Proximate and Ultimate Mechanisms of

Nestmate Recognition in Ants

by

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ABSTRACT

The most abundantly studied societies, with the exception of humans, are those of the eusocial insects, which include all ants. Eusocial insect societies are typically composed of many dozens to millions of individuals, referred to as nestmates, which require some form of communication to maintain colony cohesion and coordinate the activities within them. Nestmate recognition is the process of distinguishing between nestmates and non-nestmates, and embodies the first line of defense for social insect colonies. In ants, nestmate recognition is widely thought to occur through olfactory cues found on the exterior surfaces of individuals. These cues, called cuticular hydrocarbons (CHCs), comprise the overwhelming majority of ant nestmate profiles and help maintain colony identity. In this dissertation, I investigate how nestmate recognition is influenced by evolutionary, ontogenetic, and environmental factors. First, I contributed to the sequencing and description of three ant genomes including the red harvester ant, *Pogonomyrmex barbatus*, presented in detail here. Next, I studied how variation in nestmate cues may be shaped through evolution by comparatively studying a family of genes involved in fatty acid and hydrocarbon biosynthesis, i.e., the acyl-CoA desaturases, across seven ant species in comparison with other social and solitary insects. Then, I tested how genetic, developmental, and social factors influence CHC profile variation in *P. barbatus*, through a three-part study. (1) I conducted a descriptive, correlative study of desaturase gene expression and CHC variation in *P. barbatus* workers and queens; (2) I explored how larger-scale genetic variation in the *P. barbatus* species complex influences CHC variation across two genetically isolated lineages (J1/J2 genetic caste determining lineages); and (3) I experimentally examined how CHC development is

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influenced by an individual's social environment. In the final part of my work, I resolved discrepancies between previous findings of nestmate recognition behavior in *P. barbatus* by studying how factors of territorial experience, i.e., spatiotemporal relationships, affect aggressive behaviors among red harvester ant colonies. Through this research, I was able to identify promising methodological approaches and candidate genes, which both broadens our understanding of *P. barbatus* nestmate recognition systems and supports future functional genetic studies of CHCs in ants.

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CHAPTER 1

INTRODUCTION

Eusociality, Chemical Communication, and Nestmate Recognition

The most abundantly studied societies (with the exception of humans) are those of the highly social, or 'eusocial,' insects, which include all ants and termites, as well as some bees and wasps. Eusociality is defined by three qualities (1) concentration of reproductive functions to a limited number of group members, (2) overlap between adult generations within a group such that parents receive some form of support from offspring, and (3) cooperation between non-reproductive or less reproductive group members to care for young and contribute to other labor (Wilson 1971). Given these conditions, the societies of eusocial insects require some form of communication to maintain group (i.e., colony) cohesion, identify roles of reproductive and non-reproductive members, and coordinate activities between them. In eusocial insects, communication systems take on many forms including: visual, tactile, auditory, vibrational, and chemical.

Social communication occurs when the actions of one organism affects the probability pattern of behavior in another, conspecific organism in an adaptive manner (Wilson 1975). As such, insect societies must efficiently coordinate communication signals in space and time so that they can effectively exploit and defend the resources necessary for colony growth and reproduction. Ants, in particular, are known for their highly developed systems of communication, which are significantly mediated through chemical signals and cues, i.e., pheromones (Hölldobler 1978). Similar to many solitary insects, the pheromones of ants can function as releasers of alarm behavior, sexual attraction, and recruitment. Importantly, however, because of their eusocial lifestyle ants

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additionally use these chemical signals and cues to maintain colony organization and identity.

The capacity to identify in-groups and out-groups is a precondition of sociality that necessarily entails the operation of a complex communication system. Eusocial insects ostensibly distinguish between species (e.g., predators and prey, friends and foes), potential mates (e.g., populations, genetic lineages), kin, colonies, castes, and individuals. Furthermore, communication among most eusocial insects (with the exception of unicolonial species) implies a further layer of complexity insofar as some form of colony recognition is necessary to deter members of nearby colonies from entering and exploiting neighbor colonies (Hölldobler & Wilson 1990). This phenomenon, commonly referred to as nestmate recognition in eusocial insects, allows nestmates to collectively establish a territory from which they acquire resources vital for colony survival and reproduction, by competing with and effectively excluding non-nestmates from inter- and intra-specific colonies.

