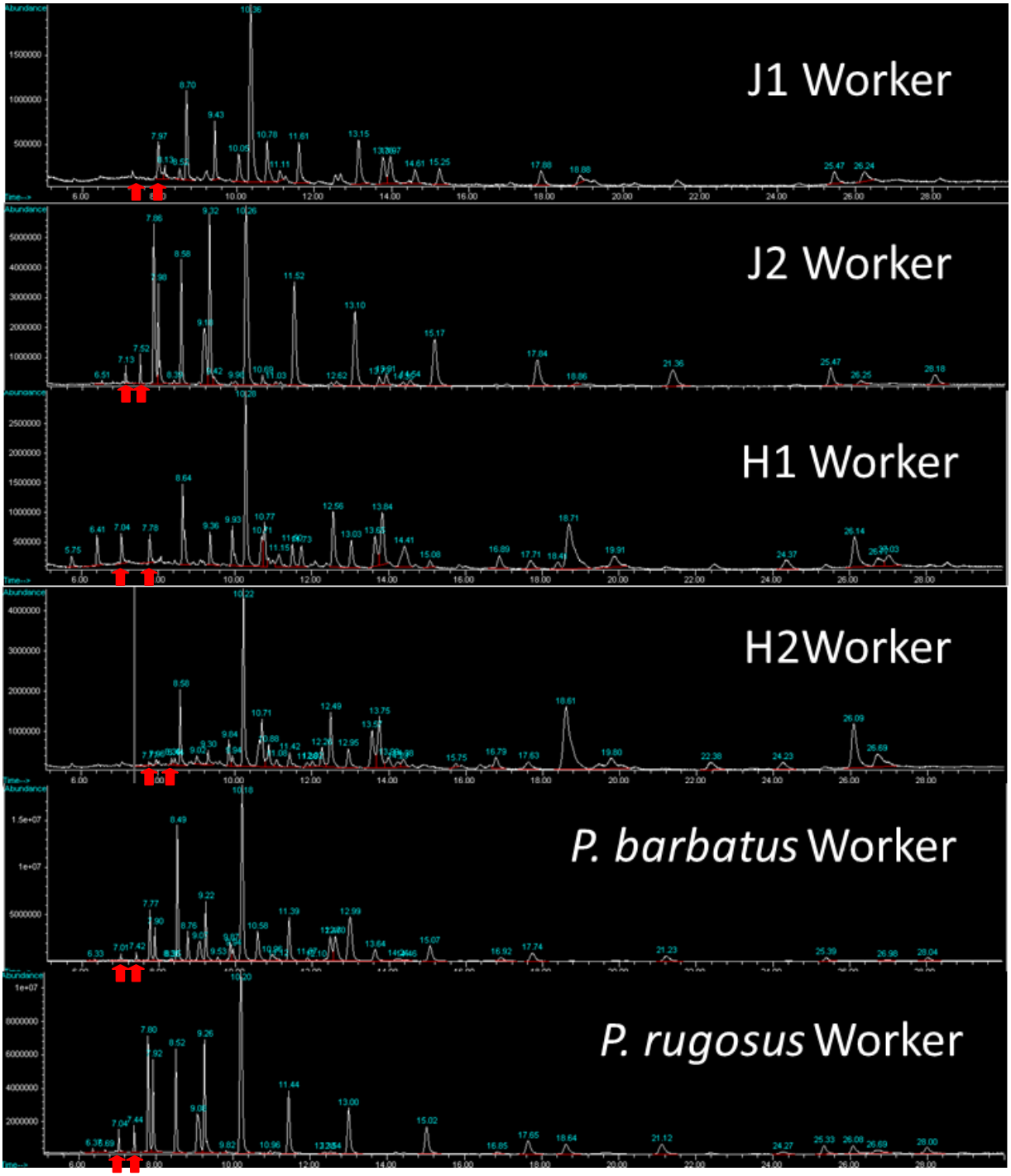

Figure 3.2 : Total ion chromatogram (TIC) via EI-GCMS traces (TIC versus elution time in minutes) from a representative worker of each species used in this study. The red arrows point towards the two smallest alkanes, n-eicosane and n-docosane, found in this study.

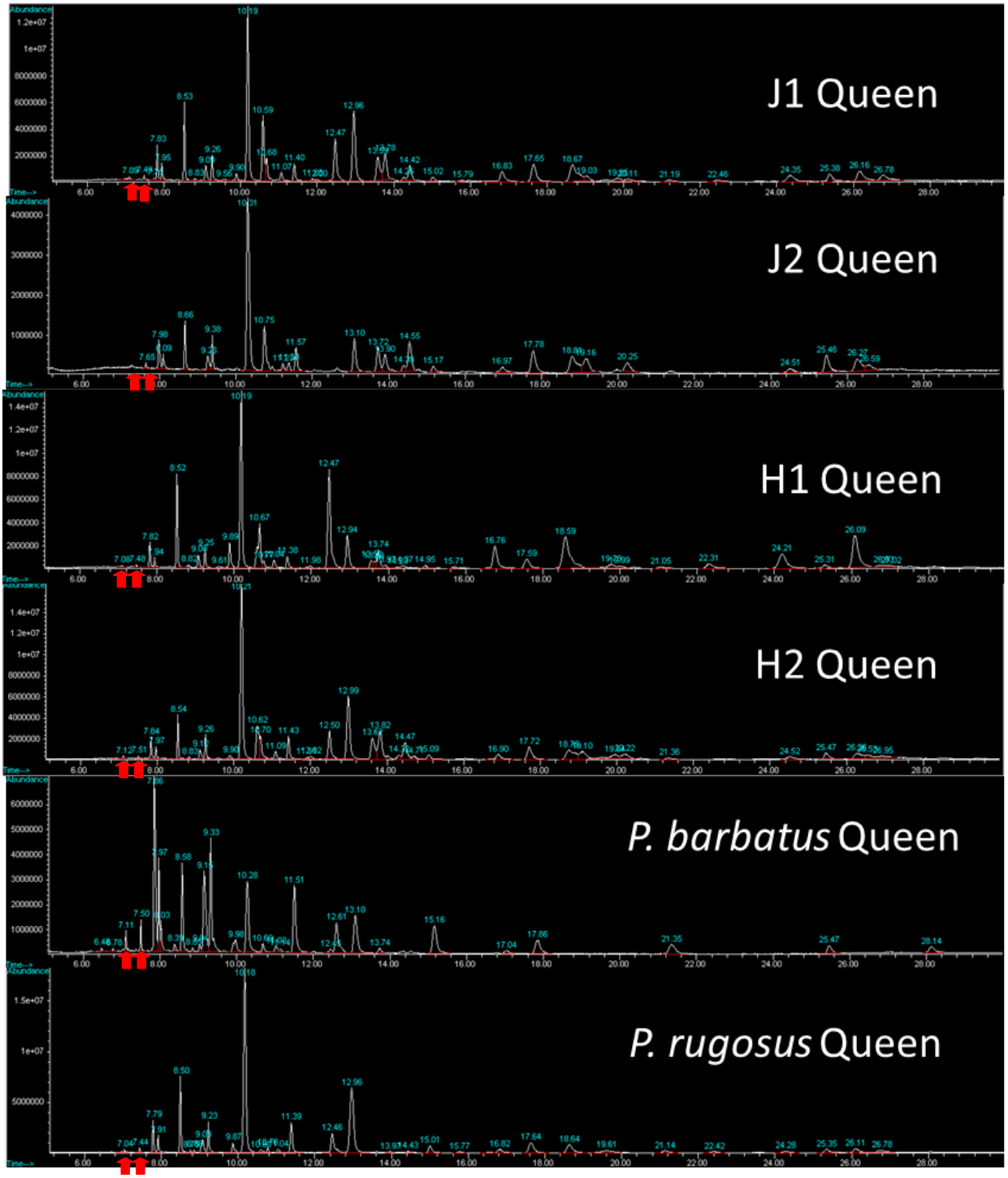

Figure 3.3 : Total ion chromatogram (TIC) via EI-GCMS traces (TIC versus elution time in minutes) for a representative queen from each species. The red arrows point towards the two smallest alkanes, n-eicosane and n-docosane, found in this study.

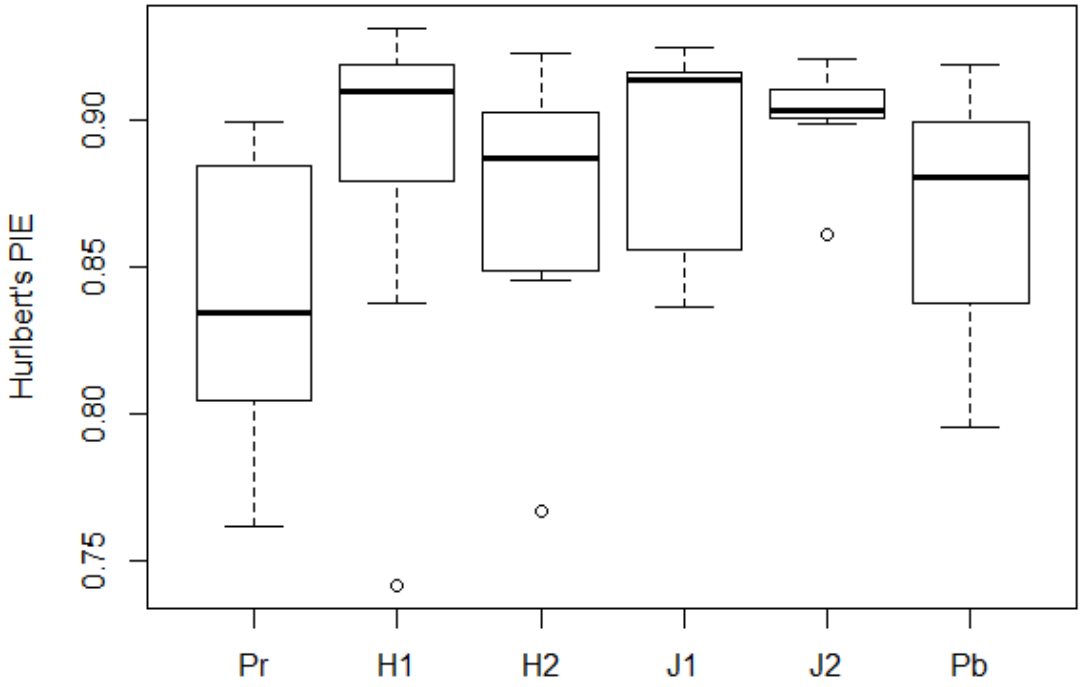

Figure 3.4 : Diversity measurements for each species, measured using Hurlbert's Probability of Interspecific Encounter diversity calculation. Pr is *P. rugosus,* and Pb is *P. barbatus*. Species showed significantly different levels of diversity in their cuticular hydrocarbon profiles.

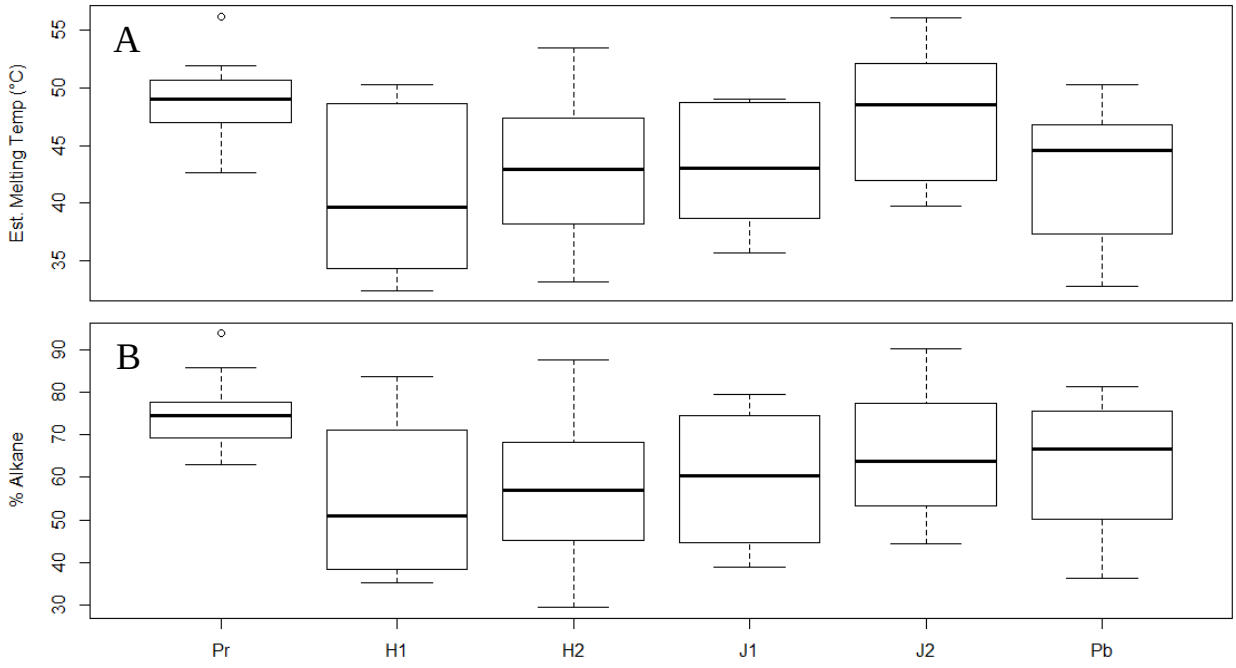

Figure 3.5 : Boxplot of the thermal traits of CHC profiles. A) the estimated melting point of the CHC profiles of each species. B) the percentage of the CHC profile comprised of straight-chain (n) alkanes.

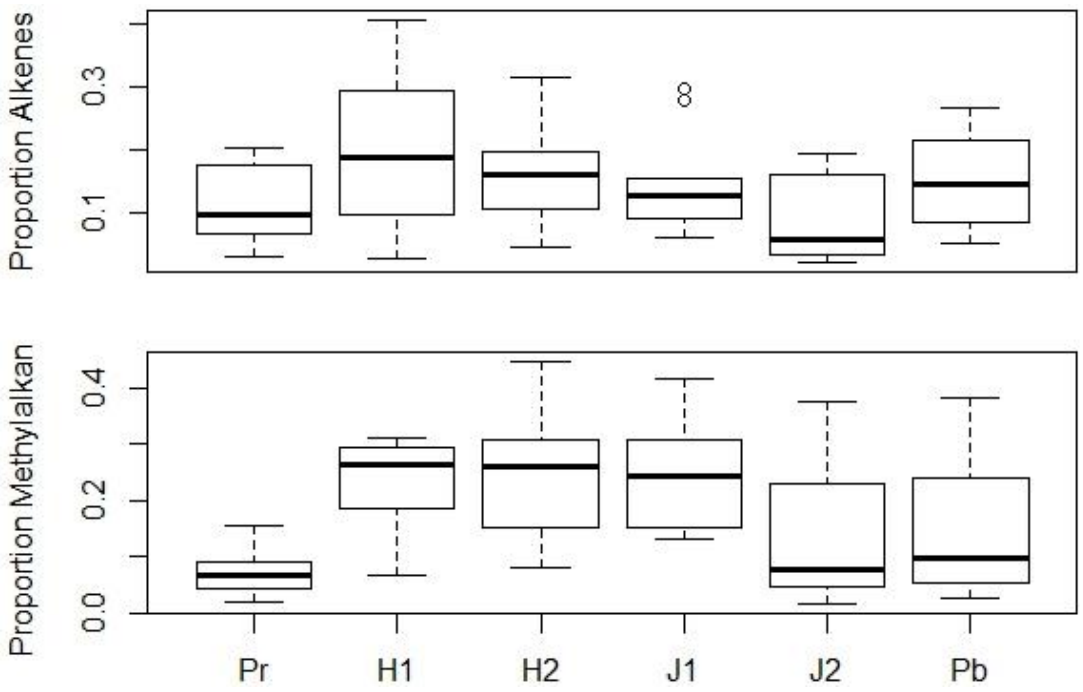

Figure 3.6 : Boxplot of the proportion of communication molecules in each species' CHC profile. A) the proportion of unsaturated alkenes in each profile. B) The proportion of methylated alkanes in each species' CHC profile.

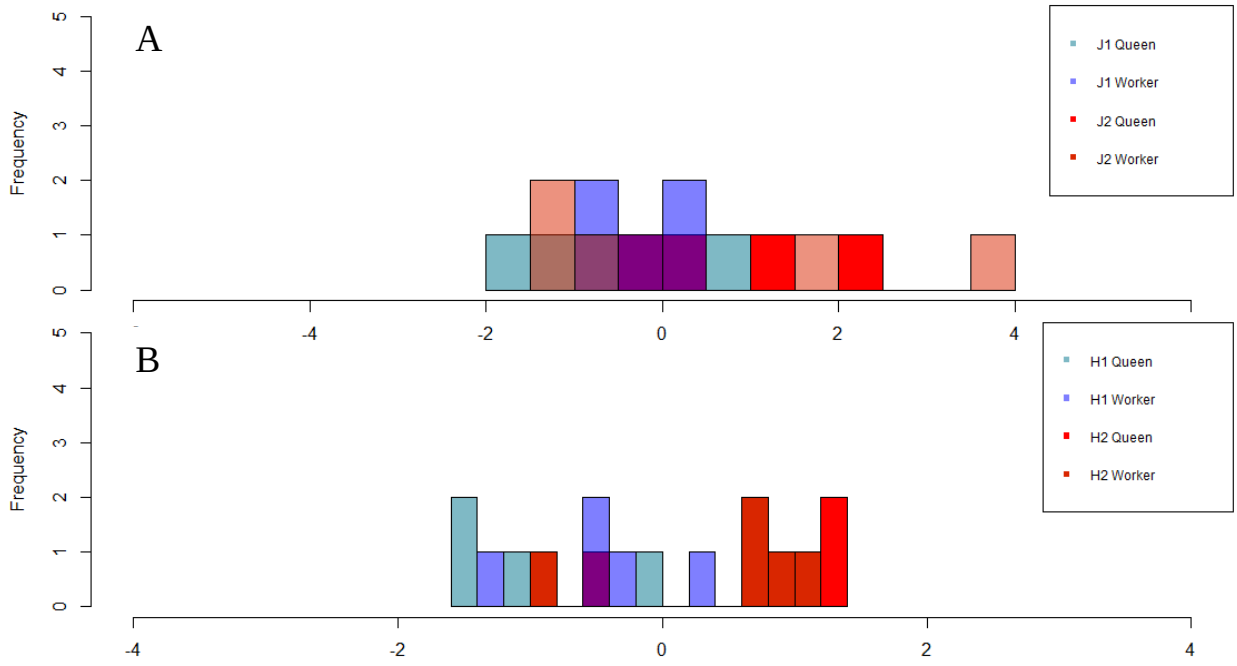

Figure 3.7 : Linear Discriminant Analysis graph of the J-lineage populations (a) and H-lineage populations (b) using queen-right workers as the training population, and isolated workers as the test population.

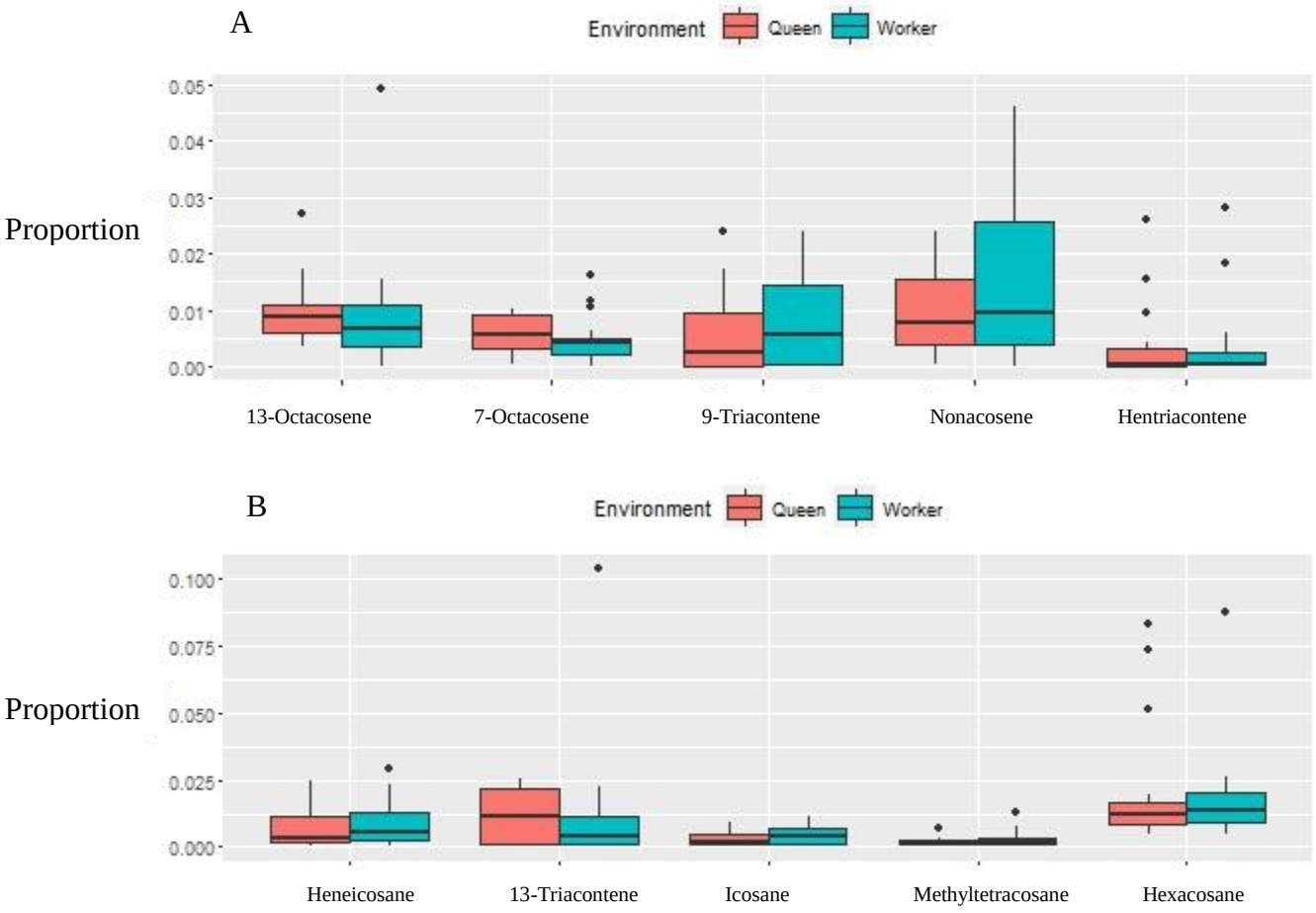

Figure 3.8 : Relative amounts of the top five queen loading (A) and top five worker-loading (B) compounds in queen-right workers and isolated workers.

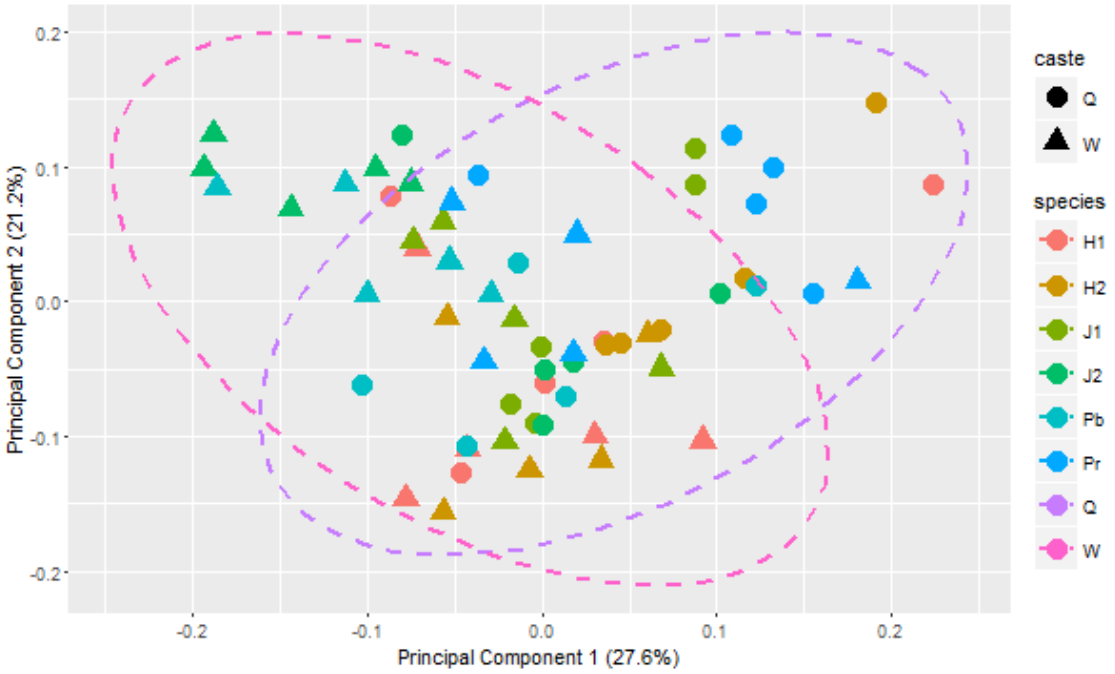

Figure 3.9 : Biplot of top two principal components of all samples. Ellipses show confidence intervals for the two castes. Although there is a large degree of overlap in the middle, queens and workers differentiate on the first principal componenent.

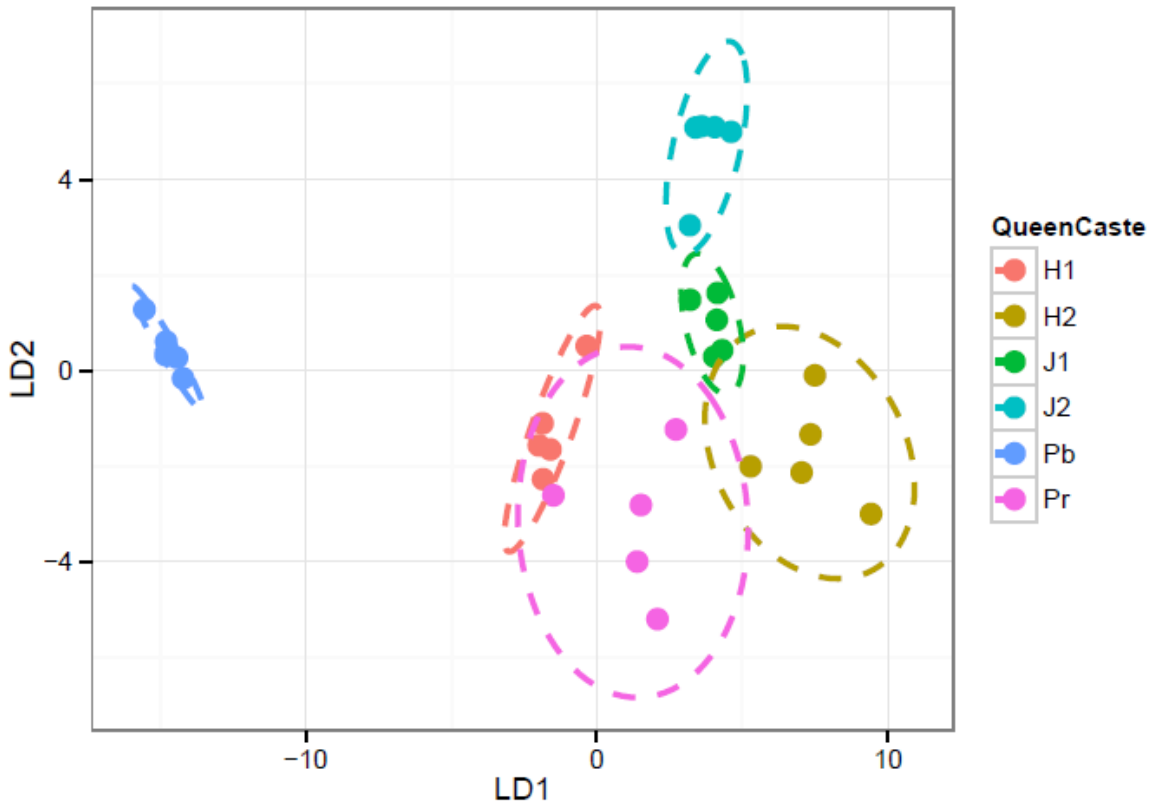

Figure 3.10 : Plot of the first two axes of the linear discriminant analysis of queens from all six species.

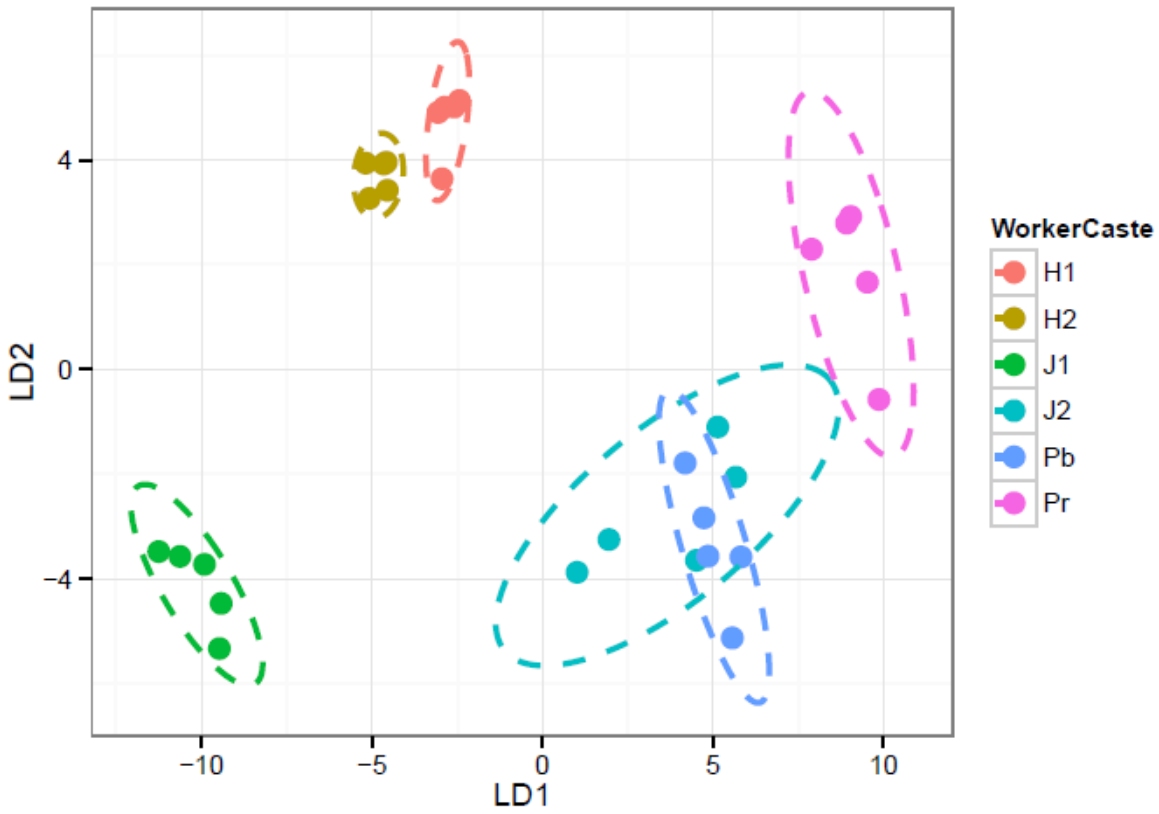

Figure 3.11 : Plot of the first two dimentions of the linear discriminant analysis using only workers of each species.

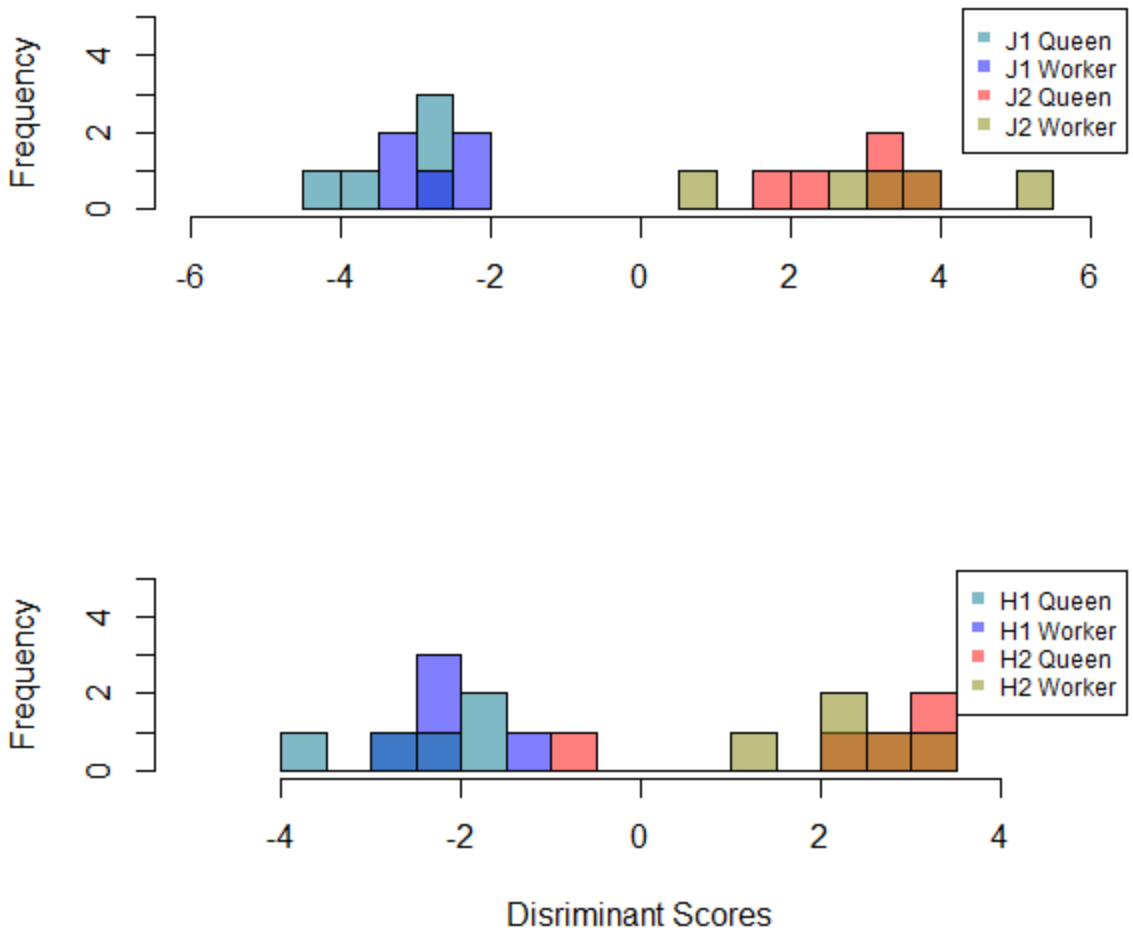

Figure 3.12 : Plot of the LDA using queens as the training population, and workers as the test population. "1" lineages are negative, "2" lineages are positive.

Table 3.1 Collection location and collection dates of colonies that were used for CHC analysis.

Species	Description	Date Collected	County	Coordinates
H	Site H	7/22/2010	Hildago Co, NM	32.466 N, -109.067 W
H	San Simon	7/30/2010	Cochise Co, AZ	32.266 N, -109.227 W
H	San Simon	7/19/2009	Cochise Co, AZ	32.266 N, -109.227 W
J	Paradise/Grill	7/20/2010	Cochise Co, AZ	31.986 N, -109.12 W
J	Sulphur Canyon	7/15/2010	Cochise Co, AZ	32.466 N, -109.2 W
J	Outside Fort Bowie	7/25/2010	Cochise Co, AZ	32.158 N, -109.455W
J	North of Deming	7/13/2009	Luna Co, NM	32.449 N, 108.664 W
J	4 miles east site H	7/12/2009	Hildago Co, NM	31.63 N, -109.671 W
P. barbatus	Welder Wildlife Reserve	6/1/2010	San Patricio Co, TX	28.115 N, -97.425 W
P. barbatus	Welder Wildlife Reserve	7/1/2010	San Patricio Co, TX	28.115 N, -97.425 W
P. barbatus	Ft Martin Scott Historical Site	7/7/2012	Gillespie Co, TX	30.249 N, -98.846 W
P. rugosus	Casa Grande	6/25/2012	Pinal Co, AZ	32.393 N, -111.281 W
P. rugosus	North of Casa Grande	2006	Pinal Co, AZ	32.938 N, -111.581 W

Table 3.2 : Compound Identification, class, and estimated melting point. Molecules are organized by the time that they elute in the gas chromatography.

Compound	*Chemical Formula*	*Class*	*Melting Point (°C)*
Icosane	**$C_{20}H_{42}$**	**Alkane**	**36.7**
Heneicosane	**$C_{21}H_{44}$**	**Alkane**	**40.5**
Butyl-n-hexadecaonate	**$C_{20}H_{40}O_2$**	**Ester**	**41**
Docosane	**$C_{22}H_{46}$**	**Alkane**	**42**
Tricosane	**$C_{23}H_{48}$**	**Alkane**	**49**
13-Methyltricosane	**$C_{24}H_{50}$**	**Methyl alkane**	**26**
9-Methyltricosane	**$C_{24}H_{50}$**	**Methyl alkane**	**12**
Butyl-n-octadecaonate	**$C_{22}H_{44}O_2$**	**Ester**	**51**
Tetracosane	**$C_{24}H_{50}$**	**Alkane**	**52**
13-Methyltetracosane	**$C_{25}H_{52}$**	**Methyl Alkane**	**29**
Pentacosene	**$C_{25}H_{50}$**	**Alkene**	**4**
Pentacosane	**$C_{25}H_{52}$**	**Alkane**	**54**
13-Methylpentacosane	**$C_{26}H_{54}$**	**Methyl Alkane**	**28.6**
7-Methylpentacosane	**$C_{26}H_{54}$**	**Methyl Alkane**	**17**
Dimethylpentacosane	**$C_{27}H_{56}$**	**Methyl Alkane**	**21**
Hexacosane	**$C_{26}H_{54}$**	**Alkane**	**56.4**
13-Methylhexacosane	**$C_{27}H_{56}$**	**Methyl Alkane**	**31**
9-Methylhexacosane	**$C_{27}H_{56}$**	**Methyl Alkane**	**19**
Heptacosene	**$C_{27}H_{54}$**	**Alkene**	**8**
Heptacosane	**$C_{27}H_{56}$**	**Alkane**	**59.5**
13-Methylheptacosane	**$C_{28}H_{58}$**	**Methyl alkane**	**36.4**
9-Methylheptacosane	**$C_{28}H_{58}$**	**Methyl alkane**	**22.5**
13-Octacosene	**$C_{28}H_{56}$**	**Alkene**	**11**
9-Octacosene	**$C_{28}H_{56}$**	**Alkene**	**11**
7-Octacosene	**$C_{28}H_{56}$**	**Alkene**	**12**
Octacosane	**$C_{28}H_{58}$**	**Alkane**	**63.25**
Nonacosene	**$C_{29}H_{58}$**	**Alkene**	**14**
Nonacosane	**$C_{29}H_{60}$**	**Alkane**	**64.5**
15-Methyl Nonacosane	**$C_{30}H_{62}$**	**Methyl alkane**	**39.1**
9-Methyl Nonacosane	**$C_{30}H_{62}$**	**Methyl alkane**	**27.5**
13-Triacontene	**$C_{30}H_{60}$**	**Alkene**	**15.5**
9-Triacontene	**$C_{30}H_{60}$**	**Alkene**	**15.5**
Triacontane	**$C_{30}H_{62}$**	**Alkane**	**65.8**
Hentriacontene	**$C_{31}H_{62}$**	**Alkene**	**16.6**
Hentriacontane	**$C_{31}H_{64}$**	**Alkane**	**67.9**
13-Dotriacontene	**$C_{32}H_{64}$**	**Alkene**	**18.5**

9-Dotriacontene	**$C_{32}H_{64}$**	**Alkene**	**18.5**
Dotriacontane	**$C_{32}H_{66}$**	**Alkane**	**69**

Table 3.3 : Top 6 caste-specific loadings from caste LDA. The numbers are the loadings of each compound in the discriminant axis.

	13-Octacosene	-239.1
	7-Octacosene	-113.4
Queen-Loading	9-Triacontene	-101.3
	13-Nonacosene	-50.7
	13-Hentriacontene	-21.1
	Triacontane	-13.3
	9-Methylhexacosane	75.4
	Hexacosane	82.8
Worker-Loading	13-Methyltetracosane	111
	Icosane	164.2
	13-Triacontene	276.8
	Heneicosane	328.1

Table 3.4 : Top 5 loading compounds for LD1 and LD2 between *P. rugosus* and *P. barbatus*.

P. rugosus loading

LD1	LD2
13-Octacosene	13-Methyltetracosane
7-Octacosene	7-Octacosene
13-Methyltetracosane	Dimethylpentacosane
Triacontane	9-Methylpentacosane
Dimethylpentacosane	Triacontane
13-Methylhexacosane	Octacosane
9-Methyltricosane	13-Methylpentacosane
Icosane	Icosane
Heneicosane	13-Methylhexacosane
13-Triacontene	9-Methyltricosane

P. barbatus loading

Table 3.4 : Cross-validation of worker LDA analysis. Predicted are in rows, while actual is the columns. Bolded numbers are individuals that are correctly placed.

		Observed					
		H1	H2	J1	J2	Pb	Pr
Predicted	H1	**4**	1	0	0	0	0
	H2	1	**1**	1	0	1	1
	J1	0	2	**0**	1	1	1
	J2	0	0	2	**1**	1	0
	Pb	0	1	2	1	**1**	0
	Pr	1	1	0	1	1	**1**

3.7 Works Cited

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CHAPTER 4: CONTACT DURING MATING REDUCES CUES FOR MATE DISCRIMINATION IN THE GENETIC CASTE DETERMINING "J" LINEAGES OF *POGONOMYRMEX BARBATUS*

4.1 Abstract

Sexual conflict arises when the reproductive interests of males and females do not align. The extent that each sex can impose their interests on their mates depends on both their power to do so and the amount of information available for making optimal decisions. In *Pogonomyrmex* harvester ants with genetic caste determination (GCD), two genetically distinct lineages interbreed with one another, and male fitness is increased only by mating with same-lineage queens. Despite the fitness benefits of positive assortative mating, males in these populations appear to initiate mating randomly. I hypothesized that the absence of male mate choice results from an inability to identify the lineage of a potential mate due to convergence in lineage-specific recognition cues, encoded by cuticular hydrocarbons, before or during the mating flight. I compared the cuticular hydrocarbon profiles of reproductive males and females of the two lineages before, during, and after the mating swarm to determine whether cues that discriminate the two lineages are available when males make mating decisions. Results show that the

two lineages differ significantly the morning of and immediately prior to a mating flight, but these differences between lineages are reduced in the swarm, possibly limiting the discrimination ability during the course of a mating swarm. Differences reappeared by two weeks following the mating swarm, but not to the extent of initial differences. This suggests that cuticular hydrocarbons are physically exchanged between queens and males during the mating swarm, making accurate discrimination between lineages difficult. Because mating opportunities are likely to be scarce, highly competitive and time-limited, males with poor information may achieve higher fitness returns from indiscriminate mating attempts. The loss of discrimination cues and higher male propensity to mate can lead to interspecies matings and hybridization in lek mating species, which could help explain the origin of some hybrid ant species.

4.2 Introduction

Sexual conflict arises when the fitness interests of males and females are not aligned (Parker 1979, Chapman et al. 2003). Because traits that benefit one sex do so at the expense of their mate, sexual conflict can lead to antagonistic coevolution between the sexes (Arnqvist and Rowe 2002). However, these traits are limited in their extent by what mechanisms are under the control of each sex (Beekman and Ratnieks 2003). In sexual conflict systems, sensory exploitation is often used to deceive a potential mate, maximizing the fitness of the deceiving organism at the expense of their mate (Arnqvist 2006). This sensory exploitation occurs when one sex takes advantage of a sensory bias that has been selected for in the opposite sex, altering the behaviors of these potential mates that ultimately results in lower fitness for the sex with the sensory bias. In rare situations, sensory exploitation can lead to cross-species mating leading to hybridization that creates asymmetric fitness benefits to males and females of different species. Examples of this type of sensory exploitation in a cross-species mating system include

amazon mollies (Schupp and Plath 2005) and hybridogenic water frogs (Engeler and Reyer 2001), where females take advantage of a lower male selectiveness for mates through exploiting the sensory system of males in closely related species.