The Regulation of Nestmate Recognition

Social communication and nestmate recognition in eusocial insects is considered an essential component of the "extended phenotype" of a colony (Dawkins 1982). Eusocial insect colonies must compete for resources, and their ability to compete, which partly depends on their ability to recognize and communicate with colony members, has some genetic basis. Success in resource competition means finding the right balance between maximizing a colony's resource acquisition through optimal foraging strategies, and minimizing resource exploitation by non-nestmates through defense of territories. Social

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insect colonies that employ the most economical combination of these strategies will ensure the best success in maximizing their reproductive output potential, and therefore the inclusive fitness of all colony members. This results in passing down some part of the colony's nestmate recognition and communication (i.e., "extended") phenotype through the colony-level genotype (Hölldobler 1999; Reeve & Hölldobler 2007).

Recognition may occur relatively quickly, using a variety of cues, expressed under a range of conditions, including exchanges between both inter- and intra-specific individuals (Crozier & Pamilo 1996). The predominant medium of recognition cues in eusocial insects is thought to be chemical substances; indeed, eusocial insects have been described as "walking chemical factories" precisely because they synthesize such a wide range of compounds for social communication and other functions (Hölldobler & Wilson 1990). The prevailing nestmate recognition model proposes that eusocial insects perceive recognition cues through a neuronal template matching system in which the detected cue is compared with an internal colony odor template, and unfamiliar cues are responded to differently than familiar ones (Lacy & Sherman 1983; Crozier 1987; Breed 1998; Lenoir *et al*. 1999; Starks 2004; Loenhardt *et al*. 2007). Once an individual encounters an unfamiliar recognition cue, they then react to that cue, most visibly, in the form of an overt, often agonistic, behavior; however, some subtler behavioral reactions can also occur that may initially go unnoticed to human observers (Breed 2003). Furthermore, these behavioral responses may vary according to the circumstances under which a cue is encountered (Reeve 1989; Buczkowski & Silverman 2005; Tanner & Adler 2009; Bos *et al*. 2010). When the recognition process functions adaptively, nestmates are accepted and allowed to move freely within a colony and its territory, engaging in behaviors such as

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foraging, brood care, trophallaxis, allogrooming, etc. By contrast, non-nestmates are rejected, and subject to behaviors including: avoidance, prolonged antennation, threat displays, physical removal, or attacking behaviors such as biting, stinging, and excreting defense chemicals (Wilson 1971). At stake, is the potential life or death of the superorganism.

Territoriality and Defense Behavior

Usually the worker caste carries out defense of colony territories. Many insect societies have unique castes for this purpose, which may be morphologically and/or behaviorally distinct, e.g., 'soldiers' in termites and some ants, 'guards' in many social bees and wasps, and 'patrollers' in harvester ants (Wilson 1971; Gordon 1987). These castes help occupy and maintain territorial boundaries through a combination of aggressive and passive defense methods (Wilson 1971). In ants, the methods and intensity of defense may vary widely between species and context: intruder type (heterospecific, conspecific, etc.), habitat, resource availability, season, colony density, colony age and size, and reproductive status. As a result, the ways in which ants defend their colony territories can range from relatively passive, as seen in crazy ants, *Paratrechina longicornis*, who primarily avoid confrontations when faced with intruders, to the overtly aggressive behaviors observed in numerous ant species when attacking intruders and competitors; in a dramatic example, it is not uncommon for pavement ants, *Tertamorium caespitum*, to leave hundreds or thousands of individuals dead in the wake of territorial battles (Hölldobler & Wilson 1990). As a strategy for avoiding such mutually harmful confrontations, some ants are also thought to use chemical cues to demarcate territory, as

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observed in the chemically marked spaces surrounding some *Pogonomyrmex* (Hölldobler 1976; Gordon 1984; Sturgis *et al.* 2011), *Oecophylla* (Hölldobler & Wilson 1977), *Messor* (Grasso *et al.* 2005), and *Lasius* (Lenoir *et al.* 2009) territories. The primary form of territorial and nest defense, however, is the ants' ability to recognize and respond to potential inter- and intra-specific intruders.