Another example of interspecific sexual conflict can be found in interbreeding lineages of genetic caste determination (GCD) *Pogonomyrmex* harvester ants (Herrmann and Cahan 2014). In these ants, two genetically distinct populations interbreed with one another in large mating swarms (Hölldobler 1976). The genetic ancestry of females determines which caste they will develop into: hybrid offspring develop into sterile workers, while non-hybrid, pure lineage offspring develop primarily into queens (Volny and Gordon 2002, Helms Cahan and Keller 2003, Anderson et al. 2006a). This system results in different fitness payoffs for males depending on their mating partner. Male sperm that is transferred to alternate-lineage queens sire sterile workers exclusively, preventing the male from obtaining fitness benefits from that mating. In contrast, sperm that is transferred to a same-lineage queen sire reproductive females, offering high fitness payoffs. The fitness differences experienced by males in this system should thus impose selection for lineage-specific mate choice by males during the mating swarm. Queens, on the other hand, must mate with opposite-lineage males to obtain sperm for the workers needed to successfully found a colony, and should be selected to counter male avoidance of alternate-lineage mating partners.

Previous work has identified cryptic methods by which males and females manipulate mating duration and sperm transfer during copulation, seeking to maximize

their fitness at the expense of their mate (Herrmann and Cahan 2014). Surprisingly, however, there is no evidence that either sex displays active mate choice prior to copulation, either in no-choice mating experiments (Herrmann and Helms Cahan 2014) or in natural mating swarms (Schwander et al. 2006). One possible explanation for the lack of mate choice is that the chemical recognition system used for mate recognition, the cuticular hydrocarbon profile, becomes difficult to interpret in a mating swarm, causing accurate recognition before mating to be difficult. Cuticular hydrocarbons are a waxy outer layer on the exoskeleton of insects, and are commonly used as species, nestmate, sex, caste, and task-specific recognition cues in social insects (Howard and Blomquist 2005). They are produced *de novo* by insects, but are also readily exchanged among colony members, forming a "gestalt" colony odor blend (van Zweden et al. 2010). These compounds also serve as species recognition cues, and species-mediated differences can maintain sexual isolation between closely-related species (Legendre et al. 2008, Zhang et al. 2014).The loss of species recognition ability could be an evolved strategy of CHC profile convergence prior to the mating flight, with common hydrocarbon signals created by female reproductives to "mask" their identity in the mating swarm. Alternatively, cuticular hydrocarbons could be physically exchanged during the mating swarm through repeated direct contact with opposite-lineage reproductives, homogenizing initially distinct cues and preventing accurate discrimination.

To test for convergence of signaling hydrocarbons in these populations before or during the mating swarm, we analyzed the CHC profiles of reproductive queens and

males of the "J" GCD lineage pair before, during, and after mating swarms. Field samples were collected in July 2011 at two time-points: 12-24 hours prior to the mating swarm, and during the mating swarm. To examine how hydrocarbon profiles change on foundress queens after the swarm, additional queens were maintained in the laboratory for two weeks following the swarm. Because the rate of hydrocarbon turnover is relatively short in insects, we expect that any CHC differences between lineages would be regenerated over this time period. We used principal component analysis and discriminant analysis to examine how well the two lineages could be discriminated from one another.

4.3 Methods

4.3.1 Field collections

Samples of reproductive male and female *Pogonomyrmex* harvester ants were collected from two sites during their annual mating swarm season in Arizona in July 2011. Both sites were north of Rodeo, NM, near the state line approximately 3 miles from one another. Mating swarms generally occur the day after the first monsoon rain in the summer, with males and females appearing at the entrance of the nest in early morning and late afternoon before the mating flight at approximately 3:30PM (Hölldobler 1976). In one of the two mating swarms, samples of 1-5 males and females were randomly collected from 10 colonies around 8:30 AM, placed into a 20mL PET plastic tube and immediately frozen by placing into a cooler filled with ice. At both mating swarms,

colonies were checked at approximately 3:00 PM, approximately a half hour before their mating swarm and a second sample of males and females were collected from colonies.

If conditions are appropriate for a mating swarm, reproductives fly from their nest, and aggregate in the thousands on the ground at a centralized swarm site (Hölldobler 1976). Flying reproductives were followed on foot to the swarm site. To allow individuals the opportunity to mate several times before being collected, 10 males and 10 females that were not in copula were collected approximately 75 minutes after mating began near the swarm center and frozen. To assess CHC profiles of founding queens, 20 additional queens were collected from the soil surface while digging founding nests after the mating swarm, and maintained in a 16x155mm culture tube with a water reservoir separated by a cotton ball for two weeks, then frozen until CHC extractions; males typically die within 1-2 days following the flight, and were not included in this final sampling point.

4.3.2 CHC extraction and analysis

Cuticular hydrocarbons were extracted from each frozen sample in 500µL of pentane. To ensure that the solvent was able to contact the entire cuticle, samples were agitated for 2 minutes, then left to soak for an additional 8 minutes. The pentane was evaporated, and the dried sample was frozen until used for GCMS analysis.

To analyze the hydrocarbon profile, each sample was reconstituted in 50 µl of hexane, placed in a 1.5 ml autosampler vial with a 300ul glass conical insert, and run through an Agilent/HP 5973 gas chromatography-mass spetrometer, using electron impact ionization (EI) and a quadrapole mass analyzer with a Zebron ZB-1 column. We used a protocol modified from Wagner (1998, 2000), with an inlet temperature of 300° C. A 5:1 split/splitless injection was used, and the carrier gas was helium at 1 cm^3/s .The oven was started at 100°C, increased at 25°C/min until 240° C, held for 15 minutes, then ramped to 320 at 25° C/min. A five-minute solvent delay was used to ensure the hexane solvent did not affect the gas chromatography.

Compounds were identified by comparing the mass spectrum of each component in each sample against published spectra from the NIST Chemistry Webbook database, then checked against the published CHC profiles of Pogonomyrmex ants in Wagner (1998, 2000) for verification. The elution profile area of each component was integrated from the total ion count for each sample using Chemstation software (Agilent co.); each component was then divided by the total ion count to obtain proportional amounts of each component. The resulting values were arcsine-transformed to normalize for statistical analysis.

4.3.3 Lineage identification

To determine lineage identity, samples were typed for lineage-diagnostic restriction sites within the *cox1* mitochondrial gene (Helms Cahan and Keller 2003). Because colonies are typically monogynous, all reproductives collected from the same colony were presumed to share the same maternal ancestry and only a single reproductive was typed. DNA was extracted with a rapid Chelex extraction protocol (Schwander et al. 2007) on tissue from two legs. Lineage identification was performed by PCR amplification of a 433bp portion of *cox1* as in Helms Cahan and Keller (2003), followed by two separate enzymatic digests using the enzymes MfeI and BsaI; MfeI matches a restriction site unique to the J1 lineage haplotype, while BsaI matches a site unique to the J2 lineage haplotype (Schwander et al. 2006). Positive J1 and J2 controls were included in each digest to confirm enzyme activity. The resulting fragments were run on a 1.5% agarose gel at 120 mV until separation could be resolved.

4.3.4 Statistical analyses

The relative quantity of each hydrocarbon was obtained by taking the proportion of each compound in a sample's profile, then normalized with an arcsin transformation. Several of the samples showed a large peak at approximately 7.4 minutes, which was identified as oleic acid, a compound that is emitted after death in ants (Wilson et al. 1958); these were excluded from the analysis. Because we predicted a convergence of CHC profiles either before or during the mating swarm, we focused on differences in chemical composition between the lineages by using discriminant analysis. To examine

how interlineage differences changed at different time periods, four total discriminant analyses were used; one on all samples, and one for each of the three periods that samples were collected. To focus on the effects of a mating swarm on CHC differences between lineages, these linear discriminant analysis were done with males and females combined, with lineage identity as the categorical variable. Leave-one-out cross-validation was used to check the accuracy of each discriminant analysis. The predicted discriminant score of each sample provided a numerical value for each sample based on a one-dimentional scale, with scores below 0 more similar to J1, while scores above 0 were more similar to J2. To test for CHC changes in each sex, males and females were subset and the LDA process was repeated for each time period. A two-way ANOVA using lineage and time as factors was used to determine whether there were significant differences between the lineages at each time point.

To examine if compounds were transferred between lineages during the mating swarm, we focused on compounds that differentiated between the two lineages before contact with alternate-lineage reproductives. This was done using a a one-way ANOVA was run on each compound using lineage identity as the factor. Compounds found to be significantly different were then compared between lineages before and during the swarm, using a two-way ANOVA using lineage and time as factors was used to check for significant differences. To check for convergence in the cuticular hydrocarbon profiles of males and females before and during the swarm, each lineage was subset into males and females and a discriminant analysis was performed on the two lineages, using sex as the

discriminating factor. The predicted discriminant score and leave-one-out cross-validation were used to analyze the differences between each sex before and during mating swarms.

4.4 Results

The discriminant score of all samples showed a significant effect of lineage ($F_{1,86} = 290$, $p < 0.001$) and a significant interaction between lineage and time ($F_{2,86} = 10.2$, $p = 0.005$), but the of time alone was not a significant ($F_{2,86} = 1.62$, $p = 0.4$). J1 and J2 reproductives differed significantly in CHC profile for pre-flight samples (ANOVA : $F_{1,47} = 1362$, $p < 0.001$, cross-validation= 84%; Fig. 4.1, Fig. 4.2A, Table 1). Although samples collected from the mating flight also differed significantly (mating ANOVA : $F_{1,21}=84$, $p < 0.01$, cross-validation=57%, Table 2), the extent of differentiation was considerably smaller, with the two discriminant score distributions adjacent to one another (Fig. 2B). Foundress queens two weeks after the mating flight showed an increase in the differences of the mean discriminant scores between lineages (5.5 in post-mating queens, compared to 2.4 in mating queens), but were not as well separated as the samples taken before the mating flight (post-flight ANOVA: $F_{1,18} = 225$, $p < 0.001$, cross-validation=45%, Fig. 4.1, Fig. 4.2A, Table 4.3). Separating samples into male and female subsets showed nearly identical discriminant analysis values for both sexes in the pre-flight and flights samples (Fig. 4.1).

Compounds that differed between the lineages before the mating flights showed signs of convergence in the mating swarm samples, although none of the interaction effects (e.g., lineage x time) were significant. Of the 8 compounds that showed significant differences before the mating swarm, six showed a reduction in the differences between lineage means, although no interaction effects were significant in the ANOVA (Table 4.4, Fig. 4.3).

Males and females were readily distinguished before the mating swarm when analyzed with linear discriminant analysis, with little to no overlap between their estimated discriminant score (ANOVA: J1 $F_{1,10} = 74$, $p < 0.001$; J2 $F_{1,35} = 8000$, $p = 0$, cross-validation = 94%, Fig. 4.3A). Samples collected during the mating swarm were still able to be successfully separated using discriminant analysis, but were more similar than before the swarm, with a lower cross-validation score (ANOVA: J1 $F_{1,7}=3.4$, $p = 0.9$; J2 $F_{1,35} = 27$, $p = 0.0002$; cross-validation = 50%, Fig. 4.3B).

4.5 Discussion

The results from this experiment suggest that although males would benefit from being able to discriminate queens based on lineage before copulation begins, the chemical cues that would allow that discrimination become blended during the mating swarm, making lineage identification more difficult. While no convergence of

hydrocarbon profiles occurs before the mating swarm, males and queens of the two lineages become more similar to the opposite lineage during the course of mating, and quantitative differences in individual CHC compounds that were significant before the mating swarm became more similar. The magnitude of differences between males and females were also significantly reduced during the course of the mating swarm. CHC transfer appeared to be long-lasting, with a residual effect on queen hydrocarbon profiles for at least two weeks following the mating swarm.

When sexual conflict creates a large fitness gap between male and female interests, both sexes can benefit from masking their identity, preventing the opposite sex from obtaining the information needed to make decisions to exert their fitness interests on mates. This masking behavior could occur before mating begins by producing non-specific hydrocarbons or mimicking the CHC profiles of the opposite lineage, or during mating through hydrocarbon transfer during physical contact. Similar mechanisms of chemical deception have been seen in social nest parasites, who attempt to avoid detection through copying the CHC profiles of their host colonies. Social parasites use two different strategies to infiltrate the nest of their host: chemical camouflage, where the parasites remove hydrocarbon compounds from members of their host colony in order prevent being expelled from the nest, and chemical mimicry, where the parasites produce common compounds from the host's profile, and use these to infiltrate the nest (Lenoir et al. 2001, van Zweden and d'Ettorre 2010). In the GCD harvester ants, CHC profiles remain distinct before a mating flight, but converge in the mating swarm itself. The lack

of convergence in CHC profiles prior to the beginning of the mating flight suggests that these ants do not produce masking hydrocarbons before the mating swarm, which suggests that chemical mimicry is not seen in this system.

Despite the lack of chemical mimicry, the results suggest that chemical camouflage is a possible cause of CHC profile convergence in these ants. Once the mating swarm commences, the CHC profiles of males and queens converge and sex-specific differences are reduced compared to pre-swarm levels (Fig. 4.1), likely due to CHC exchange during contact in the mating swarm. Whether this hydrocarbon exchange is an active process, with queens removing hydrocarbons from other ants in the swarm, or a passive process with compounds exchanged through repeated contact, is not known. Again, both of these processes have been observed in the nest invasion behaviors of social nest parasites, which mimic the CHC profiles of their host colony in order to infiltrate the host nest. If this camouflage is due to active hydrocarbon exchange, we would see queens seeking to obtain compounds during the first stage of a mating swarm by actively exchanging hydrocarbons, possibly through a behavior similar to allogrooming (Howard 1993). Alternatively, if the exchange of hydrocarbons is passive, there would be no hydrocarbon exchange behaviors at the beginning of a mating swarm, and CHCs would be transferred during mating attempts (Kather et al. 2015).

Changes in sex-specific differences that occur during the mating swam, however, suggest that the transfer of CHC compounds is a passive effect, rather than an

active one. In addition to reducing lineage differences, interactions between ants in the mating swarm, also reduced differences between the sexes. This can lead to mating mistakes, and could explain observations of same-sex copulation attempts during mating (M. Herrmann, personal observation, Hölldobler 1976). Although not significant, nearly all compounds that differentiated the sexes prior to mating showed a reduction in the mean differences between lineages during mating (Fig. 4.3). The results show that hydrocarbon compounds are exchanged between mating swarm participants, regardless of the sex of the ant or the signaling function of the compounds. This would suggest that rather than an active strategy to conceal identity, the exchange of hydrocarbons and limiting of discriminating potential is a side-effect of the mating swarm.

The blending of CHC profiles of all participants in the mating swarm calls into question the reliability of CHC profiles for accurate mate and species recognition, especially in insects that mate in large aggregations. In non-social insects, such as flies, beetles and cockroaches, CHC are passively exchanged between males and females during mating attempts, and provide information to future potential mates on the mating history of potential mates (Harris and Moore 2005, Scott et al. 2008). However, queens from the species *Leptothorax gredleri* showed no changes in their hydrocarbon profiles immediately before and after mating, suggesting that hydrocarbon transfer during mating may be limited to only some taxa (Oppelt and Heinze 2009). Since mating swarms in these species of *Pogonomyrmex* ants often involve thousands of reproductives from

hundreds of colonies forming “mating balls” on the ground, repeated contact with other reproductive ants from both species is common, especially in P. barbatus and P. rugosus which show the highest competition for mates among males (Hölldobler 1976). This increasing the changes of limited discrimination ability based on CHCs, possibly leading to hybridization.

The lack of pre-copulatory discriminatory ability created by the convergence of CHC profiles during mating swarms could preempt any ability for males to identify high-value mates, removing male power to mate selectively (Beekman and Ratnieks 2003). Without reliable discrimination cues, males should instead adopt a mating strategy where they mate with any queen they come into contact with, rather than risk losing a mating opportunity by attempting to identify the lineage of possible mates first. Previous work on mate choice in the J lineage population has shown that in a one-on-one no-choice context, males are similarly willing to mate with females of either lineage (Herrmann and Cahan 2014). In contrast, studies of another GCD lineage of harvester ants found evidence of assortative mating between the GCD ants and a closely related ECD species, suggesting that mate discrimination is generally seen in these ants when a third species is involved, but not within interbreeding GCD lineages (Schwander et al. 2008). This suggests that the expected CHC exchanges seen in these swarms does not remove all recognition ability, possibly due to an additional sex pheromone cue other than cuticular hydrocarbons. In the GCD lineages, both sexes appear to be able to identify the lineage of

their mate after copulation begins based on the differential mating time and sperm transfer rate observed in these ants (Herrmann and Cahan 2014). This could be a result of new discriminating cues becoming available to each sex during copulation, such as lineage-specific pheromones, that are difficult to detect before mating begins. In *Pogonomyrmex*, sex pheromones excreted from queen poison glands excite males (Hölldobler 1976), and if these compounds differentiate by lineage, could serve as discrimination cues for males after copulation begins.Lineage specific sex pheromones have been seen in some vertebrates and insects, and can serve as a mechanism for sexual isolation, leading to speciation (Palmer et al. 2005).

With few informative cues available to males, the power of control in the sexual conflict is shifted in favor of queens. Without the ability to discriminate between lineages, males will mate randomly, thereby reinforcing the survival of the GCD populations. If male discrimination were to evolve, males who selectively breed would have a fitness advantage over indiscriminant males, leading to fixation of genes that allow for this discrimination (Linksvayer et al. 2006). The lack of non-discriminating males would prevent new queens from producing workers, and the interbreeding GCD populations would quickly crash. Because informative species identification cues seem to be limited during the mating swarm, the potential for male-mediated selection is reduced, possibly serving a stabilizing selection force on the lineage frequencies by ensuring that the rarer lineage queens obtain more workers, increasing their likelihood of survival while decreasing the survival of the common lineage queens.

After the swarm, queen CHC profiles differentiated again, likely from compounds produced *de novo* by the foundress queens. In these queens, the two lineages began to diverge from one another, although not to the extent seen before the mating swarm. These foundress queens are fully claustral and remain isolated until their first worker cohort has eclosed, which would mean that any changes in the CHC profiles of these ants are an effect of newly produced hydrocarbons, not from an environmental component (Johnson 2002, Brown and Bonhoeffer 2003). Since these foundress queens do not have a colony, nestmate recognition cues are not needed. However, in a closely related species of harvester ant, foundress queens have been shown to damage and lose a proportion of their hydrocarbon profile while digging their nest, resulting in increased water loss (Johnson et al. 2011). Although the ants in this study were captured before digging a founding nest, hydrocarbon production may still be triggered following copulation as a mechanisms to restore the integrity of the cuticular water barrier.

These results suggest a possible mechanism for the origin of the GCD lineages. Based on phylogenies generated from mitochondrial, microsatellite and allozyme sequence data, the GCD lineages were likely created through an initial hybridization event between two species with environmental caste determination, *P. rugosus* and *P. barbatus*, that led to this species complex (Helms Cahan and Keller 2003 but see Anderson et al. 2006). Because selection for more desiccation-resistance hydrocarbons

imposed by the desert environment also limits the number of hydrocarbons that can be utilized for communication purposes (Herrmann, in prep), the blending of discriminating compounds between these two species could have led to a collapse of mating barriers after secondary contact due to a lack of signals available to discriminate lineage identity. This limitation of recognition cues could allow cross-lineage mating to become more common in sympatric species. Over evolutionary time, repeated hybridization events could have produced stable populations of hybrids that evolved into the GCD lineages. On a larger scale, we can hypothesize that the desiccation limitations of hydrocarbon profiles would lead to a higher rate of hybridization in any insects that live in a dry environment, resulting in an increase in hybrids in deserts. Although a lek mating system can increase the probability of hybridization occurring due to CHC exchange, it would not be required for this constraint to increase hybridization. In ants, the three ant hybridizations that occur at the highest incidence are found in xeric environments, suggesting that this idea should be investigated (Feldhaar et al. 2008).

4.6 Figures and Tables

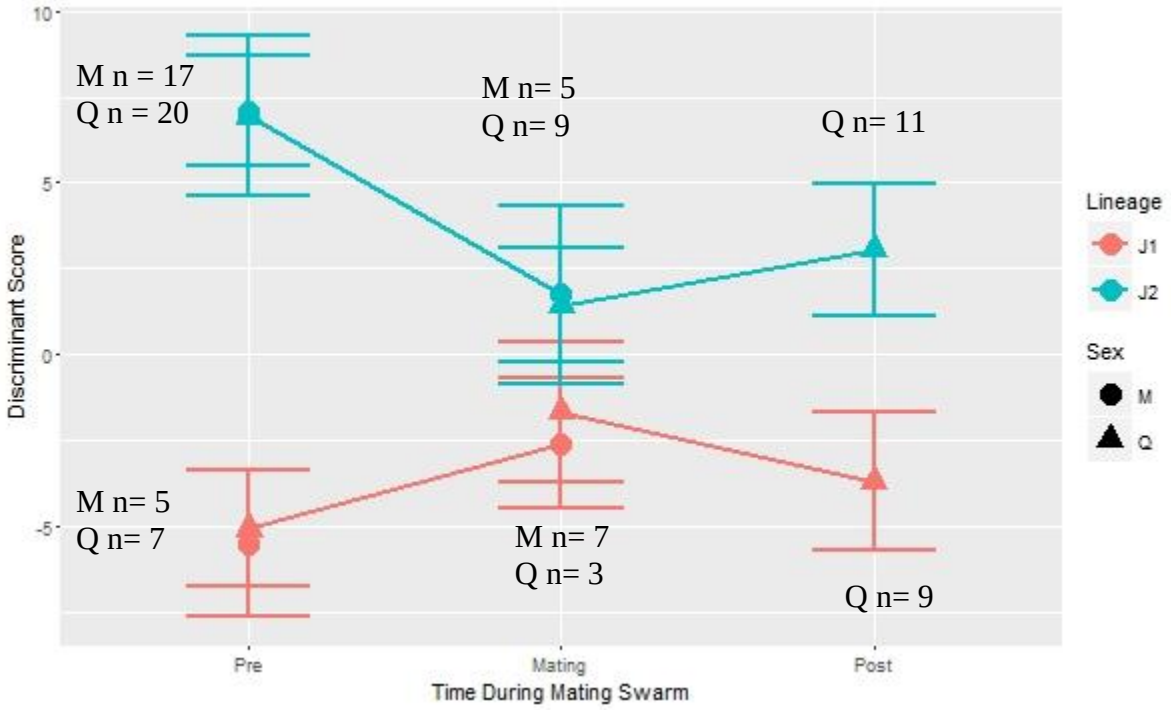

Figure 4.1 : Discriminant analysis scores of J1 and J2 reproductives before, during, and after the mating swarms. Each lineage is split by sex. Error bars represent the 95% confidence interval.

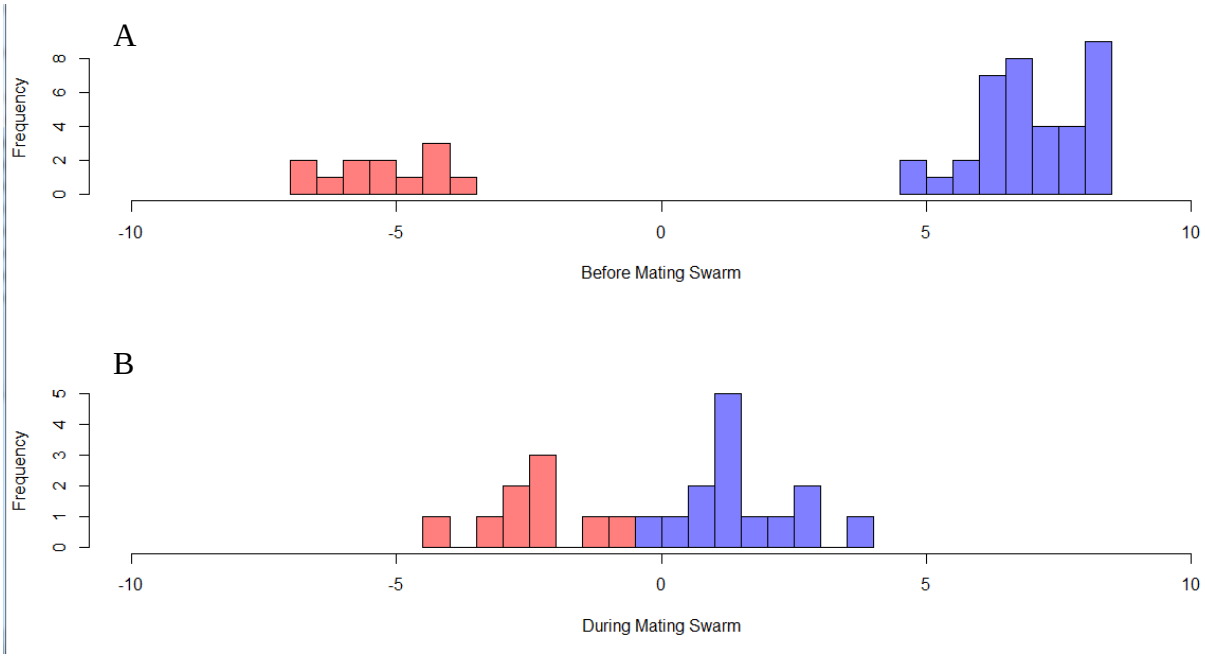

Figure 4.2 : Histograms of LDA results separating J1 lineage from J2 lineage ants prior to the mating swarm (A) and during the mating swarm (B). Red represents the J1 lineage, while blue is the J2 lineage.

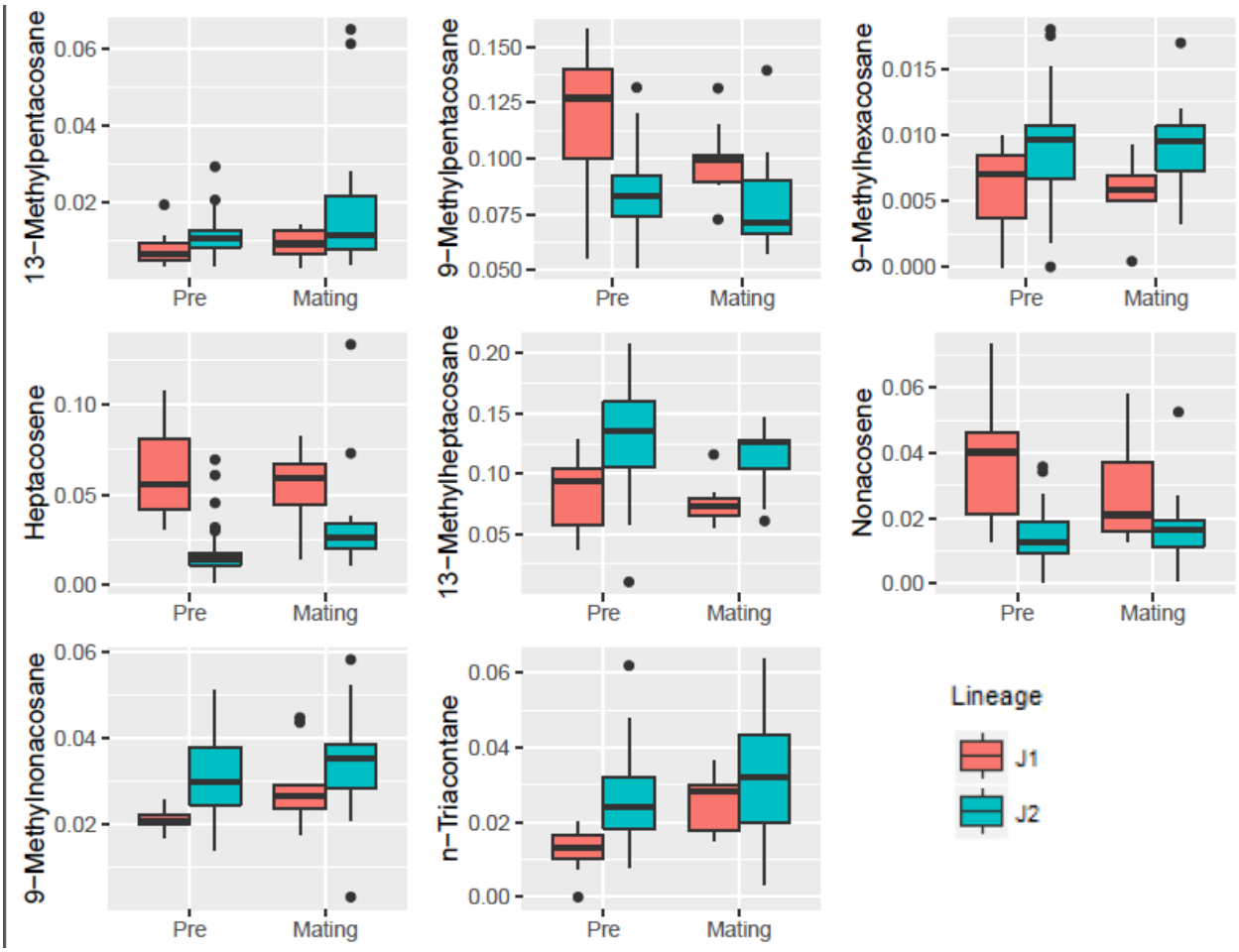

Figure 4.3 : Boxplots of relative abundances of 8 compounds that differed significantly in pre-mating swarm samples. Boxes represent the 0.25 and 0.75 interquartile range, while whiskers show the furthest point within 1.5 interquartile ranges. Points outside of the range are outliers, shown as black dots. Y-axis represents the proportion of the total CHC profiles.

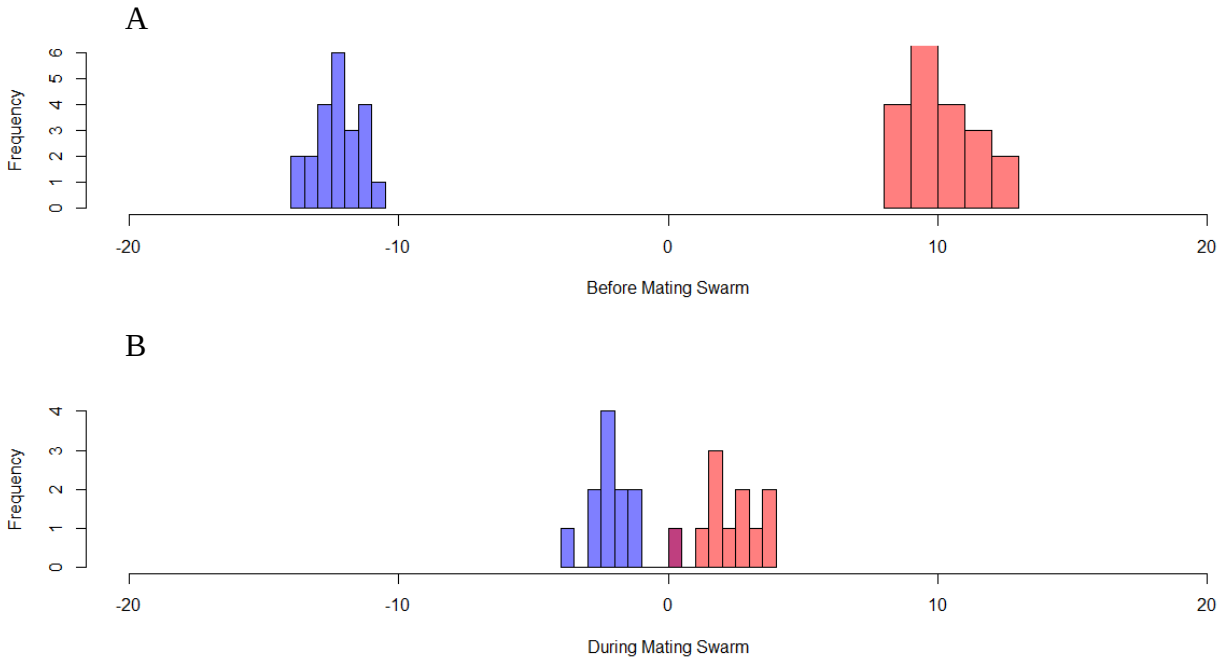

Figure 4.4 : LDA discriminant scores of males and females before (A) and during (B) mating swarm. Queens are blue, while male ants are red.

Table 4.1 : Cross-validation results for the pre-flight samples. Observed numbers are in rows, while predicted are in columns. Proportion of correctly classified ants in J1 (83%) and J2 (86%) were roughly equal. Leave-one-out cross-validation correctly identified 41 of 49 samples (83.6%).

Observed/Predicted	J1	J2
J1	10	2
J2	6	31

Table 4.2 : Cross-validation results for samples collected during the mating swarm. Observed numbers are in rows, while predicted are in columns. J1 ants were correctly classified (44%) slightly less often than J2 (64%). Leave-one-out cross-validation correctly identified 13 of 23 samples (56.5%).

Observed/Predicted	J1	J2
J1	4	5
J2	5	9

Table 4.3 : Cross-validation results for foundress queens sampled two weeks after the mating flight. Observed numbers are in rows, while predicted are in columns. Proportion of correctly classified ants in J1 (44%) and J2 (36%) were roughly equal. Leave-one-out cross-validation correctly identified 8 of the 20 samples (40%).

Observed/Predicted	J1	J2
J1	4	5
J2	7	4

Table 4.4 : ANOVA results of CHC compounds tested by time (before vs during mating swarm) and lineage (J1 or J2), given in p-values. Significant results are in bold.

Compound	Lineage	Time	Lineage x Time
13-Methylpentacosane	**0.04**	**0.02**	0.2
9-Methylpentacosane	**0.001**	0.09	0.11
9-Methylheptacosane	**0.002**	0.92	0.86
Heptacosene	**0.0001**	0.11	0.053
13-Methylheptacosane	**0.0001**	0.13	0.84
Nonacosene	**0.0001**	0.75	0.15
9-Methylnonacosane	**0.004**	**0.04**	0.33
n-Triacontane	**0.002**	**0.007**	0.38

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CHAPTER 5: GENETIC LINKAGE MAPPING AND HYDROCARBON QTL ANALYSIS IN J-LINEAGE *POGONOMYRMEX* HARVESTER ANTS

5.1 Abstract:

Cuticular hydrocarbon compounds (CHCs) play multiple communication roles in ants, including serving as species, kin, and caste recognition cues. However, the genetic basis of variation in the hydrocarbon profile in ants is unknown. In this study, we identified genomic regions associated with elements of the CHC profile in *Pogonomyrmex* harvester ants. We exploited the unusual mating system of obligately hybridogenetic "J" populations of *P. barbatus*, which are composed of two genetically distinct genetic lineages with quantitatively different CHC profiles that interbreed and produce F1 hybrid workers. Without a queen present, workers will produce haploid, F2 male progeny. These were used to generate a ddRADseq-based genetic linkage map and conduct QTL analysis on CHC profile components that differentiate the lineages. Results showed a high recombination rate and 19 linkage groups, slightly more than the actual chromosome number (n=16). Of the eleven compounds tested, two compounds, 13-methyl-nonacosane and n-pentacosane, were each significantly associated with a single genomic region. The significant region in n-pentacosane contained an enzyme involved in the CHC production pathway, as well as regulatory genes that may be responsible for modulation of CHC production. 13-methyl-nonacosane has been identified as a highly conserved queen-specific hydrocarbon signal in ants. Interestingly, the QTL for this compound also included multiple odorant receptor genes. Physical linkage between genes involved in signal production and their reception can potentially resolve the expected decline in effectiveness of kin-selected altruism as signal alleles increase in frequency, known as Crozier's Paradox. In addition, this linkage could be the first step in the emergence of large non-recombining "social supergenes" that underlie both queen social form and worker acceptance of queens in the ants *Solenopsis invicta* and *Formica selysi*.

5.2 Introduction

Cuticular hydrocarbons serve two primary functions in insects: they act as desiccation resistance molecules that form a waterproof outer layer on the cuticle, and also as tactile communication cues between individuals (Blomquist 2010b). Cuticular hydrocarbons are the primary form of kin recognition, especially in eusocial insects (Nehring et al. 2011). An honest relatedness signal permits targeting of altruistic actions only towards related individuals, increasing the inclusive fitness benefits of sociality (Hamilton 1964). To function as a relatedness indicator, a signal must be complex enough to permit fine-scale distinction of relative similarity, and variation in the signal must be genetically based (Rousset and Roze 2007). The chemical profiles of insect cuticular hydrocarbons (CHC's) serve as an ideal medium for assessing genetic relatedness, as CHC compounds have nearly unlimited potential for variability in chain length, functional group types, and functional group position, although environmental constraints can limit the diversity of these compounds in an insect's hydrocarbon profile (Howard and Blomquist 2005).

Although CHC profiles are influenced by both environmental and social factors (Liang and Silverman 2000, van Zweden et al. 2010), they are primarily produced *de novo* (Blomquist 2010a) and variation in CHC profile is heritable (Thomas and Simmons 2008, van Zweden et al. 2009, van Zweden et al. 2010, Nehring et al. 2011). Cuticular hydrcocarbons are produced from the breakdown and combination of fatty acids, a

process controlled through a well-understood pathway involving five key enzymes: fatty-acid synthetases, elongases, reductases, desaturases, and decarboxylases (Wicker-Thomas and Chertemps 2010). The desaturases in *Drosophila melanogaster* have two positional isomers of heptacosadiene are produced by two different desaturase alleles, showing that different genes are responsible for the generation of different CHC compounds (Coyne et al. 1999). While desaturases tend to be relatively conserved across animals, elongases have undergone significant expansion, with over 19 different elongase genes identified in *Drosophila* alone (Wicker-Thomas and Chertemps 2010). In ants, desaturases have also undergone gene expansion, with many ant species having between 10 and 24 different desaturases, compared to seven in *Drosophila* (Simola et al. 2013). Such diversification increases the complexity of CHC compounds that can be simultaneously produced, allowing them to serve as effective communication signals of diverse identity-related information.

The genetic architecture of CHC production has been well-studied in non-eusocial model organisms (de Renobales and Blomquist 1983, Gleason et al. 2005, Gleason et al. 2009, Blomquist 2010a), but mapping CHC profiles in ants presents a considerable technical challenge. Relatively few ant species mate readily under lab conditions, and for those that do, both long generation times and behavioral inbreeding avoidance make inbred lines impossible (Boomsma et al. 2005). One system that can potentially overcome both of these problems is the Genetic Caste Determination (GCD) system in the harvester ant genus *Pogonomyrmex*. GCD populations are composed of two genetically distinct lineages originally derived from an interspecific hybrid zone

(Helms Cahan and Keller 2003, Schwander et al. 2007, Sirvio et al. 2011), which retain species-level diagnostic differences throughout the genome at multiple classes of genetic marker (Helms Cahan et al. 2002, Julian et al. 2002, Volny and Gordon 2002, Sirvio et al. 2011). The unusual mating system employed by GCD lineages generates natural crosses that can yield appropriate recombinant mapping populations (Fig. 5.1). Queens of both lineages obligately hybridize with one or more males of the alternate lineage to produce a uniformly F1 hybrid workforce; although workers cannot mate and are sterile under queenright conditions, they do possess functional ovarioles and are capable of producing recombinant F2 haploid males when isolated from the queen (Curry et al. 2010). Males of the two interbreeding lineages of the "J" GCD population have quantitative differences in their cuticular hydrocarbon profiles, providing phenotypic variation that can be investigated (Volny et al. 2006).

In this study, we conducted QTL mapping of the CHC profile in the J population of *Pogonomyrmex barbatus*. We used double-digest RADtag sequencing to generate a set of lineage-diagnostic single nucleotide polymorphism (SNP) markers. These were employed to generate a linkage map for *P. barbatus*, whose genome has been sequenced but to date has only been assembled into 4,646 unordered scaffolds (Smith et al. 2011b). A recombinant F2 mapping population was generated in the laboratory from worker-laid males; by isolating the males before they eclosed into adults, we limited their hydrocarbons to only molecules that they themselves produced, minimizing environmental and social transfer effects on CHC profiles. The markers were used in

QTL analyses to identify the number and effects of genomic regions associated with the overall patterns and individual elements of the CHC profile.

5.3 Methods

5.3.1 Recombinant Worker-laid males

To obtain recombinant males, 45 queenless worker colony fragments were created from lab-reared J-lineage colonies. The source colonies were collected from natural populations in Arizona and New Mexico by collecting foundress queens in summer field seasons between 2005 and 2010 (Table 5.1). Colony fragments consisting of 50-100 workers were isolated from the queen and maintained until all queen-laid female brood had eclosed, then checked 3-4 times a week for worker-laid eggs and larvae, and then daily once male pupae were observed. Colony fragments were raised in common-garden conditions in a steady temperature environment at 28°C with a 12-hour light/dark cycle. Individual colonies were housed inside 13x17.5x7 cm plastic boxes with two 16x155mm culture tubes filled approximately 1/3 full of water held in place by a small cotton ball in order to provide a humid nesting area. All colonies were fed an identical diet of seed mixture (wheat germ, cornmeal, and oat bran) and two mealworms per week. Individual pupae were isolated when they had initiated melanization of the exoskeleton. Once isolated, males were maintained individually in a 16x155mm test tube and checked daily until eclosion. Males were collected and frozen at -20°C four days following eclosion to allow deposition of hydrocarbons on the exoskeleton for analysis. A total of 118 recombinant males from 29 colonies were sampled between winter 2011 and spring 2013.

Colonies produced an average of four males, although some colonies produced up to 15 males.

To obtain sufficient numbers of recombinant males for linkage and QTL analyses, it was necessary to pool the F2 recombinant offspring of multiple queenless colony fragments into a single mapping population. This is potentially problematic if mothers vary genetically, which would alter the phase relationships of alleles across loci and lead to over-estimation of recombination rates. To minimize this problem, only those markers that differed diagnostically between lineages, and therefore could be presumed to show the same genotype, phase and linkage relationships across all matrilines, were used in the analyses. In order to assay lineage allele frequencies and select appropriate markers, a single newly-mated queen of each lineage was collected for genotyping following mating flights at three sites spanning the geographic range of the J-lineage population: Sonoita, Santa Cruz Co., Arizona; Fort Bowie, Cochise Co., Arizona; and north of Rodeo, Hidalgo Co., New Mexico.

5.3.2 CHC Extraction and Analysis

Cuticular hydrocarbons were extracted from each frozen sample in 500µL of pentane. To ensure that the solvent was able to contact the entire cuticle, samples were agitated for 2 minutes, then left to soak for an additional 8 minutes. The pentane was evaporated, and the dried sample used for GCMS analysis.

To analyze the hydrocarbon profile, each sample was reconstituted in 50 µl of hexane, placed in a 1.5 ml autosampler vial with a 300ul glass conical insert, and run through an Agilent/HP 5973 gas chromatography-mass spetrometer, using electron impact ionization (EI) and a quadrapole mass analyzer with a Zebron ZB-1 column. We used a protocol modified from Wagner (1998, 2000), with an inlet temperature of 300° C. A 5:1 split/splitless injection was used, and the carrier gas was helium at 1 cm^3/s .The oven was started at 100°C, increased at 25°C/min until 240° C, held for 15 minutes, then ramped to 320 at 25° C/min. A five-minute solvent delay was used to ensure the hexane solvent did not affect the gas chromatography.

Compounds were identified by comparing the mass spectrum of each component in each sample against published spectra from the NIST Chemistry Webbook database, then checked against the published CHC profiles of Pogonomyrmex ants in Wagner (1998, 2000) for verification. The elution profile area of each component was integrated from the total ion count for each sample using Chemstation software (Agilent co.); each component was then divided by the total ion count to obtain proportional amounts of each component. The resulting values were arcsine-transformed to normalize for statistical analysis. A composite measurement of hydrocarbon profile was obtained by using field-collected males (n=22, collection details in chapter 4) from each lineage to create a linear discriminant analysis training data set, which was able to correctly place 64% of the field-collected samples using leave-one-out cross-validation; worker-laid males were compared to this dataset as unknowns, and assigned a score calculated from the discriminant function. Seven individual compounds that were determined to differ

significantly between the J1 and J2 field-collected males were analyzed: 13-methylpentacosane, 9-methylpentacosane, heptacosene, n-heptacosane, 13-methylheptacosane, n-octacosane, and 13-methylnonacosane. We also tested four additional compounds that were widely variable across F2 samples: n-tricosane, n-pentacosane, 9-methylheptacosane, and 9-methylnonacosane.

5.3.3 DNA Extraction and ddRAD-tag sequencing

DNA was extracted from the F2 males and the six reference queens from the gaster (males) or thorax (queens) using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol, and eluted into 45µl of AE buffer. Samples were quantified using a Qubit dsDNA assay (Life Technologies, Carlsbad, California). Double-digest RADseq library construction was based on the protocol of Recknagel et al. (2015). Briefly, samples were standardized to 200ng of DNA, and double-digested with the enzymes NlaIII and MluCI by incubating with both enzymes at 37°C for 2 hours, then at 60°C for 12 hours. The enzymes were deactivated by incubating at 90°C for 20 minutes. Samples were purified using 1.5XAMPure beads, according to the manufacturer's instructions (New England Biolabs (NEB), Ipswich, MA) and requantified. Forty-eight individually barcoded P1 adaptors, differing from one another by two bases to minimize miscalling, as well as a universal P2 adaptor, were ligated to the digested DNA using T4 ligase mix according to NEB guidelines (NEB, Ipswich, MA). Each set of 48 samples was pooled by combining 40mg of DNA from each barcoded sample. The pooled DNA was amplified using Phusion High-Fidelity polymerase for an empirically-determined 13 cycles, and was size-selected for 300-400bp

fragments (including adaptors) from a 1.5% agarose gel and gel extracted with the QIEXII gel extraction kit (Qiagen). Completed libraries were sequenced with 100 bp single-end runs on a HiSeq 2000 at the Vermont Cancer Center Advanced Genome Technologies Core facility.