Nestmate Recognition Cues

Over the past few decades, with advances in chemical detection and analyses, biologists have come to a consensus that communication among eusocial insects (and particularly ants) is predominantly olfactory in nature (Hölldobler 1999). Furthermore, colony recognition in ants is widely thought to occur through chemical cues found on the bodies of individuals. These cues, called cuticular hydrocarbons (CHCs) (Lahav *et al.* 1999; Wagner *et al.* 2000; Akino *et al.* 2004; Ozaki *et al.* 2005; Martin *et al.* 2008), may also coincide with other organic compounds such as aldehydes, alcohols, fatty acids, ketones, and esters (reviewed by Richard & Hunt 2013). However, CHCs are distinctly associated with colony recognition insofar as these compounds comprise the overwhelming majority of cuticular profiles (reviewed by Martin & Drijfhout 2009). Colony recognition cues have been shown to be complex mixtures of the intrinsic chemical profiles of all workers (Hölldobler & Michener 1980; van Zweden *et al.* 2009), the queen (Carlin & Hölldobler 1986; Liebig *et al.* 2000), and the environment (Liang & Silverman 2000; Tissot *et al.* 2001, Wagner *et al.* 2001). The result of the combination of different cues is a gestalt odor that is generally thought to be unique from one colony to another (Crozier & Dix 1979).

Insect CHCs are synthesized in specialized cells called oenocytes during adult developmental stages (reviewed by Martins & Ramalho-Ortigão 2012). After biosynthesis, the CHCs are then transported from oenocytes via hemolymph lipophorins, and delivered to the cuticular surface (reviewed by Schal *et al*. 1998), or in the case of ants, transported into the postpharyngeal gland for storage and distribution (Soroker *et al*. 1994; Blomquist & Vogt 2003; Lucas *et al*. 2004). The gestalt colony odor arises, in part, from the combined contribution of each individual's innately developed CHCs. Individual ants may distribute CHCs during selfgrooming, allogrooming, trophallaxis, and direct body contact (Soroker *et al*. 1995; Lahav *et al*. 1998; Dahbi *et al*. 1999; Hefetz *et al.* 2001; Ichinose & Lenoir 2009). Each of these functions help to generate a homogenized colony wide recognition cue.

To date, nearly 1,000 CHC compounds including: *n*-alkanes, methylalkanes, alkenes and, to a lesser extent, methylalkenes have been identified across 78 ant species studied (Martin & Drijfhout 2009). Ant CHC compounds are typically odd-numbered and 19-35 carbons in length; however, several studies have found evidence of ants with longer-chain CHCs in the C_{37}-C_{47} range (Nelson *et al*. 2001; Akino 2006). The preponderance of CHCs documented on insect cuticles, including ants, are *n*-alkanes and mono-methylalkanes (Martin & Drijfhout 2009) (Table 1.1).

Given their ubiquity in insects, hydrophobic properties, and their flexible, solid-liquid phase transition state (Gibbs 1995; Gibbs & Pomonis 1995), cuticular *n*-alkanes and mono-methylalkanes are most often associated with the function of maintaining water balance (Gibbs 1998). Ants (and many other insects), however, show an abundance of other CHC compound-classes – e.g., di-, tri-, and tetra- methylalkanes as well as

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mono-, di- and tri- alkenes – and evidence suggests that this otherwise unexplained complexity is important for facilitating the recognition systems of eusocial insects (Lucas *et al.* 2005; Dani *et al.* 2001; Meskali *et al.* 1995; Martin *et al.* 2008, van Wilgenburg *et al.* 2011; but see Greene & Gordon 2003; Greene & Gordon 2007). Yet despite such detailed studies into the roles of CHCs in eusocial insect chemical communication, little is known about genetic basis of CHCs and the genetic architecture of colony recognition molecules. What is known, however, indicates a strong correlation between the genetic relatedness of individuals and their CHC profile similarity (Espelie *et al.* 1994; Vander Meer & Morel 1998; Vasquez *et al.* 2009; Drescher *et al.* 2010; Nehring *et al.* 2011).

Cuticular Hydrocarbon Variation

Generally, CHC compounds vary qualitatively between ant species, with some degree of interspecific overlap in the types of compounds present. Some of the 'simplest' ant CHC profiles identified to date have been reported in studies of *Formica exsecta* and *Formica hayashi*, both of which possess only 9 types of CHCs in some populations (Akino *et al.* 2002; Martin *et al.* 2008), while many ant species show more complex CHC profiles and typically possess 50-60 CHC compounds (Martin & Drijfhout 2009). When comparing samples from within a species, however, variation is mainly quantitative (Martin & Drijfhout 2009). Accordingly, CHC variation among caste groups at the colony level usually demonstrate a difference of degree (quantity), rather than kind (quality). For example, reproductive castes may exhibit higher quantities of certain CHCs that are otherwise also present in other castes, albeit in smaller quantities (Monnin *et al.* 1998; Liebig *et al.* 2000, Denis *et a*l. 2006; Lommelen *et al.* 2006; Smith *et al.* 2009).

Considered from the standpoint of individual development (ontogeny), however, CHCs follow a slightly different pattern wherein different developmental stages may be characterized by qualitatively distinctive profiles. For example, young callows register low quantities of CHCs, in general, and occasionally exhibit callow specific compounds (Dahbi *et al*. 1998; Ichinose & Lenoir 2009). Gradually, however, the individual callow proceeds to maturity and develops or acquires a CHC profile that is concurrent with the given species, colony, and caste (Ichinose & Lenoir 2009). Furthermore, colony-level development may also contribute to CHC variation such that smaller (typically younger) colonies can be more strongly influenced by the queen's (or queens') profile(s) (Carlin & Hölldobler 1983, 1986; but see Crosland 1990; Lahav *et al*. 1998), or colony CHC profiles may change as colonies grow larger (Endler *et al*. 2006), and in the case of colony fissions, queen loss can also affect colony CHC profiles (Ichinose *et al*. 2009).

Another dimension of eusocial insect CHC variation comes from genetic variation within and between colonies. For example, intra-colonial genetic variation can result when queens have mated with multiple males (i.e., polyandry), or when multiple queens live in colonies together (i.e., polygyny), and the resultant workers produced from different patrilines/matrilines show CHC differences that strongly correlate with their respective parentage (Page *et al*. 1991; Arnold *et al*. 1996; Boomsma *et al*. 2003; Dani *et al*. 2004; Nehring *et al*. 2011; van Zweden *et al*. 2011). Furthermore, comparisons between colonies with high genetic relatedness (e.g., monoandrous/monogynous) show more similar CHC patterns than comparisons between colonies with low relatedness (e.g., polyandrous and/or polygynous) (Vander Meer & Morel 1998; Vasquez *et al*. 2009; van Zweden *et al*. 2011). Findings such as these, although not directly connected to the roles

of specific genes, show the significant influence of genetic variability on nestmate recognition cues.

Finally, a large variety of other life history and environmental factors may also contribute to CHC variation. Colony CHC patterns can vary with season (Nielsen *et al.* 1999; Liu *et al.* 2001), temporal differences (Vander Meer *et al.* 1989), nesting conditions (Heinze *et al.* 1996; Tissot *et al.* 2001), and food sources (Liang & Silverman 2000; Richard *et al.* 2004). Additional factors may include microbial endosymbionts, which have been demonstrated to affect nestmate recognition behaviors in termites (Matsuura 2001), and larger scale biological symbioses such as insect mutualisms and social parasitisms. The specific influences of symbiotic organisms on eusocial insect CHC patterns, however, are not yet known. Consequently, when attempting to uncover the genetic bases of CHC variation, it is important to consider and/or control for these and other factors, which can have confounding effects on eusocial insect CHC patterns.

The Red Harvester Ant, *Pogonomyrmex barbatus*

In identifying a model system to study the evolution of eusocial insect nestmate recognition I first consider a number of features, i.e., measurable variation in nestmate recognition behavior, a good foundational understanding of the recognition cues, and the availability of genetic tools. Given this, my research on nestmate recognition primarily uses *Pogonomyrmex barbatus,* a species of harvester ant distributed throughout the desert-southwest of United States and Mexico. Aside from its accessibility throughout Arizona and my ability to rear colonies in the laboratory, *P. barbatus* makes an ideal study system of nestmate recognition for several reasons. First, *P. barbatus* is highly
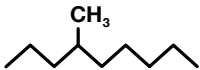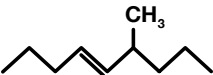
aggressive and territorial between neighboring colonies along foraging trails (Hölldobler 1974), indicating strong selection for an effective means of recognizing and maintaining colony identity and boundaries. Second, CHCs in this species have been documented as influencing both colony recognition and task-group differentiation (Wagner *et al*. 1998, Wagner *et al*. 2000). Third, the recently completed genome for *P. barbatus* (Smith *et al*. 2011b) as well as six other ant species comprising four sub-families (Bonasio *et al*. 2010; Nygaard *et al*. 2011; Smith *et al*. 2011a; Suen *et al*. 2011; Wurm *et al.* 2011) provide the necessary and heretofore unavailable tools for exploring the evolution and genetic basis of CHCs in eusocial insects.