5.3.4 Sequence contig mapping, diagnostic loci, and quality control

All bioinformatics analyses were conducted on the NCGAS Mason computer cluster at Indiana University. Sequences were demultiplexed using the program Sabre (Nikhil Joshi, UC Davis Bioinformatics Core), allowing up to a single base mismatch in the barcode sequence. The restriction site sequence was removed and all sequences were trimmed to 90bp using the FastQ Trimmer tool from the Fastx Toolkit v. 0.0.13, and the FastQ Quality Filter tool was used to filter out all sequences containing one or more base call at any point in the sequence whose quality score fell below 10. Reads from the reference queens were aligned to the *Pogonomyrmex barbatus* genome using Bowtie (Langmead et al. 2009), then were clustered into homologous tags with the ref_map function in STACKS. SNP loci were considered diagnostic and retained for analysis if the genotype was called in at least two of the three queens of each lineage, and was homozygous for one allele in all queens of the the J1 lineage and an alternate allele in the J2 lineage. F2 males were mapped and genotyped for the subset of diagnostic tags with the same Bowtie-STACKS pipeline.

5.3.5 Linkage Mapping

Linkage mapping was performed using JoinMap 4.1 Software (Kyazma B.V., Wageningen, Netherlands). To prevent over-saturation of markers (Cruz et al. 2007, Illa et al. 2009), we reduced the number of markers randomly by 75%, from 23,600 to 5,900; this number provides a resolution of approximately 3.2cM/marker, which is close to the maximum resolution that can be mapped with a mapping population size around 100 (Cruz et al. 2007). Markers for which fewer than 80% of the samples possessed a genotype call were removed, resulting in a final marker count of 2619. Two males with genotype calls for fewer than 50% of the markers were also removed from the analysis.

The remaining markers were assembled into linkage groups using JoinMap's grouping function, using a starting independence LOD score of 2.0, and increasing to a LOD-score of 10 with an increment of 1.0. A LOD score of 6.0 was used as the cutoff point to determine which markers formed linkage groups; since extremely small linkage groups are likely caused by genotype errors, two groups with only two markers were discarded, resulting in a final marker count of 2615.Once groups were established, markers within linkage groups were ordered in JoinMap by reanalyzing the data with the mapped scaffold locations. Maximum likelihood was used to map linkage groups and estimate map distances. Map order and positioning were obtained using three rounds of optimization per sample, and multipoint recombination frequencies were estimated with a burn-in chain length of 10,000.

5.3.6 QTL Analysis

QTL analysis was performed using the Rqtl package (Broman et al. 2003) in Rstudio (Revolution Analytics,Redmond, WA). Each of the eleven compounds and the discriminant scores were analyzed separately using the scanone function. In order to prevent genotyping errors from causing false-positive LOD scores, both the Haley-Knotts method and the EM Maximum Likelihood method were used. Seven samples that showed an excessive level of recombination, with over 200 events, were excluded. A genome-wide LOD score significance level was determined using a permutation test, and applied to identify genomic intervals significantly associated with the trait of interest. Genes falling within these intervals were identified from the annotated genome of *Pogonomyrmex barbatus* (Smith et al. 2011b), using the most recent gene annotation of Pbar UMD V03 (Accession no. GCF 000187915.1). Gene ontogeny terms were derived from the GO terms listed on the ID page from this gene annotation on the NCBI website.

5.4 Results

5.4.1 Linkage Mapping

Using a linkage LOD score of 6.0, 19 linkage groups were identified, three more than the number of chromosomes reported for the genus *Pogonomyrmex* (n=16;Taber et al. 1988). The number of markers in each linkage group ranged from 15 to 334, and the number of scaffolds per linkage group ranged from two to 58. Linkage group size ranged

from 42 to 953 cM (Fig 5.2A, B). Average mapping distance between markers was 3.05 ± 3.6 cM (Supplemental table 5.1). Several regions in the center of linkage groups did not clearly resolve, resulting in a large area of linkage with uncertain ordering (Fig. 5.3).

5.4.2 CHC QTL Analysis

Of the eleven hydrocarbons and predicted discriminant score analyzed, only two compounds, 13-Methylnonacosane and n-pentacosane, had a LOD-score region above the genome-wide threshold level of 3.45 (Fig. 5.5, 5.6). 13-Methylnonacosane showed a single significant peak in linkage group 11, around the 100cM region (Fig. 5.4). The significant region encompasses an interval of approximately 400,000 base pairs. Fifty-six genes were found in this region, 49 of which were annotated protein coding genes. The identified genes were primarily odorant binding protein genes, with 42 of the 49 having odorant binding domains (Fig 5.7A). For n-pentacosane, the significant region was found on linkage group one, at around 300cM (Fig. 5.6). In this region, 30 protein coding genes were found, with eleven of the genes related to transcription or transcriptional regulation (Fig. 5.7B). In addition, one gene, carnitine O-palmitoyltransferase 2, is an acytltransferase gene that creates an acytl-CoA compound, which is a molecule utilized in the elongating step in cuticular hydrocarbon synthesis (Blomquist 2010a).

5.5 Discussion

In this experiment, we tested for genetic regions significantly associated with the production of cuticular hydrocarbons. We created a high-resolution genetic map using 2,600 markers, dispersed over 19 linkage groups. Two compounds, 13-methylnonacosane and n-pentacosane, showed a genetic region above the significance threshold. The 13-methylnonacosane region contained 56 annotated genes, most of which were odorant receptor genes, while the significant region in n-pentacosane contained 28 genes, with many of the genes involved in transcription. This work has created a detailed linkage map of a GCD lineage of harvester ants, with a map size of 7,917 cM, one of the largest values found in insects. The linkage map showed a highly recombinant rate within the GCD harvester ants, and Quantitative Trait Loci analysis identified two significantly associated genomic regions that likely play a role in gene expression and hydrocarbon reception. These results could provide clues to the underlying theoretical issues of genetic kin recognition, and how the higher recombination rates can affect the kin recognition ability of genetically encoded signals.

The linkage groupings separated into 19 total groups, three more than the number of chromosomes in *Pogonomyrmex* ants. The three additional linkage groups are likely smaller regions of other linkage groups that appeared unlinked in this analysis, possibly due to regions with sparse markers or poor sequencing coverage. Most of our linkage groups therefore likely represent entire chromosomes, with three chromosomes being broken into multiple groups. The less-resolved regions near the center of several of the linkage could reflect the lower recombination rates typical around the centromeres, making higher resolution of map positions difficult (Johnson et al. 1996). The overall

map size was quite large, with a total map size of 7,917 and the largest linkage groups measuring over 950 cM, despite having an average genome size for ants (Tsutsui et al. 2008, Smith et al. 2011b). Although this map size is large, linkage mapping of one of the parental species of these lineages, *Pogonomyrmex rugosus*, have shown the highest rates of recombination found in insects, with a total estimated map length of 2,129 cM using 751 markers (Sirvio et al. 2011). This is nearly half of the size of using a similar number of markers in our data, which gives a map length of 4500, suggesting that while marker number can explain some of the difference, a higher recombination rate in the GCD lineages appears likely. Given that the population of ants used in this analysis are derived from hybrids between these highly recombinant species, it is possible that the GCD lineage ants have an even greater recombination rate, creating the larger map size. This is the opposite of patterns of reduced recombination rates often seen in other hybrid species, where genomic differences between species limits the regions that can successfully recombine (McGaugh and Noor 2012).

Despite using eleven compounds and the predicted loadings of discriminant analysis as phenotypes for QTL analysis, only two compounds, 13-methylnonacosane and n-pentacosane, had a LOD score above the genome-wide significance threshold, while the other nine compounds and LDA loading did not show any significant region. One possible reason for this lack of significant LOD regions could be a result of low CHC concentrations that dampened phenotypic variation across samples. Since the males were newly eclosed, they had a limited amount of hydrocarbon deposited on their cuticle, leading to much lower concentrations of ions to be analyzed in the GCMS analysis, with

male ion counts approximately 30 times smaller than lab-reared queens and workers (M. Herrmann, unpublished data). Another possible reason is the relatively small sample size used in this analysis; ideally, thousands of samples would provide better resolution in the genetic map and allow higher resolution of genetic regions with small to moderate individual effects (Illa et al. 2009). In addition, the differences between the two lineages were quantitative in nature and occurred in a small proportion of compounds in the CHC profile, making the detection of QTLs in these species even more difficult (Chapter 3). These deficiencies in the phenotype measurement, coupled with the sensitivity of quantitative trait loci method to genotyping errors and smaller phenotypic differences, could have prevented more significant genomic regions from being identified in this analysis.

N-pentacosane is a straight-chain cuticular hydrocarbon, and is the most abundant compound in the cuticular hydrocarbon profile of *Pogonomyrmex* (Wagner et al. 1998). It is primarily a desiccation-resistance compound, preventing water from freely transferring across the cuticle (Greene 2010). Differences in expression of desiccation-resistance compounds could trace back to the historic ancestors of the GCD populations, *P. rugosus* and P. *barbatus* (Helms Cahan and Keller 2003). *Pogonomyrmex rugosus* lives in a drier, more xeric environment, which would select for higher levels of desiccation-resistance molecules than the more mesic *P. barbatus*. Our results show that n-pentacosane was significantly associated with a genomic region on linkage group 1, which contained numerous transcription and RNA modification genes. This suggests that this genomic region may be involved in controlling expression of n-pentacosane. As

evolutionary changes can occur faster through changes in regulation of gene expression rather than through novel alleles, control of CHC production would be better maintained through interactions in regulatory genes (Wittkopp et al. 2004). Alternatively, several genes in this region play a role in development, and can possibly alter the speed that development occurs in these ants. Since adult ants eclose with few hydrocarbons (van Zweden et al. 2010), an increased speed of development would allow ants to melanize their cuticle and deposit more hydrocarbons on their exoskeleton at the three-day period that the males were kept for, giving these males more time to differentiate their CHC profile which would result in differential express of n-pentacosane.

13-methylnonacosane is a compound that has recently been identified as a conserved queen-specific signaling molecule in numerous ant species (Van Oystaeyen et al. 2014). The large number and diversity of odorant receptors that are found in this significant region are likely associated with the detection of chemical signals, making this genetic region important for chemical communication. Since ant males derive their fitness only through mating opportunities in a mating swarm and die soon after mating (Boomsma et al. 2005), the ability to produce and interpret chemical signals, especially one that is related to a fecundity signal, is especially important for both male identification of queens, and queen willingness to mate with males. In non-recombinant field-collected males, 13-methylnonacosane was significantly higher in queens than in males, and was also significantly different between the males of the two lineages. Since the males used for mapping are recombinants between the two lineages, the difference in expression seen here could also be a result of dysregulation in the F2 males (Landry et al.

2005); regulatory genes found in one lineage could be incompatible with or superseded by other genes in the hybrid, resulting in transgressive expression not seen in the parentals. This is especially relevant in haploid males, since they lack any trans-gene regulation, increasing the probability of gene dysregulation if part of a gene's regulatory pathway is lost or altered. This could have implications for mating success in hybrid males in these populations; dysregulation could increase male fitness by creating a rare phenotype, increasing their attractiveness in females seeking inbreeding avoidance (Zhang et al. 2014), or it could decrease hybrid fitness by altering cues used by females for species identification. As introgression between interbreeding populations is low, a reduction in fitness for recombinant males seems likely (Schwander et al. 2007).

In order for these compounds to be effective at communicating numerous different recognition, a high level of diversity in the CHC profiles of these ants must be maintained. However, genetically based kin recognition cues are thought to be unstable over time, since recognition signals that enhance cooperation will be highly selected for and become more common, reducing polymorphism needed for diversity and eliminating the discrimination ability of the recognition cue (Rousset and Roze 2007). This problem is often referred to as Crozier's paradox, where the lack of additional diversifying selection on kin recognition cues will lead to fixation within a population due to the benefits of shared altruistic behaviors, leading to universal cues that have no kin-discrimination ability(Holman et al. 2013). The loss of genetic polymorphism also allows parasites and non-cooperators to be recognized as members, further decreasing the effectiveness of these discrimination cues (Gardner and West 2007). As CHC's are

utilized in nestmate recognition, species recognition and mate identification, how is enough variation maintained within a species to allow enough differentiation in these regions to accurately use these compounds for discrimination and recognition purposes? One hypothesis is that the alternative selective pressures imposed by the differing functions of communication-specific compounds results in the balancing selection pressures necessary for the maintenance of different CHC compounds for discrimination (Crozier 1986, Holman et al 2013). Kin recognition cues that promote altruistic behavior would be under positive selection due to the benefits provided through cooperation, while selection to avoid inbreeding will select for recognition cues that differentiate between families, resulting in diversifying selection. Since cuticular hydrocarbons are utilized as both kin recognition cues as well as sexual selection and inbreeding avoidance cues, these forces could exert the balancing selection pressures on CHC diversity that are required to maintain the diversity needed for kin recognition. An alternative hypothesis that could explain this diversity is that the genes that control the production and modification of communication molecules could be located in a hypervariable region, allowing for higher rates of mutation and recombination that can maintain a high level of genetic diversity in these compounds. As our linkage map reinforces, *Pogonomyrmex* ants appear to have one of the highest rates of recombination in insects (Sirvio et al. 2011), which could help maintain the diversity required for colony-level discrimination. However, the significant QTL regions found for 13-methylnonacosane and n-pentacosane are not in a highly-recombinant region, as would be expected if high levels of recombination stabilized the kin-recognition ability of communication cues (Rousset and Roze 2007). In addition, neither significant region contained any of the five classes of enzymes involved in the

production and modification of cuticular hydrocarbons, suggesting that these genomic regions are further up the CHC pathway, rather than the actual production enzymes themselves (Blomquist 2010a).

Finally, physical linkage between genes that promote social behavior and recognition cues could result in tightly-linked clusters of genes involved in the generation and reception of specific rare signals. This linkage could circumvent the issues of losing signal specificity predicted in Crozier's paradox by creating a genetic region with little to no recombination between the behavior and reception of cues associated with that behavior, preventing the signal and behavior from being decoupled due to recombination. This would allow these signals to serve as effective genetic kin recognition cues without a loss of signal specificity, maintaining the honesty of the signal. The genomic region associated with 13-Methylnonacosane contained a high density of odorant binding genes, suggesting the possibility of an interesting interaction between the production/presence of a specific communication cue and the proteins needed to receive and interpret that cue. These reception proteins serve as the receiving mechanism of chemical communication in ants and are equally as important as the production of the hydrocarbon signals themselves, since communication modalities are only useful if they can be received and interpreted by other organisms. The genus *Pogonomyrmex* shows a large gene expansion in odorant receptor proteins, allowing for a greater number of receptors and communication molecule reception specificity in these ants (Smith et al. 2011b). In *Solenopsis invicta*, proteomics analysis of the antennae showed that a high level of

odorant-binding receptors were localized there, suggesting that these genes are used for chemical reception in ants (Gonzalez et al. 2009).

The coupling of signal receptors and production would ensure that individuals possessing a signaling variant can both generate and interpret the signal; if reception triggers performance of a socially altruistic behavior, it would effectively create a "green beard" type of social gene, where the expression of a specific signal can serve as an honest indicator of relatedness at the receptor gene as well, encouraging social behaviors among any organisms with this gene complex (Keller and Ross 1998). This significant region could offer important insights into the evolution of linked genomic regions associated with social structure variation that have been found in ants, such as the "supergene" complexes found in *Solenopsis invicta* and *Formica selysi* (Nipitwattanaphon et al. 2013, Purcell et al. 2014). These so-called "social genes" are linked to several social traits; in *S. invicta*, this region both includes odorant binding receptors and is associated with upregulation of genes from the cuticular hydrocarbon synthesis pathway (Nipitwattanaphon et al. 2013). "Supergenes" tend to show extremely low levels of recombination, maintaining the specific combination of genes on the same haplotype (Keller and Parker 2002). Although not found in a region with low levels of recombination, the genetic regions associated with CHC expression found in our study could show the evolutionary first step in the creation of a social gene complex, linking social traits to communication expression and reception to allow for honest signaling of fecundity-associated hydrocarbons (Holman 2012). Future work could be done to investigate these regions in relation to social behaviors, as well as to hydrocarbon

production, to understand how expression of these compounds can be linked to other traits, and how diversity in the CHC profiles are maintained.

5.6 Figures and Tables

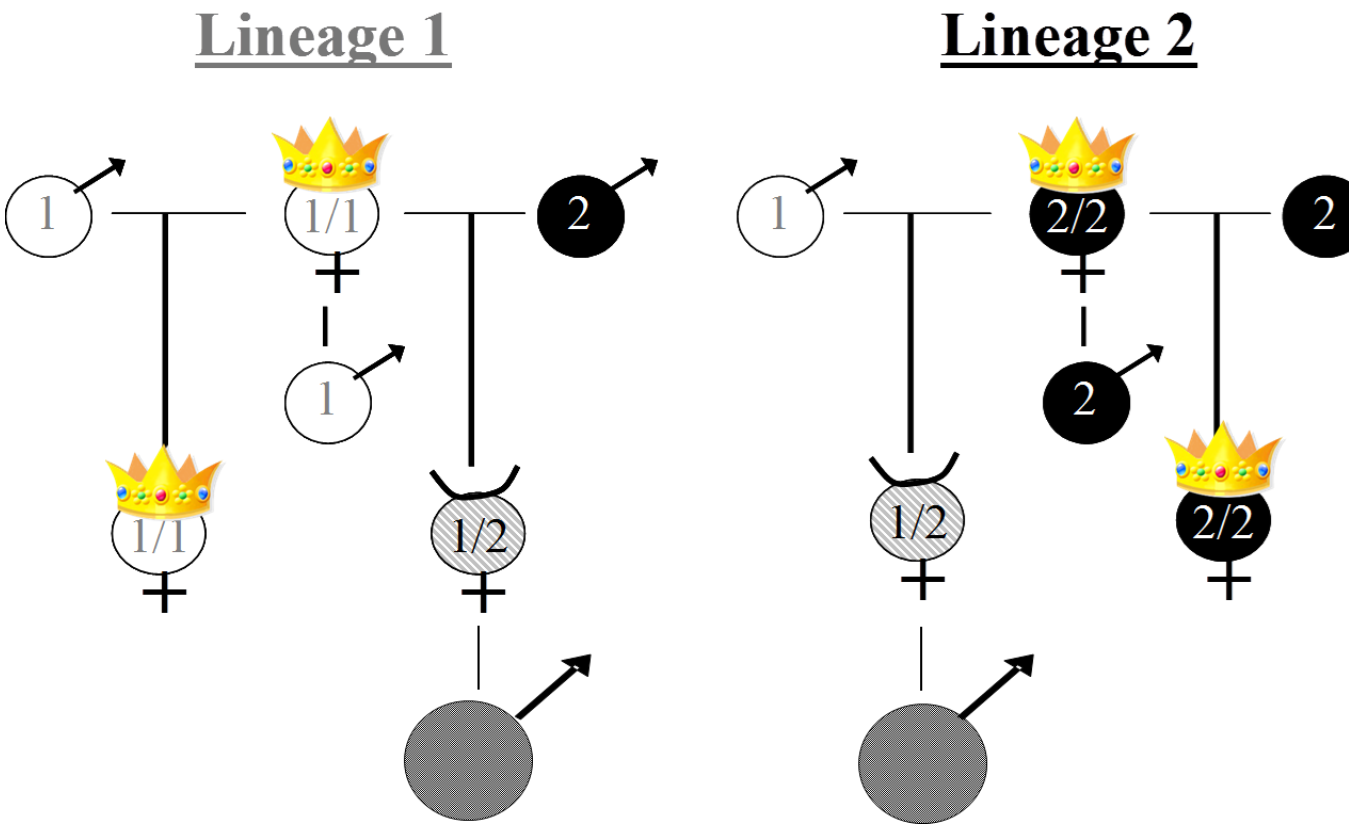

Figure 5.1 : The breeding design of the males used in this experiment. Male symbols represent males, crowns represent queens, and female symbols with hatches over it represent workers. The numbers refer to the lineage chromosomes found within each group. When isolated, F1 hybrid workers will activate their ovaries, producing recombinant haploid males. Since these haploid males are produced from a hybrid worker, the overall design closely resembles a double-haploid mating design. Numbers refer to lineage origin of chromosomes; the worker-laid males at the bottom are recombinant.

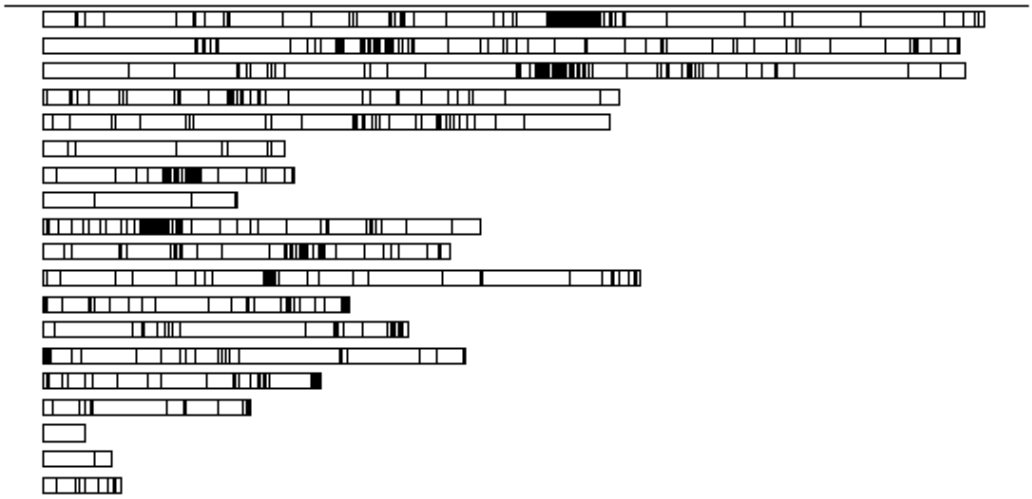

Figure 5.2 : Linkage groups from *Pogonomyrmex*, drawn by continuous scaffold. Black lines represent boarders between two different scaffolds, while black blocks represent numerous smaller scaffolds that occur in succession.