Although certain key features of nestmate recognition have already been identified in *P. barbatus* (i.e., the role of CHCs, colony aggression and territoriality, as previously noted), several important attributes have yet to be examined, including: genetic bases for CHC variation, CHC ontogeny, as well as the effects of intercolony dynamics and prior experience on aggressive behavior and nestmate recognition. Given this, my work offers a better understanding of the nestmate recognition system in *P. barbatus*, and helps identify methodological approaches and candidate desaturase genes involved in CHC alkene biosynthesis; how these genes are evolving, and how they affect nestmate recognition cues are also questions I investigated in this dissertation. My research, presented below, provides a better understanding of nestmate recognition in *P. barbatus* using a combination of approaches and techniques, namely: comparative genomics, genetics, developmental, chemical, and behavioral ecology.

Overview

In this dissertation, I researched how nestmate recognition is influenced by evolutionary, genetic, ontogenetic, and behavioral factors, with a special focus on the red harvester ant, *Pogonomyrmex barbatus*. As a first step to investigating evolutionary and genetic components of nestmate recognition, I present a detailed description of the sequenced genome of *P. barbatus* (Chapter 2). Next, I studied how variation in nestmate cues may be shaped through evolution by comparatively studying a family of genes involved in fatty acid and hydrocarbon biosynthesis. I investigated these genes, the acyl-CoA desaturases, across seven ant species in comparison with other social and solitary insects (Chapter 3). After identifying over 170 CHC-related candidate genes in ants, I then tested how genetic, developmental, and social factors influence CHC profile variation in *P. barbatus*, through a three-part study. In this research I explored: (1) how genetic variation in the *P. barbatus* species complex influences CHC variation across two genetically isolated lineages (i.e., J1 and J2 genetic caste determining lineages); (2) how desaturase gene expression correlates with CHC variation in *P. barbatus* workers and queens; and (3) how CHC profile development is influenced by an individual's social environment (Chapter 4). In the final part of my work, I resolved discrepancies between previous findings of nestmate recognition behavior in *P. barbatus* by studying how factors of territorial experience, i.e., spatiotemporal relationships such as colony proximity and seasonality, affect aggressive behaviors among red harvester ant colonies (Chapter 5). In my final chapter (6), I summarize how this work broadens our understanding of *P. barbatus* nestmate recognition systems and supports future functional genetic studies of CHCs in ants.

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Table 1.1. The classes, structures, numbers, and frequencies (% distribution) of cuticular hydrocarbons found in 78 ant species reviewed to date (modified from Martin & Drijfhout 2009).

Name	Chemical Structure	Number of Compounds	Frequency
n-Alkanes		23	97%
Methylalkanes			
mono-		194	96%
di-	CH_3	602	84%
tri-		48	22%
tetra-		4	1%
Alkenes			
mono-		47	73%
di-		51	27%
tri-		1	1%
Methylalkenes	CH_3		
mono-		17	4%
di-		6	1%
Totals		993	

CHAPTER 2

A DRAFT GENOME OF THE RED HARVESTER ANT, *POGONOMYRMEX*

BARBATUS: A MODEL FOR REPRODUCTIVE DIVISION OF LABOR AND

SOCIAL COMPLEXITY

Abstract

We report the draft genome sequence of the red harvester ant, *Pogonomyrmex barbatus*. The genome was sequenced using 454 pyrosequencing, and the current assembly and annotation were completed in less than one year and for under $100,000. Analyses of conserved gene groups (more than 1200 manually annotated genes to date) suggest a high quality assembly and annotation comparable to recently sequenced insect genomes using Sanger sequencing. The red harvester ant is a model for studying reproductive division of labor, phenotypic plasticity, and sociogenomics. Although the genome of *P. barbatus* is similar to other sequenced hymenopterans (*Apis mellifera* and *Nasonia vitripennis*) in GC content and compositional organization, and possesses a complete CpG methylation toolkit, its predicted genomic CpG content differs markedly from the other hymenopterans. Gene networks involved in generating key differences between the queen and worker castes (e.g., wings and ovaries) show signatures of increased methylation and suggest that ants and bees may have independently co-opted the same gene regulatory mechanisms for reproductive division of labor. Gene family expansions (e.g., 343 functional odorant receptors) and pseudogene accumulation in chemoreception and P450 genes compared to *A. mellifera* and *N. vitripennis* are consistent with major life history changes during the adaptive radiation of *Pogonomyrmex* spp., perhaps in parallel with the development of the North American deserts. Future comparisons with other ant and

social insect genomes will provide insights into novel and shared molecular mechanisms for the evolution of sociality, as well as ecological adaptation.