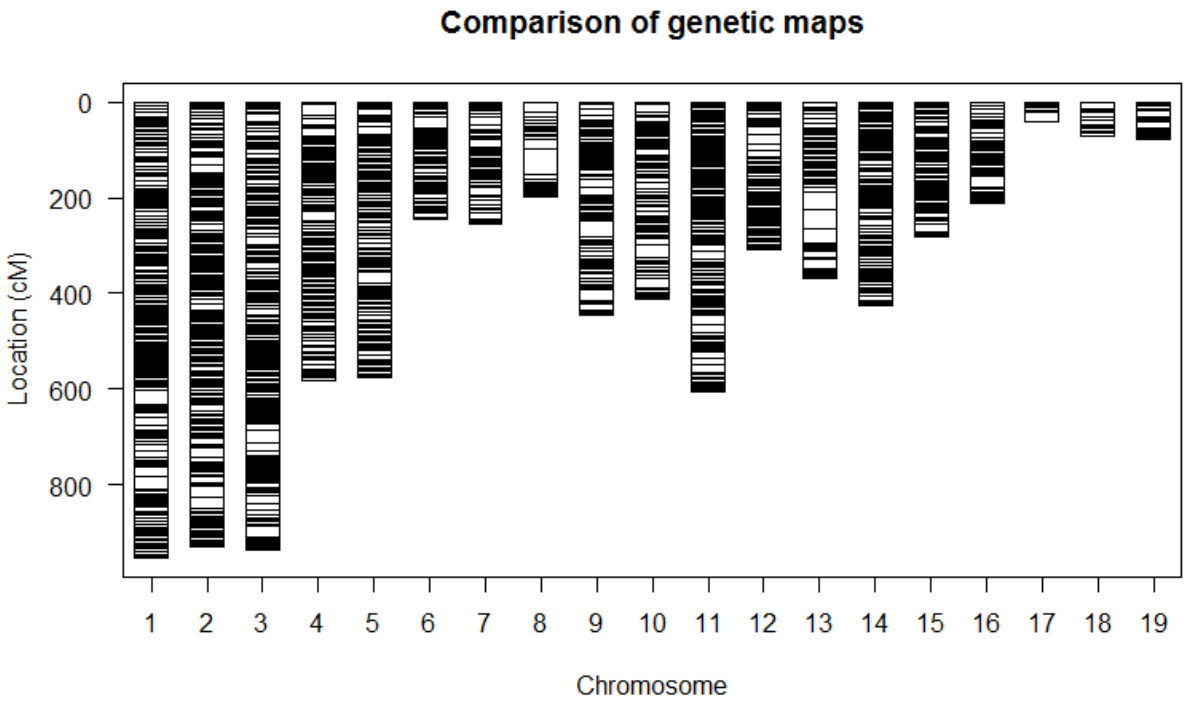

Figure 5.3 : Marker locations (black bands) in mapped linkage groups.

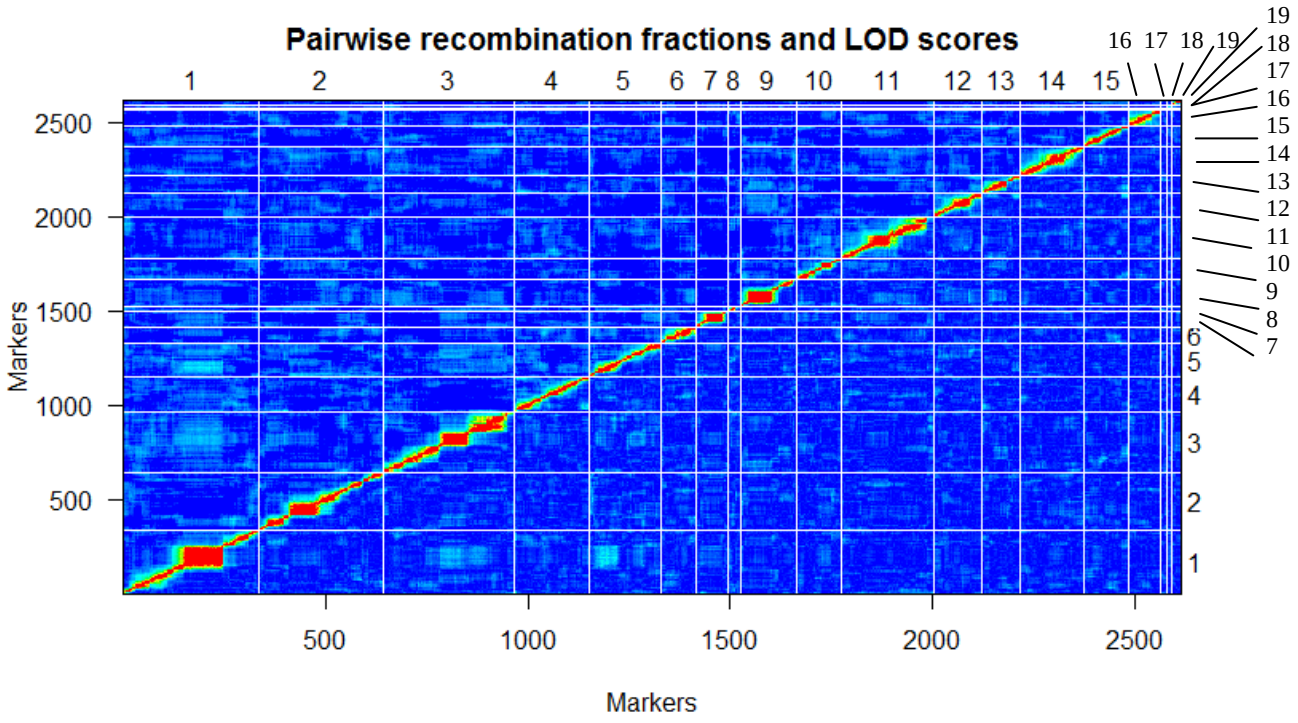

Figure 5.4 : Recombination heat-map across linkage groups. Red represents high linkage/low recombination, while dark blue represents low linkage/high recombination. The area of high linkage (red) that is seen diagonally through of the plot shows regions of low recombination that were mapped close together.

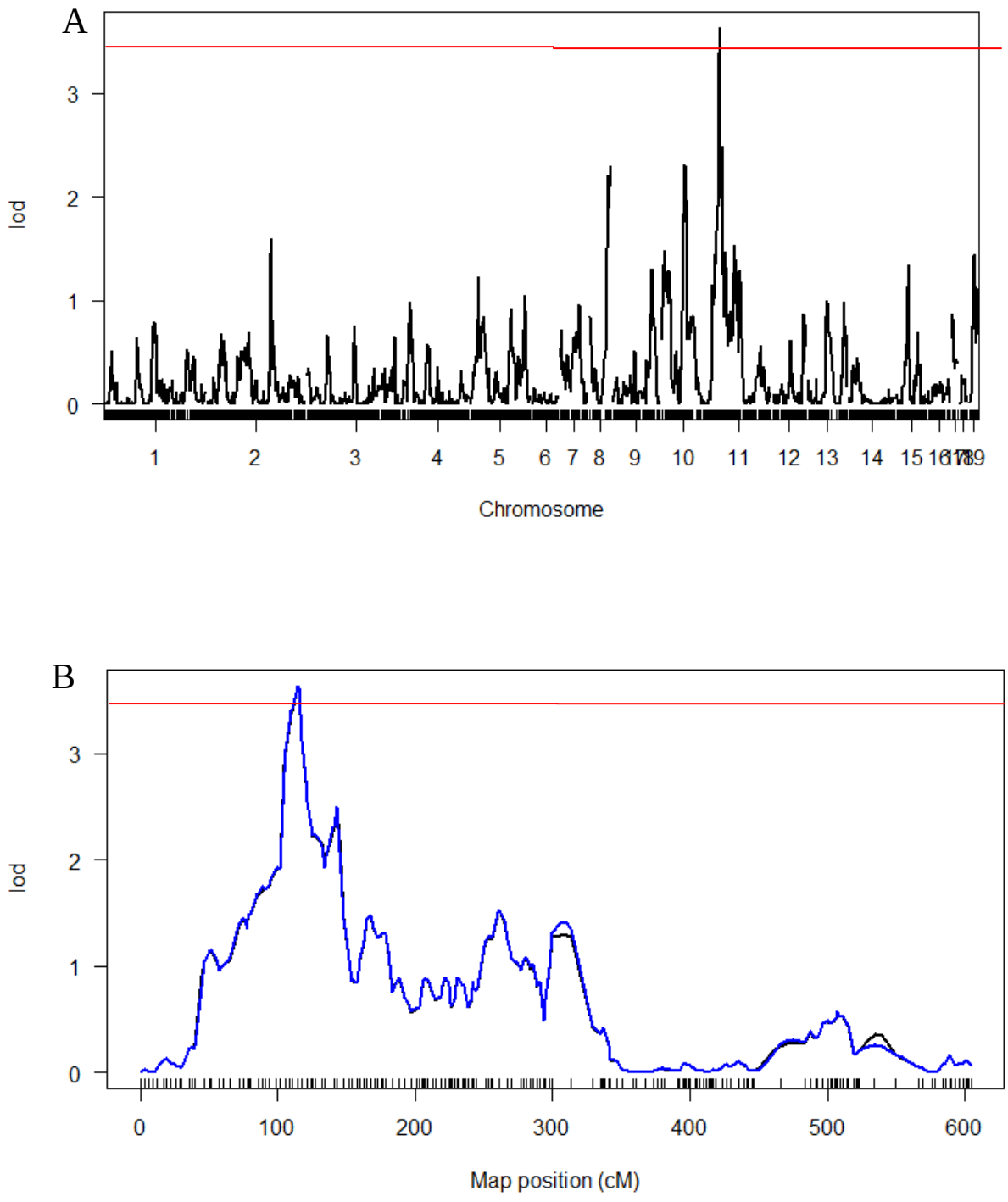

Figure 5.5 : Plot of QTL LOD score for 13-Methylnonacosane. A) LOD scrore across all linkage groups. B) linkage group 11. Black ticks at the bottom indicate marker location, and the red line indicates the genome-wide significance threshold.

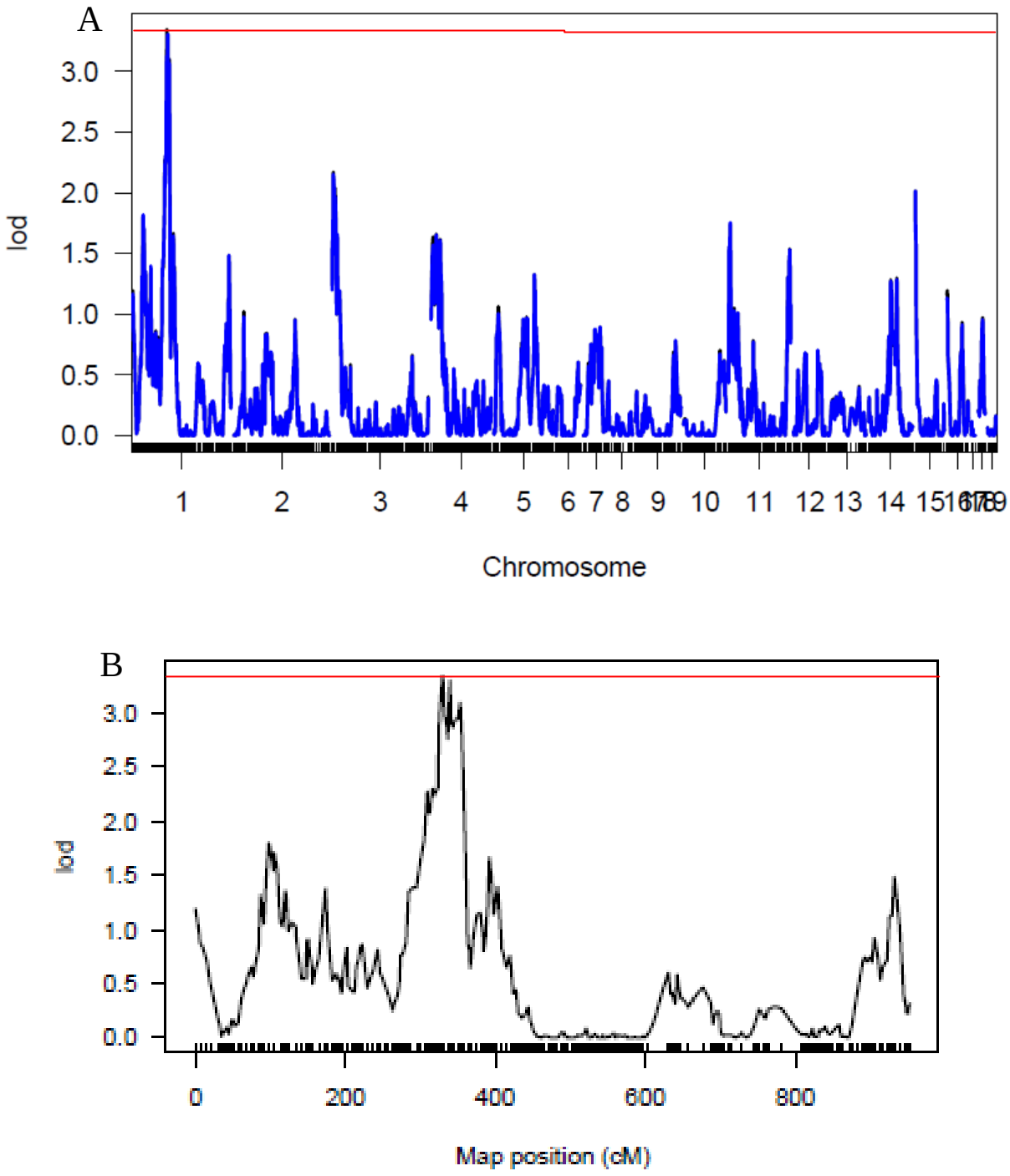

Figure 5.6 : Plot of the significant QTL LOD score for n-pentacosane A) across all linkage groups and B) linkage group 1. The red line indicates the genome-wide significance threshold of 3.45. The black ticks at the bottom indicate marker locations within this linkage group.

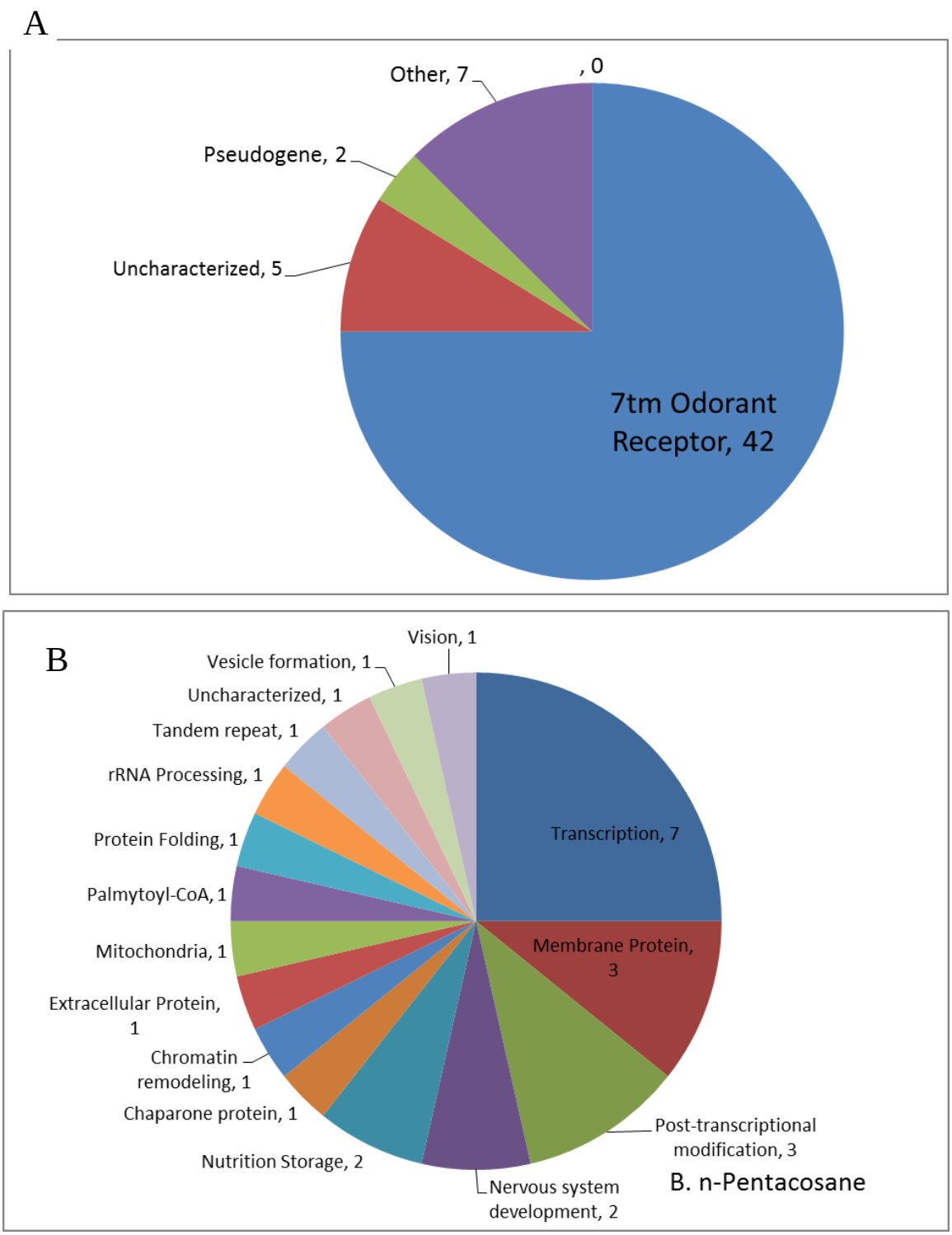

Figure 5.7 : Breakdown of gene products in the significantly associated genomic region for A. 13-Methylnonacosane, and B. n-Pentacosane. Numbers are based on unique genes identified within the significant region, with a total of 56 in the 13-Methylnonacosane region, and 30 in the n-pentacosane region

.

Table 5.1 : Collection information for colony fragments that produced the males used in this experiment.

Number of Males	Location	County	Coordinates
20	Paradise/Grill, AZ	Cochise Co, AZ	31.986 N, -109.12 W
40	Outside Ft Bowie	Cochise Co, AZ	32.158 N, -109.455 W
14	Site SH	Cochise Co, AZ	31.845 N -109.049 W
36	North of Lordsburg	Grant Co, NM	32.428 N, -108.673 W
8	4 Miles east of site H	Hildago Co, NM	31.63 N, -109.671 W

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CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

Sexual conflict resolves around differences in optimal reproductive fitness strategies of males and females(Parker 1979). However, sexual selection can be very difficult to detect without a thorough investigation, since control of sexual conflict often relies on cryptic mechanisms (Cox and Calsbeek 2010). This dissertation has provided evidence of the cryptic mechanisms of interspecies sexual conflict that occurs in hybridizing harvester ants. In these populations, male and female fitness interests differ, since males only produce fertile offspring by mating with their own lineage, while queens must mate with opposite-lineage males in order to survive (Julian et al. 2002, Volny and Gordon 2002, Helms Cahan and Keller 2003). Our results show that copulation duration appears to be under queen control, while males can control the rate of sperm transfer (Chapter 2). The result of these counteracting strategies is essentially equal amounts of sperm being transferred during each mating, despite the differences in copulation duration. This means that if only using sperm transferred as a measure of fitness, sexual conflict would not be observed at all. Therefore, the resolution at which sexual conflict is examined could determine if the conflict is seen or not, and future studies should keep this in mind when examining systems where sexual conflict is thought to occur.

These ants are also an example of antagonistic coevolution occurring between males and females in different species, with an asymmetrical fitness benefit to males or females depending on the lineage of their mate (Schupp and Plath 2005, Pfennig 2007).

This leads to antagonistic coevolution between the sexes of two different isolated genomes, resulting in an unusual case of what is called interlocus sexual conflict, where the genes controlling the conflicted trait are not linked (Rice and Holland 1997). The interlocus conflict removes the limits on the extent of antagonistic coevolution, allowing one sex to "win" and exert its will on the other. Males that are able to selectively mate with their own lineage queens will have an increased fitness over indiscriminant males, since all of their offspring will be reproductive queens. This fitness benefit would quickly go to fixation, preventing queens from obtaining and producing workers and causing the hybridizing system to quickly collapse. However, this does not seem to occur, as random mating is seen in these swarms. This random mating is reinforced by the combination of thermal constraints on signaling molecules, as well as the homogenization of CHC compounds between lineages in the mating swarm.

Cuticular hydrocarbon molecules serve as both desiccation-resistance molecules and communication signals, and are the primary cues used in nest, species, and mate recognition in ants (Gibbs 1998, Lahav et al. 1999). They form a mixture of many different hydrocarbons on the exoskeleton of insects, creating a waxy layer between the cuticle and the environment (Howard and Blomquist 2005). Hydrocarbons that contain functional groups have greater variation and can carry more complex communication cues but are less effective in desiccation resistance, while straight-chain alkane compounds with no functional groups increase the desiccation-resistance ability of insects yet have limited usefulness for communication (Gibbs and Pomonis 1995, Greene

and Gordon 2007). In areas with a high risk of desiccation, such as deserts, a tradeoff between molecules that are effective for communication and ones that are effective for desiccation resistance is expected (Rouault et al. 2004). Our work supports this idea, with the species that exist in the driest environments having a greater percentage of straight-chain alkanes and a higher composite melting point for the molecules in their CHC profiles (Chapter 3). This constraint can also play a role in the lack of mate choice during mating swarms, since a limited amount of compounds available for communication could reduce the recognition ability during mating. The desiccation resistance tradeoff could lead to a higher level of nest parasitism and hybridization in drier environments, since the limits would lower the variability and reliability of correct identification of insects that rely on cuticular hydrocarbons.

This dissertation also shows that the differences between lineages and sexes before a mating swarm are reduced during the swarm itself, limiting the information cues available to identify the lineage of potential mates (Ch. 4). This convergence occurs during the mating swarm, likely due to exchanging of hydrocarbons between ants in the mating swarm similar to the exchange of hydrocarbons and formation of a gestalt odor within ant colonies (van Zweden et al. 2010). The limiting of reliable discriminating hydrocarbons during the swarm would lead to indiscriminate mating in order to maximize their fitness, allowing for the stabilization and survival of the interbreeding system. The convergence of CHC profiles between the two lineages suggests that this dynamic could play a role in the origin and maintenance of hybridizing systems.