Introduction

The formation of higher-level organization from independently functioning elements has resulted in some of the most significant transitions in biological evolution (Maynard Smith & Szathmáry 1995). These include the transition from prokaryotes to eukaryotes and from uni- to multicellular organisms, as well as the formation of complex animal societies with sophisticated division of labor among individuals. In eusocial insects such as ants, distinct morphological castes specialize in either reproduction or labor (Hölldobler & Wilson 1990). Currently, very little is known of the genetic basis of caste and reproductive division of labor in these societies, where individuals follow different developmental trajectories, much like distinct cell lines in an organism (Smith *et al*. 2008). The resulting phenotypes, queens and workers, can differ greatly in morphology, physiology and behavior, as well as having orders of magnitude differences in lifespan and reproductive potential (Hölldobler & Wilson 1990). Ants, of all social insects, arguably exhibit the highest diversity in social complexity, such as queen number, mating frequency, and the degree of complexity of division of labor (Hölldobler & Wilson 1990), and most social traits have independent origins within the ants, making them well suited to comparative genomic analyses.

The sequencing of the honey bee (*Apis mellifera*) genome marked a milestone in sociogenomics (Robinson *et al*. 2005; HBGSC 2006), facilitating research on the evolution and maintenance of sociality from its molecular building blocks. Since then,

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genomes of three closely related species of solitary parasitic hymenopterans, *Nasonia* spp., were published and similarities and differences were extensively discussed in the context of the evolution of eusociality (Werren *et al.* 2012). However, *A. mellifera* represents only one of at least 10 independent evolutionary origins of eusociality within the order Hymenoptera (Hines *et al.* 2007; Brady *et al.* 2006a; Brady *et al.* 2006b; Schwarz *et al.* 2007; Moreau *et al.* 2006), and thus it remains unclear whether differences between the honey bee and *Nasonia* spp. truly reflect differences inherent to sociality. With at least six ant genomes on the horizon (Smith *et al.* 2010), among other solitary and social insects, sociogenomic comparisons are likely to yield exciting new insights into the common molecular basis for the social lifestyle. Ant genomics will also allow us to gain a better understanding of variation in social organization, elaborate variations of physical and behavioral division of labor, invasion biology, and convergent evolution of life histories and diets. It also remains a major question whether there are many evolutionary routes to eusociality, especially at the molecular level, or whether we can extract generalities and rules for the molecular evolution of eusociality (Smith *et al.* 2008; Robinson *et al.* 2005; Toth & Robinson 2007). While it is likely that much variation in social structure is due to changes in the regulation of conserved pathways, it is undetermined what, if any, role novel genes or pathways have played in the solitary to social transition and diversification of social phenotypes (Page & Amdam 2007).

The genus *Pogonomyrmex* contains species that vary greatly in social organization (Johnson 2000), is among the best studied of ant genera (Taber 1998, Gordon 1999), is sister to almost all other genera in the diverse subfamily Myrmicinae (Brady *et al.* 2006a, Moreau *et al.* 2006), and contains species of major ecological

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importance as granivores in both North and South America (Pirk *et al*. 2006, MacMahon *et al*. 2000). Colonies can contain over ten thousand workers, and a single multiply-mated queen that may live for decades. Some *P. barbatus* populations have a unique system of genetic queen-worker caste determination (Figure 2.1) where individuals are essentially hard-wired to develop as either queens or workers compared to environmentally determined diphenism (Anderson *et al*. 2008; Helms Cahan *et al*. 2002; Julian *et al*. 2002; Cahan *et al*. 2004; SI Appendix, Chapter 1). As a consequence, individuals can be genotyped using genetic markers to determine their caste even prior to caste differentiation. This unique system of caste determination provides a means of studying the genes and regulatory networks employed in caste determination.