Finally, this dissertation looks at the evolution and maintenance of hydrocarbons that are used in genetic kin recognition. Genetic kin recognition cues are thought to be unstable due to the difficulty of maintaining variation in recognition signals in a population, especially if altruistic behaviors directed towards organisms with shared recognition cues results in fitness benefits (Gardner and West 2007). This problem is known as "Crozier's Paradox", where genetic-based kin recognition cues would undergo positive selection from the benefits of altruistic acts and go to fixation, unless other disruptive selective forces act on that cue (Holman et al. 2013). In this dissertation, I examined the evolution of genetic kin recognition by creating a linkage map of the genome of the hybridizing harvester ant and using quantitative trait loci analysis to identify genetic regions associated with expression of specific hydrocarbons (Chapter 5). Our results showed significant QTLs for two compounds, one a fecundity signaling molecule, and another a common desiccation-resistance molecule. The fecundity molecule appeared to be closely linked to multiple odorant-binding receptors, directly linking expression of the fecundity molecule to reception. This linkage would couple genes utilized in reproduction and signal reception genes, creating an honest fecundity signal (Holman 2012). The linking of functional genes to signals also suggests a possible evolutionary origin of "super genes," genomic regions with little recombination that contain several genes linked to social behavior (Keller and Parker 2002, Nipitwattanaphon et al. 2013). Future research could use these ants as a model for the linkage of signals and reception, using the genetic region identified as a target for

studying the link between traits, communication signaling, and reception over evolutionary time.

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SUPPLEMENTAL MATERIALS

Table S3.1 : Collinear compounds in LDA analysis from chapter 2. Collinear compound 2 was set to null to remove the collinearity.

LDA	Collinear Compound 1	Collinear Compound 2	Corr. Coefficeint
J-lineages	Hexacosane	Octacosane	0.989
J-lineages	13-Methylhexacosane	13-Methylheptacosane	0.912
H-lineages	Icosane	13-Octacosene	0.922
Queens	Hexacosane	Octacosane	0.971
Workers	Tetracosane	Hexacosane	0.916
Workers	13-Methylheptacosane	9-Methylheptacosane	0.921

Table S3.2: LDA Loadings from Chapter 3, with up to the first three linear discriminant axis ordered by LD1.

Caste LDA	LD1	LD2	LD3
13-Octacosene	-22.8156		
Heneicosane	-21.4268		
Nonacosene	-17.1146		
Hentriacontene	-15.4143		
9-MethylTricosane	-13.5016		
9Methyl.Pentacosane	-13.3584		
13-Methyl.Heptacosane	-13.1141		
Heptacosene	-13.0607		
Hentriacontane	-11.7673		
Hexacosane	-11.4248		
Pentacosane	-11.3247		
Heptacosane	-11.2194		
13Dotriacontene.1	-10.8179		
9Triacontene	-9.57686		
Ester	-8.65587		
Octacosene.3.check.	-7.83364		
Nonacosane	-6.27143		
13Methyl.Pentacosane.1	-5.33245		
Tetracosane	-4.82617		

Octacosene.2..check.	-3.28908		
Dotriacontene.2	-0.93696		
Dimethylpentacosane..check.	-0.78532		
Methyl.Hexacosane.1	-0.47623		
Octacosane	0.692298		
Methyl.Nonacosane.1	1.061003		
Methyl.Tricosane.1	1.254907		
Triacontane..c30.	1.725477		
Methyl.Nonacosane.2	3.272536		
Dotriacontane	5.321728		
Docosane	6.102371		
Methyl.Tetracosane	9.267188		
Tricosane	12.15294		
Methyl.Hexacosane.2	12.62489		
Pentacosene	14.63395		
Methyl.Heptacosane.2	18.14712		
Triacontene.1	33.97565		
Icosane	39.31865		
J-lineage LDA			
Heneicosane	-928.898	-159.661	221.204
Triacontene.1	-315.826	-73.9496	59.07692
Methyl.Tricosane.2	-191.52	-62.0641	10.53607
Methyl.Tricosane.1	-120.43	-225.903	329.9974
Methyl.Tetracosane	-64.7753	66.09554	9.778121
Octacosene.2..check.	-51.5126	-17.5134	-3.52652
Pentacosene	-36.6896	100.995	-2.67975
Methyl.Hexacosane.2	-32.581	-40.2378	20.74614
Hexacosane	-31.3319	-4.21137	-3.11936
Methyl.Nonacosane.1	-31.2628	-6.15136	8.021152
Hentriacontene	-30.532	-43.6459	31.16118
Nonacosene	-27.3869	26.64022	-63.1637
Dotriacontene.2	-24.8698	-33.2761	15.83348
Methyl.Pentacosane.1	-12.489	-8.9905	10.07291
Methyl.Heptacosane.2	-9.8419	8.116636	-4.25096
Pentacosane	-8.36109	3.287175	-5.74856
Tetracosane	-5.25052	0.695727	-0.74228
Dotriacontane	-3.39632	-29.7417	19.28871
Heptacosane	0.990094	-0.31328	1.225774
Nonacosane	1.166957	-21.3057	0.896486
Octacosene.1..check.	1.238609	-71.4252	53.45352

Docosane	3.236006	88.31307	-0.88121
Ester.2	23.80464	17.01075	0.466953
Hentriacontane	33.36958	0.201909	-20.7134
Dotriacontene.1	38.19197	-24.5018	-2.4133
Heptacosene..check.	44.19338	0.410471	15.52211
Methyl.Nonacosane.2	51.65206	-25.6421	18.3718
Triacontane..c30.	57.69316	10.33359	-13.1201
Methyl.Pentacosane.2	83.02137	-52.8033	38.48199
Triacontene.2	93.09948	17.22878	-59.3825
Tricosane	185.6596	44.67723	-20.518
Methyl.Hexacosane.1	268.3047	219.6226	-180.019
Dimethylpentacosane..check.	309.4773	28.28725	49.36349
Octacosene.3.check.	316.5089	66.62771	-734.747

H-lineage			
Methyl.Tricosane.2	-287.385	-278.861	53.27036
Methyl.Tetracosane	-130.367	152.259	53.47755
Docosane	-81.5425	-83.055	36.50983
Methyl.Pentacosane.1	-71.6428	4.323801	-20.4149
Ester	-61.2361	26.17083	19.11181
Hentriacontene	-52.3972	33.57923	-46.0655
Ester.2	-51.8518	19.55366	29.87606
Nonacosene	-34.0491	-30.212	26.29068
Dimethylpentacosane..check.	-23.4599	0.697749	-8.97655
Heptacosene..check.	-13.1127	-0.60228	9.196205
Methyl.Heptacosane.1	-11.8774	-45.8061	-127.451
Dotriacontene.1	-11.1603	3.264402	-10.9257
Methyl.Pentacosane.2	-9.10161	-7.26435	3.404653
Octacosene.3.check.	-6.36526	20.6368	46.174
Dotriacontane	-6.29656	-32.0445	-9.48411
Hexacosane	-5.42217	-13.4212	-42.6093
Pentacosane	-4.87587	1.489623	5.761583
Nonacosane	1.48246	7.825324	6.045705
Heptacosane	1.79373	3.889106	-6.30993
Methyl.Tricosane.1	2.644433	-44.0061	-35.1398
Octacosene.2..check.	3.052143	25.13327	11.92881
Triacontane..c30.	4.78033	-48.8135	-70.7178
Tricosane	5.246214	-31.8708	-1.62607
Octacosane	9.917159	-0.28737	-19.6421
Methyl.Nonacosane.1	10.55419	-16.5713	-2.22324
Hentriacontane	13.31028	9.303892	1.547896

Pentacosene	13.90239	-7.72048	-9.74388
Methyl.Nonacosane.2	17.51086	13.9224	20.0272
Dotriacontene.2	23.99929	-1.31613	96.69905
Tetracosane	24.59267	-9.25491	33.47403
Heneicosane	26.90995	-12.8002	-8.34607
Icosane	27.03126	14.57775	-13.9524
Methyl.Heptacosane.2	54.2715	62.78296	11.00803
Methyl.Hexacosane.2	73.63173	-21.0344	-60.3701
Triacontene.1	125.3379	-100.339	30.89331
Triacontene.2	126.8885	233.8465	84.15
Methyl.Hexacosane.1	272.3984	143.3462	215.9081
Isolated vs. Queenright Workers			
Triacontene.2	-1361.99	1361.99	
Octacosene.1	-1319.78	1319.781	
Heneicosane	-985.126	985.1261	
Pentacosene	-861.515	861.5149	
Tricosane	-481.798	481.798	
Ester	-399.152	399.1519	
Methyl.Heptacosane.1	-380.153	380.1526	
Methyl.Nonacosane.2	-214.502	214.5024	
Methyl.Hexacosane.1	-167.658	167.6582	
Dotriacontene.2	-161.758	161.7578	
Methyl.Hexacosane.2	117.982	117.982	
Methyl.Tricosane.2	130.7374	130.7374	
Triacontane..c30.	298.8177	298.8177	
Methyl.Heptacosane.2	519.7709	519.7709	
Nonacosane	536.7203	536.7203	
Nonacosene	606.6819	606.6819	
Octacosene.2..check.	667.3452	667.3452	
Ester.2	832.9271	832.9271	
Methyl.Nonacosane.1	939.1593	939.1593	
Pentacosane	956.9396	956.9396	
Tetracosane	1016.402	1016.402	
Docosane	1183.476	1183.476	
Triacontene.1	1210.66	1210.66	
Octacosane	1251.908	1251.908	
Icosane	1345.155	1345.155	
Hentriacontane	1381.982	1381.982	
Heptacosene..check.	1398.279	1398.279	
Methyl.Tricosane.1	1809.863	1809.863	

Methyl.Pentacosane.2	1887.668	1887.668	
Heptacosane	2032.277	2032.277	
Methyl.Pentacosane.1	2085.843	2085.843	
Hexacosane	2303.765	2303.765	
Dotriacontene.1	2325.741	2325.741	
Dimethylpentacosane..check.	2620.824	2620.824	
Hentriacontene	3097.493	3097.493	
Octacosene.3.check.	6805.909	6805.909	
Methyl.Tetracosane	10167.32	10167.32	

Queen LDA with all Species			
Triacontene.2	-1200.46	-238.602	1439.061
Icosane	-609.101	-402.007	1011.107
Methyl.Hexacosane.1	-399.296	-372.348	771.6443
Triacontene.1	-334.475	-199.136	533.6112
QueenCompMethyl.Hexacosane.2	-141.693	-49.8363	191.5296
QueenCompMethyl.Tricosane.2	-83.2534	-117.558	200.8115
QueenCompTriacontane..c30.	-80.3969	-57.7992	138.1961
Methyl.Pentacosane.1	-70.5288	-174.693	245.2218
QueenCompMethyl.Nonacosane.2	-57.3756	-3.42876	60.80434
QueenCompOctacosane	-48.5969	-52.8128	101.4097
QueenCompNonacosene	-48.5876	54.02922	102.6168
QueenCompHentriacontane	-42.2022	4.495078	46.69729
QueenCompMethyl.Heptacosane.1	-41.9323	37.84572	79.77801
QueenCompTricosane	-17.4502	-12.0065	29.45673
QueenCompPentacosene	-13.9521	32.00791	45.95996
QueenCompNonacosane	-12.8541	7.643028	20.49709
QueenCompTetracosane	-10.2193	11.60126	21.82059
QueenCompMethyl.Nonacosane.1	-2.95197	6.877759	9.829731
QueenCompDotriacontene.1	0.336961	17.26254	17.5995
QueenCompMethyl.Pentacosane.2	3.607975	87.53395	91.14193
QueenCompPentacosane	15.759	4.460194	20.2192
QueenCompHeptacosene..check.	19.55962	8.385107	27.94472
QueenCompMethyl.Tricosane.1	21.37204	-2.53722	23.90925
QueenCompEster.2	21.69098	-14.4814	36.17238
QueenCompEster	28.06517	16.26896	44.33412
QueenCompDotriacontene.2	37.1255	45.50802	82.63352
QueenCompDocosane	50.92364	-2.20211	53.12574
QueenCompHeptacosane	64.17552	21.51302	85.68855
Octacosene.2	85.06849	150.6493	235.7178
QueenCompDimethylpentacosane..check.	94.54379	51.32012	145.8639

Heneicosane	122.5604	222.0484	344.6088
QueenCompDotriacontane	130.2261	-3.5727	133.7988
Octacosene.1	230.6552	158.5326	389.1878
Methyl.Tetracosane	358.5089	-58.1934	416.7023
Hentriacontene	408.6939	138.5871	547.2809
Octacosene.3.check.	570.9199	136.1229	707.0429

Table 3.3 : Proportional amounts of CHC compounds found in all samples used in chapter 3.

Sample_ID	Species	Caste	Clade	Parental	Icosane	Heneicosar	Ester	Docosane
H1DQ	H1	Q	Prug	Lin	0.0004	0.0008	0.0155	0.0038
H1BQ	H1	Q	Prug	Lin	0.0006	0.0014	0.0149	0.0050
H1CQ	H1	Q	Prug	Lin	0.0008	0.0013	0.0141	0.0059
H1EQ	H1	Q	Prug	Lin	0.0029	0.0052	0.0710	0.0193
H1AQ	H1	Q	Prug	Lin	0.0024	0.0003	0.0012	0.0022
H1AW	H1	W	Prug	Lin	0.0019	0.0003	0.0032	0.0049
H1EW	H1	W	Prug	Lin	0.0206	0.0175	0.0222	0.0009
H1CW	H1	W	Prug	Lin	0.0023	0.0007	0.0015	0.0008
H1DW	H1	W	Prug	Lin	0.0014	0.0008	0.0011	0.0142
H1RW	H1	W	Prug	Lin	0.0018	0.0030	0.0029	0.0143
H2DQ	H2	Q	Pbar	Lin	0.0012	0.0014	0.0146	0.0070
H2EQ	H2	Q	Pbar	Lin	0.0023	0.0040	0.0006	0.0007
H2BQ	H2	Q	Pbar	Lin	0.0007	0.0010	0.0145	0.0044
H2CQ	H2	Q	Pbar	Lin	0.0003	0.0003	0.0064	0.0023
H2AQ	H2	Q	Pbar	Lin	0.0008	0.0011	0.0135	0.0066
H2BW	H2	W	Pbar	Lin	0.0054	0.0005	0.0052	0.0064
H2EW	H2	W	Pbar	Lin	0.0078	0.0093	0.0099	0.0017
H2CW	H2	W	Pbar	Lin	0.0059	0.0006	0.0019	0.0044
H2RW	H2	W	Pbar	Lin	0.0006	0.0003	0.0026	0.0014
H2R2W	H2	W	Pbar	Lin	0.0015	0.0007	0.0024	0.0017
J1DQ	J1	Q	Prug	Lin	0.0005	0.0019	0.0247	0.0075
J1CQ	J1	Q	Prug	Lin	0.0003	0.0006	0.0064	0.0030
J1BQ	J1	Q	Prug	Lin	0.0003	0.0000	0.0046	0.0019
J1RQ	J1	Q	Prug	Lin	0.0001	0.0006	0.0003	0.0004
J1R2Q	J1	Q	Prug	Lin	0.0001	0.0002	0.0003	0.0005
J1BW	J1	W	Prug	Lin	0.0035	0.0005	0.0312	0.0045
J1CW	J1	W	Prug	Lin	0.0016	0.0011	0.0022	0.0150
J1EW	J1	W	Prug	Lin	0.0005	0.0008	0.0029	0.0027
J1AW	J1	W	Prug	Lin	0.0086	0.0023	0.0073	0.0027
J1R1W	J1	W	Prug	Lin	0.0178	0.0041	0.0105	0.0211
J2CQ	J2	Q	Pbar	Lin	0.0006	0.0036	0.0000	0.0026
J2BQ	J2	Q	Pbar	Lin	0.0039	0.0022	0.0275	0.0098
J2EQ	J2	Q	Pbar	Lin	0.0017	0.0032	0.0236	0.0198
J2AQ	J2	Q	Pbar	Lin	0.0002	0.0003	0.0052	0.0025
J2RQ	J2	Q	Pbar	Lin	0.0005	0.0003	0.0001	0.0009
J2EW	J2	W	Pbar	Lin	0.0082	0.0121	0.1610	0.0420
J2CW	J2	W	Pbar	Lin	0.0047	0.0079	0.0898	0.0320
J2BW	J2	W	Pbar	Lin	0.0040	0.0072	0.0555	0.0199
J2DW	J2	W	Pbar	Lin	0.0067	0.0102	0.1488	0.0300
J2AW	J2	W	Pbar	Lin	0.0058	0.0113	0.1180	0.0368
PbarEQ	Pb	Q	Pbar	Pb	0.0027	0.0038	0.0421	0.0162
PbarAQ	Pb	Q	Pbar	Pb	0.0025	0.0035	0.0386	0.0179
PbarDQ	Pb	Q	Pbar	Pb	0.0004	0.0009	0.0128	0.0029
PbARRQ	Pb	Q	Pbar	Pb	0.0000	0.0002	0.0001	0.0005
PbARR2Q	Pb	Q	Pbar	Pb	0.0001	0.0002	0.0008	0.0008
PbarEW	Pb	W	Pbar	Pb	0.0080	0.0124	0.1361	0.0436

PbarAW	Pb	W	Pbar	Pb	0.0031	0.0054	0.0791	0.0236
PbarCW	Pb	W	Pbar	Pb	0.0015	0.0030	0.0286	0.0068
PbarDW	Pb	W	Pbar	Pb	0.0082	0.0077	0.0664	0.0567
PbarBW	Pb	W	Pbar	Pb	0.0036	0.0057	0.0698	0.0259
PrugBQ	Pr	Q	Prug	Pr	0.0015	0.0020	0.0277	0.0083
PrugAQ	Pr	Q	Prug	Pr	0.0034	0.0058	0.0669	0.0294
PrugBQ2	Pr	Q	Prug	Pr	0.0003	0.0009	0.0087	0.0049
PrugEQ	Pr	Q	Prug	Pr	0.0010	0.0021	0.0266	0.0130
PrugDQ	Pr	Q	Prug	Pr	0.0013	0.0023	0.0304	0.0107
PrugBW	Pr	W	Prug	Pr	0.0049	0.0072	0.0957	0.0253
PrugAW	Pr	W	Prug	Pr	0.0078	0.0103	0.0880	0.0570
PrugCW	Pr	W	Prug	Pr	0.0068	0.0030	0.0111	0.0015
PrugFW	Pr	W	Prug	Pr	0.0021	0.0024	0.0240	0.0049
PrUGRW	Pr	W	Prug	Pr	0.0000	0.0017	0.0016	0.0095

Tricosane	Methyl Tric	Methyl Tric	Ester 2	Tetracosan	Methyl Tet	Pentacoser	Pentacosar	Methyl Per
0.0539	0.0023	0.0010	0.0118	0.0117	0.0019	0.0227	0.1749	0.0188
0.0349	0.0055	0.0100	0.0119	0.0154	0.0059	0.0225	0.2415	0.0626
0.0552	0.0014	0.0042	0.0107	0.0224	0.0022	0.0151	0.2294	0.0342
0.0337	0.0000	0.0000	0.0530	0.0528	0.0044	0.0130	0.1667	0.0067
0.0437	0.0000	0.0046	0.0000	0.0054	0.0005	0.0000	0.4402	0.0492
0.0601	0.0000	0.0001	0.0000	0.0143	0.0001	0.0062	0.3384	0.0137
0.0396	0.0151	0.0008	0.0042	0.0190	0.0012	0.0365	0.1883	0.0251
0.0804	0.0019	0.0014	0.0113	0.0145	0.0000	0.0443	0.2784	0.0160
0.0344	0.0051	0.0042	0.0011	0.0248	0.0077	0.1138	0.1439	0.0228
0.0393	0.0034	0.0022	0.0192	0.1007	0.0050	0.0031	0.1755	0.0047
0.0290	0.0006	0.0014	0.0109	0.0164	0.0012	0.0060	0.2461	0.0534
0.0127	0.0023	0.0019	0.0000	0.0078	0.0028	0.0041	0.3202	0.0178
0.0353	0.0004	0.0007	0.0095	0.0148	0.0006	0.0025	0.3236	0.0211
0.0350	0.0006	0.0013	0.0049	0.0122	0.0018	0.0019	0.2386	0.0262
0.0415	0.0006	0.0018	0.0100	0.0201	0.0009	0.0045	0.2887	0.0269
0.0065	0.0299	0.0032	0.0122	0.0124	0.0023	0.0219	0.2073	0.0125
0.0573	0.0100	0.0008	0.0038	0.0136	0.0001	0.0171	0.2750	0.0144
0.0371	0.0031	0.0009	0.0084	0.0108	0.0035	0.0107	0.1570	0.0282
0.0464	0.0007	0.0021	0.0051	0.0103	0.0033	0.0041	0.1666	0.0042
0.0895	0.0001	0.0012	0.0012	0.0152	0.0007	0.0051	0.3197	0.0072
0.0525	0.0010	0.0014	0.0176	0.0179	0.0006	0.0078	0.1965	0.0798
0.0317	0.0009	0.0011	0.0043	0.0138	0.0004	0.0020	0.2023	0.0438
0.0199	0.0000	0.0000	0.0001	0.0054	0.0009	0.0094	0.1795	0.0790
0.0260	0.0015	0.0006	0.0003	0.0062	0.0018	0.0100	0.1760	0.1020
0.0330	0.0013	0.0006	0.0000	0.0089	0.0008	0.0031	0.2021	0.0594
0.0718	0.0013	0.0048	0.0146	0.0414	0.0003	0.0392	0.2338	0.0516
0.0764	0.0013	0.0018	0.0016	0.0904	0.0028	0.0075	0.1670	0.0310
0.0685	0.0022	0.0050	0.0063	0.0053	0.0200	0.0154	0.2086	0.0756
0.0954	0.0066	0.0038	0.0037	0.0336	0.0011	0.0166	0.3330	0.0620
0.1016	0.0007	0.0155	0.0033	0.1072	0.0079	0.0079	0.2003	0.0007
0.0289	0.0005	0.0003	0.0129	0.0284	0.0014	0.0063	0.2307	0.0617
0.0333	0.0005	0.0006	0.0164	0.0218	0.0047	0.0051	0.2621	0.0521
0.0339	0.0000	0.0002	0.0187	0.0765	0.0041	0.0021	0.1572	0.0042
0.0181	0.0000	0.0000	0.0034	0.0116	0.0016	0.0052	0.3129	0.0431
0.0189	0.0007	0.0011	0.0001	0.0114	0.0009	0.0074	0.2172	0.0622
0.0629	0.0000	0.0008	0.1204	0.0830	0.0086	0.0091	0.1101	0.0086
0.0673	0.0000	0.0017	0.0592	0.0885	0.0076	0.0044	0.1955	0.0069
0.0516	0.0002	0.0029	0.0425	0.0659	0.0008	0.0191	0.1897	0.0202
0.0536	0.0000	0.0003	0.1125	0.0831	0.0030	0.0005	0.1099	0.0033
0.0777	0.0012	0.0018	0.0904	0.0762	0.0061	0.0109	0.1484	0.0138
0.1043	0.0011	0.0245	0.0339	0.0420	0.0042	0.0286	0.2475	0.0447
0.0670	0.0259	0.0050	0.0332	0.0344	0.0069	0.0386	0.1393	0.0823
0.1556	0.0049	0.0078	0.0053	0.0205	0.0019	0.0140	0.3738	0.0339
0.0821	0.0443	0.0074	0.0008	0.0085	0.0109	0.0330	0.1730	0.1380
0.1226	0.0281	0.0051	0.0037	0.0140	0.0070	0.0417	0.2472	0.1097
0.0652	0.0029	0.0039	0.1037	0.0799	0.0109	0.0215	0.1125	0.0094

0.2606	0.0031	0.0070	0.0266	0.0496	0.0087	0.0093	0.2538	0.0184
0.2635	0.0553	0.0053	0.0000	0.0740	0.0052	0.0171	0.2713	0.0151
0.1351	0.0008	0.0006	0.0438	0.1144	0.0023	0.0062	0.1795	0.0057
0.2245	0.0030	0.0042	0.0555	0.0526	0.0036	0.0182	0.2002	0.0130
0.0618	0.0019	0.0011	0.0160	0.0180	0.0006	0.0166	0.4149	0.0145
0.0487	0.0003	0.0003	0.0508	0.0891	0.0100	0.0014	0.2120	0.0051
0.0254	0.0006	0.0001	0.0071	0.0363	0.0000	0.0007	0.3303	0.0006
0.0681	0.0005	0.0006	0.0160	0.0403	0.0000	0.0046	0.3662	0.0046
0.0675	0.0020	0.0018	0.0233	0.0301	0.0001	0.0144	0.3492	0.0041
0.0691	0.0000	0.0006	0.0477	0.0592	0.0061	0.0035	0.3269	0.0002
0.0682	0.0000	0.0008	0.0647	0.0727	0.0106	0.0009	0.2498	0.0000
0.0399	0.0021	0.0026	0.0021	0.0090	0.0003	0.0152	0.2156	0.0072
0.0629	0.0018	0.0016	0.0103	0.0247	0.0030	0.0159	0.4477	0.0121
0.0929	0.0048	0.0209	0.0115	0.0167	0.0111	0.0926	0.2752	0.0105

Methyl Per	Dimethylpe	Hexacosan	Methyl He	Methyl He	Heptacose	Heptacosa	Methyl He	Methyl He
0.0435	0.0088	0.0113	0.0005	0.0035	0.1510	0.0502	0.0061	0.0182
0.0821	0.0154	0.0227	0.0036	0.0073	0.0721	0.1260	0.0240	0.0311
0.0560	0.0083	0.0270	0.0010	0.0042	0.0821	0.0786	0.0166	0.0295
0.0131	0.0033	0.0914	0.0000	0.0003	0.0341	0.1117	0.0028	0.0019
0.0524	0.0033	0.0596	0.0009	0.0018	0.0193	0.2119	0.0003	0.0144
0.0219	0.0035	0.0126	0.0007	0.0037	0.0010	0.0457	0.0277	0.0521
0.0318	0.0095	0.0178	0.0004	0.0063	0.0552	0.0326	0.0256	0.0537
0.0150	0.0357	0.0147	0.0024	0.0362	0.0245	0.0337	0.0189	0.0303
0.0227	0.0014	0.0220	0.0069	0.0052	0.1080	0.0307	0.0393	0.0466
0.0353	0.0199	0.0724	0.0060	0.0172	0.0029	0.0690	0.0352	0.0279
0.0282	0.0089	0.0279	0.0024	0.0061	0.0460	0.1159	0.0488	0.0581
0.0085	0.0039	0.0410	0.0012	0.0007	0.0105	0.3398	0.0101	0.0170
0.0123	0.0034	0.0277	0.0012	0.0036	0.0179	0.1574	0.0206	0.0521
0.0297	0.0052	0.0261	0.0017	0.0076	0.0243	0.1313	0.0445	0.0718
0.0228	0.0050	0.0326	0.0012	0.0047	0.0368	0.1151	0.0313	0.0468
0.0183	0.0038	0.0163	0.0063	0.0031	0.0210	0.0450	0.0303	0.0304
0.0186	0.0084	0.0166	0.0060	0.0035	0.0413	0.0367	0.0325	0.0534
0.0382	0.0069	0.0146	0.0043	0.0057	0.0628	0.0258	0.0437	0.0523
0.0243	0.0398	0.0072	0.0016	0.0062	0.0603	0.0629	0.0039	0.0144
0.0034	0.0179	0.0192	0.0012	0.0049	0.0485	0.0366	0.0297	0.0547
0.0221	0.0098	0.0189	0.0022	0.0037	0.0005	0.1203	0.0499	0.0503
0.0214	0.0053	0.0361	0.0006	0.0267	0.0331	0.3206	0.0132	0.0126
0.0246	0.0096	0.0127	0.0034	0.0018	0.1041	0.1137	0.0395	0.0440
0.0294	0.0148	0.0111	0.0036	0.0040	0.1306	0.1012	0.0428	0.0493
0.0235	0.0070	0.0291	0.0020	0.0021	0.0436	0.3062	0.0323	0.0219
0.0172	0.0119	0.0480	0.0025	0.0139	0.0131	0.0692	0.0344	0.0355
0.0074	0.0091	0.0983	0.0045	0.0024	0.0104	0.0937	0.0330	0.0281
0.0156	0.0101	0.0201	0.0055	0.0032	0.0193	0.0317	0.0874	0.0900
0.0083	0.0088	0.0234	0.0037	0.0013	0.0205	0.0477	0.0525	0.0634
0.0348	0.0059	0.0964	0.0030	0.0031	0.0087	0.0927	0.0253	0.0203
0.0145	0.0101	0.0294	0.0062	0.0028	0.0155	0.0682	0.1284	0.0801
0.0091	0.0071	0.0280	0.0020	0.0005	0.0062	0.0507	0.0319	0.0234
0.0008	0.0009	0.1284	0.0005	0.0001	0.0014	0.1403	0.0068	0.0018
0.0062	0.0068	0.0266	0.0044	0.0021	0.0131	0.1363	0.0556	0.0318
0.0065	0.0099	0.0157	0.0068	0.0033	0.0174	0.0578	0.1351	0.0751
0.0041	0.0035	0.0883	0.0008	0.0048	0.0046	0.0650	0.0086	0.0080
0.0016	0.0021	0.1056	0.0001	0.0029	0.0032	0.0837	0.0075	0.0091
0.0047	0.0041	0.1022	0.0006	0.0029	0.0046	0.0995	0.0112	0.0057
0.0023	0.0019	0.1073	0.0001	0.0010	0.0018	0.0820	0.0028	0.0014
0.0060	0.0041	0.0726	0.0013	0.0066	0.0077	0.0630	0.0166	0.0171
0.0000	0.0106	0.0572	0.0049	0.0022	0.0825	0.0982	0.0200	0.0014
0.0123	0.0289	0.0381	0.0127	0.0007	0.1939	0.0647	0.0560	0.0064
0.0079	0.0021	0.0242	0.0051	0.0011	0.1147	0.1345	0.0301	0.0038
0.0130	0.0328	0.0123	0.0023	0.0194	0.1802	0.0727	0.0992	0.0054
0.0135	0.0057	0.0163	0.0125	0.0003	0.1415	0.0847	0.0858	0.0065
0.0063	0.0042	0.0904	0.0010	0.0047	0.0447	0.0656	0.0065	0.0000

0.0038	0.0029	0.0466	0.0024	0.0022	0.0665	0.0471	0.0026	0.0017
0.0012	0.0000	0.0433	0.0018	0.0027	0.0750	0.0377	0.0108	0.0000
0.0044	0.0034	0.0937	0.0000	0.0000	0.0239	0.0765	0.0049	0.0032
0.0133	0.0034	0.0453	0.0032	0.0003	0.1331	0.0410	0.0065	0.0012
0.0087	0.0064	0.0225	0.0004	0.0001	0.0493	0.1142	0.0046	0.0039
0.0008	0.0019	0.0657	0.0002	0.0015	0.0182	0.1432	0.0028	0.0021
0.0002	0.0004	0.0853	0.0000	0.0000	0.0127	0.2002	0.0009	0.0002
0.0025	0.0044	0.0630	0.0011	0.0000	0.0241	0.1912	0.0018	0.0001
0.0017	0.0060	0.0480	0.0002	0.0001	0.0423	0.1873	0.0022	0.0036
0.0006	0.0015	0.0532	0.0001	0.0023	0.0026	0.0596	0.0001	0.0002
0.0000	0.0018	0.0772	0.0004	0.0021	0.0022	0.0674	0.0000	0.0000
0.0064	0.0266	0.0169	0.0085	0.0113	0.0031	0.0127	0.0048	0.0029
0.0071	0.0051	0.0218	0.0003	0.0002	0.0480	0.1152	0.0044	0.0052
0.0187	0.0093	0.0405	0.0084	0.0011	0.0457	0.0563	0.0014	0.0007

0.0003	0.0018	0.0000	0.0264	0.0049	0.0160	0.0000	0.0000	0.0001
0.0000	0.0000	0.0001	0.0244	0.0032	0.0132	0.0000	0.0000	0.0002
0.0050	0.0034	0.0007	0.0564	0.0113	0.0312	0.0003	0.0004	0.0003
0.0003	0.0001	0.0001	0.0263	0.0065	0.0146	0.0001	0.0003	0.0001
0.0008	0.0019	0.0000	0.0031	0.0162	0.0243	0.0494	0.0000	0.0157
0.0014	0.0026	0.0001	0.0413	0.0121	0.0488	0.0182	0.0038	0.0020
0.0010	0.0015	0.0001	0.0665	0.0022	0.0679	0.0074	0.0077	0.0018
0.0037	0.0015	0.0043	0.0263	0.0045	0.0465	0.0153	0.0033	0.0056
0.0013	0.0034	0.0003	0.0176	0.0107	0.0380	0.0287	0.0109	0.0034
0.0016	0.0011	0.0003	0.0341	0.0053	0.0236	0.0283	0.0305	0.0071
0.0017	0.0013	0.0000	0.0512	0.0043	0.0297	0.0258	0.0033	0.0027
0.0026	0.0140	0.0037	0.0145	0.0078	0.1186	0.0397	0.0050	0.0083
0.0013	0.0020	0.0000	0.0036	0.0110	0.0195	0.0454	0.0015	0.0172
0.0014	0.0018	0.0008	0.0145	0.0166	0.0172	0.0664	0.0007	0.0016

Triacontene	Triacontane	Hentriacon	Hentriacon	Dotriacont	Dotriacont	Dotriacontane
0.0060	0.0053	0.0563	0.0071	0.0984	0.0080	0.0050
0.0071	0.0051	0.0099	0.0053	0.0199	0.0092	0.0026
0.0074	0.0126	0.0162	0.0128	0.0371	0.0104	0.0082
0.0060	0.0418	0.0055	0.0339	0.0113	0.0038	0.0249
0.0002	0.0000	0.0001	0.0000	0.0001	0.0002	0.0002
0.0049	0.0169	0.0105	0.0123	0.1049	0.0192	0.0032
0.0030	0.0074	0.0272	0.0097	0.0576	0.0201	0.0080
0.0009	0.0000	0.0065	0.0697	0.0720	0.0117	0.0014
0.0058	0.0134	0.0075	0.0738	0.0197	0.0005	0.0005
0.0080	0.0142	0.0005	0.0009	0.0071	0.0056	0.0648
0.0157	0.0039	0.0067	0.0147	0.0266	0.0164	0.0133
0.0045	0.0070	0.0001	0.0325	0.0033	0.0049	0.0046
0.0046	0.0046	0.0046	0.0304	0.0216	0.0103	0.0022
0.0063	0.0050	0.0208	0.0185	0.0481	0.0116	0.0065
0.0029	0.0051	0.0112	0.0130	0.0288	0.0117	0.0145
0.0048	0.0002	0.0176	0.0057	0.0704	0.0108	0.0031
0.0065	0.0025	0.0132	0.0001	0.0844	0.0167	0.0041
0.0109	0.0001	0.0230	0.0031	0.0970	0.0357	0.0141
0.0072	0.0195	0.0025	0.0891	0.0000	0.0064	0.0417
0.0033	0.0000	0.0005	0.0845	0.0000	0.0011	0.0071
0.0086	0.0057	0.0254	0.0179	0.0345	0.0178	0.0028
0.0034	0.0086	0.0095	0.0226	0.0172	0.0090	0.0035
0.0185	0.0074	0.0199	0.0126	0.0447	0.0350	0.0065
0.0065	0.0108	0.0081	0.0542	0.0000	0.0358	0.0358
0.0049	0.0060	0.0005	0.0204	0.0023	0.0043	0.0143
0.0069	0.0170	0.0080	0.0301	0.0367	0.0097	0.0127
0.0078	0.0360	0.0045	0.0366	0.0141	0.0142	0.0227
0.0139	0.0039	0.0158	0.0701	0.0058	0.0082	0.0029
0.0056	0.0033	0.0130	0.0424	0.0053	0.0001	0.0027
0.0054	0.0247	0.0050	0.0199	0.0083	0.0017	0.0117
0.0001	0.0074	0.0001	0.0233	0.0231	0.0233	0.0557
0.0335	0.0083	0.0162	0.0427	0.0406	0.0182	0.0041
0.0055	0.0677	0.0015	0.0539	0.0030	0.0021	0.0398
0.0329	0.0063	0.0002	0.0503	0.0132	0.0119	0.0039
0.0096	0.0341	0.0270	0.0531	0.0056	0.0123	0.0098
0.0037	0.0259	0.0001	0.0171	0.0055	0.0072	0.0144
0.0016	0.0344	0.0023	0.0268	0.0064	0.0030	0.0211
0.0001	0.0467	0.0002	0.0394	0.0039	0.0001	0.0270
0.0014	0.0433	0.0011	0.0264	0.0041	0.0010	0.0247
0.0016	0.0285	0.0035	0.0167	0.0151	0.0066	0.0166
0.0009	0.0187	0.0016	0.0087	0.0041	0.0006	0.0114
0.0004	0.0100	0.0025	0.0041	0.0005	0.0007	0.0051
0.0000	0.0014	0.0031	0.0000	0.0021	0.0018	0.0010
0.0000	0.0001	0.0003	0.0053	0.0010	0.0006	0.0013
0.0000	0.0002	0.0001	0.0038	0.0002	0.0005	0.0033
0.0017	0.0306	0.0000	0.0161	0.0001	0.0001	0.0175

0.0000	0.0119	0.0020	0.0054	0.0000	0.0001	0.0069
0.0003	0.0118	0.0088	0.0109	0.0004	0.0002	0.0074
0.0004	0.0240	0.0002	0.0138	0.0002	0.0009	0.0143
0.0001	0.0111	0.0022	0.0053	0.0000	0.0001	0.0056
0.0048	0.0042	0.0196	0.0132	0.0266	0.0233	0.0019
0.0020	0.0250	0.0246	0.0234	0.0133	0.0085	0.0131
0.0019	0.0502	0.0020	0.0436	0.0031	0.0024	0.0248
0.0006	0.0149	0.0044	0.0164	0.0068	0.0057	0.0083
0.0008	0.0086	0.0077	0.0119	0.0139	0.0107	0.0036
0.0020	0.0200	0.0119	0.0189	0.0224	0.0157	0.0104
0.0014	0.0293	0.0077	0.0175	0.0170	0.0095	0.0155
0.0014	0.0214	0.0152	0.1170	0.0629	0.0439	0.1147
0.0026	0.0043	0.0048	0.0139	0.0109	0.0222	0.0189
0.0000	0.0144	0.0000	0.0386	0.0019	0.0394	0.0538

Octacosene	Octacosene	Octacosene	Octacosane	Nonacosen	Nonacosan	Methyl Noı	Methyl Noı	Triacontene
0.0018	0.0033	0.0006	0.0030	0.0472	0.0248	0.1027	0.0084	0.0096
0.0039	0.0102	0.0029	0.0065	0.0170	0.0300	0.0392	0.0057	0.0095
0.0066	0.0079	0.0013	0.0159	0.0266	0.0336	0.0788	0.0093	0.0147
0.0031	0.0020	0.0001	0.0684	0.0090	0.0588	0.0157	0.0205	0.0081
0.0004	0.0023	0.0002	0.0234	0.0038	0.0457	0.0094	0.0002	0.0000
0.0013	0.0040	0.0011	0.0020	0.0132	0.0177	0.1488	0.0202	0.0077
0.0410	0.0039	0.0003	0.0083	0.0197	0.0158	0.0963	0.0272	0.0307
0.0089	0.0052	0.0001	0.0041	0.0090	0.0140	0.1162	0.0085	0.0070
0.0144	0.0090	0.0128	0.0027	0.0120	0.0077	0.1427	0.0059	0.0136
0.0013	0.0280	0.0000	0.0493	0.0069	0.1184	0.0150	0.0147	0.0045
0.0069	0.0302	0.0042	0.0080	0.0110	0.0317	0.0390	0.0284	0.0116
0.0028	0.0085	0.0009	0.0120	0.0017	0.0906	0.0063	0.0068	0.0034
0.0011	0.0122	0.0013	0.0090	0.0106	0.0619	0.0472	0.0491	0.0038
0.0047	0.0138	0.0018	0.0080	0.0165	0.0571	0.0587	0.0437	0.0049
0.0030	0.0130	0.0017	0.0110	0.0171	0.0481	0.0664	0.0333	0.0058
0.0118	0.0091	0.0071	0.0073	0.0120	0.0234	0.1507	0.1536	0.0120
0.0140	0.0021	0.0009	0.0068	0.0191	0.0210	0.1458	0.0131	0.0118
0.0142	0.0073	0.0111	0.0074	0.0187	0.0148	0.1496	0.0431	0.0229
0.0016	0.0456	0.0041	0.0751	0.0137	0.1614	0.0379	0.0050	0.0209
0.0050	0.0069	0.0045	0.0063	0.0199	0.1574	0.0212	0.0085	0.0112
0.0037	0.0270	0.0021	0.0073	0.0228	0.0459	0.0694	0.0161	0.0053
0.0011	0.0087	0.0005	0.0152	0.0044	0.0879	0.0149	0.0105	0.0029
0.0067	0.0193	0.0018	0.0029	0.0246	0.0276	0.0853	0.0201	0.0129
0.0072	0.0258	0.0018	0.0002	0.0363	0.0254	0.0174	0.0045	0.0179
0.0038	0.0117	0.0004	0.0108	0.0041	0.0910	0.0303	0.0124	0.0048
0.0024	0.0191	0.0000	0.0282	0.0049	0.0323	0.0253	0.0183	0.0041
0.0062	0.0197	0.0013	0.0626	0.0042	0.0558	0.0142	0.0052	0.0082
0.0073	0.0415	0.0023	0.0067	0.0092	0.0096	0.0543	0.0474	0.0044
0.0042	0.0258	0.0010	0.0066	0.0103	0.0118	0.0315	0.0232	0.0070
0.0042	0.0157	0.0006	0.0553	0.0000	0.0421	0.0117	0.0028	0.0024
0.0114	0.0667	0.0055	0.0120	0.0101	0.0282	0.0001	0.0000	0.0000
0.0064	0.0476	0.0010	0.0112	0.0102	0.0554	0.0568	0.0399	0.0090
0.0053	0.0061	0.0017	0.1010	0.0016	0.0784	0.0015	0.0013	0.0037
0.0082	0.0560	0.0023	0.0068	0.0112	0.0602	0.0199	0.0217	0.0080
0.0138	0.0681	0.0035	0.0034	0.0116	0.0225	0.0520	0.0215	0.0028
0.0024	0.0071	0.0001	0.0551	0.0029	0.0313	0.0049	0.0020	0.0060
0.0030	0.0039	0.0000	0.0672	0.0027	0.0381	0.0031	0.0033	0.0018
0.0030	0.0092	0.0007	0.0767	0.0049	0.0595	0.0054	0.0044	0.0040
0.0038	0.0015	0.0007	0.0767	0.0018	0.0472	0.0000	0.0001	0.0036
0.0020	0.0100	0.0011	0.0503	0.0028	0.0335	0.0080	0.0073	0.0029
0.0075	0.0026	0.0004	0.0330	0.0098	0.0262	0.0001	0.0000	0.0020
0.0071	0.0027	0.0003	0.0188	0.0190	0.0155	0.0036	0.0002	0.0008
0.0017	0.0014	0.0014	0.0042	0.0109	0.0108	0.0014	0.0006	0.0000
0.0096	0.0062	0.0033	0.0014	0.0216	0.0056	0.0064	0.0009	0.0001
0.0051	0.0040	0.0053	0.0025	0.0172	0.0054	0.0045	0.0001	0.0002
0.0023	0.0020	0.0001	0.0583	0.0066	0.0296	0.0001	0.0002	0.0013

Table S5.1 : Map size and resolution with different number of markers.

Percent of Markers	Total Markers	Final Markers	LOD Score Used	Number of Groups	Total cM	cM/Marker average
1	258	215	3	37	3558	16.54883721
10	2336	1491	4	15	5073	3.402414487
20	4672	3014	5	18	7230	2.398805574
25	5916	2650	10	19	6154	2.322264151
30	7008	4495	6	17	9032	2.009343715
40	9464	5919	5	16	10801	1.824801487
50	13315	7490	6	17	12752	1.702536716
60	14016	9041	6	18	13064	1.444972901
70	16352	10512	6	17	16143	1.535673516
80	18688	11884	7	19	17577	1.479047459
90	21024	13298	9	20	16532	1.243194465
100	23361	11944	10	16	29118	2.437876758