The goals of this paper are to 1) demonstrate the quality of one of the first *de novo* genome assemblies of an eukaryotic species solely using pyrosequencing, and 2) present genomic and evolutionary findings derived from the red harvester ant (*Pogonomyrmex barbatus*) genome.

Results and Discussion

Genome coverage is 10.5-12X based on the estimates of genome size for *Pogonomyrmex* ants being 250-284 Mb (Tsutsui *et al*. 2008). The assembly consists of 4,646 scaffolds (mean contig/scaffold = 7.22) spanning 235 Mb (~88%) of the genome and harbors 220 Mb (~83%) of DNA sequence (15 Mb are of gaps within scaffolds). The N50 scaffold size of the assembly is 793 kb and the largest scaffold is 3.8 Mb in length; the N50 contig size is 11.6 kb. The transcriptome assembly yielded 7,400 isogroups with a N50 contig size of 1.3 kb.

The MAKER annotation pipeline predicted 16,331 genes and 16,404 transcripts. InterProScan (Quevillon *et al.* 2005) identified additional genes from the *in silico* prediction programs, which were added to the MAKER predicted genes. The final official gene set, OGS1.1, which was used for computational analyses, consisted of 17,177 genes encoding 17,250 transcripts. Out of these, 7,958 (> 46%) had complete or partial EST support from the *P. barbatus* transcriptome assembly. The results of the assembly and annotation of the *P. barbatus* genome are well within the range of other insect genomes (Table 2.1).

More than 1200 genes have been manually annotated to improve models generated by MAKER (SI Appendix, Chapter2) and were used in gene family centered analyses (see discussion below and SI Appendix, Chapters 3, 6-8, 14, and 16-29). There were two fundamentally different reasons for our choice of gene families. One set comprises highly conserved gene families for quality assessment (e.g., sequencing error, genome completeness) whereas the second set was based on biologically interesting functional groups associated with the evolution and regulation of social behavior or adaptations of *P. barbatus* to a desert seed-harvesting lifestyle. These manual annotations are included in the *P. barbatus* OGS1.2, which is being submitted to NCBI (Genome Project #45803, Assembly Project ID 45797, Transcriptome Project ID 46577), and is currently available, along with other data and tools, at the Hymenoptera Genome Database (http://HymenopteraGenome.org/pogonomyrmex).

Quality of genome assembly

The core eukaryotic gene mapping approach (CEGMA) (Parra *et al*. 2007) provides a method to rapidly assess genome completeness because it comprises a set of highly conserved, single copy genes, present in all eukaryotes. In *P. barbatus*, 245 of the 248 (99%) CEGMA genes were found and 229 of 248 were complete (92%). Cytoplasmic ribosomal protein genes are another highly conserved set of genes which are widely distributed across the physical genome in animals (Uechi *et al*. 2001; Marygold *et al*. 2007). A full complement of 79 proteins was found within the *P. barbatus* genome encoded by 86 genes (SI Appendix, Chapter 6). Because ribosomal proteins are highly conserved, their manual annotation also provided an estimate of sequencing errors, such as frameshift-inducing homopolymers (a potential problem inherent to pyrosequencing) (Huse *et al*. 2007). Six erroneous frameshifts were found in ribosomal protein genes (only one homopolymer); extrapolating from the number of nucleotides encoding the ribosomal genes suggests that 1 in 7,200 coding nucleotide positions (0.014%) may be affected by frameshifts. Analyses of other highly conserved gene families, including the oxidative phosphorylation (Saraste 1999) pathway and the *Hox* gene cluster (Hughes & Kaufman 2002; Gellon & McGinnis 1998), also suggest high coverage and good genome assembly (SI Appendix, Chapters 7 and 8). Interestingly, the mitochondrial genome did not auto-assemble into scaffolds greater than 2 kb, but 71% of the mitochondrial genome could be manually assembled with the longest contig containing 5,835 bp (SI Appendix, Chapter 9; Dataset S1). The largest missing fragment of the mitochondrial genome is typically very high in AT content (96% in *A. mellifera ligustica*) (Crozier & Crozier 1993) and may not have sequenced due to PCR biases.

In silico predicted gene models gain significant support through EST sequences. Another way to confirm predicted gene models is a proteomics approach, which has the additional benefit that it demonstrates that a gene is not only transcribed but also translated. A proteomic analysis of the poison gland and antennae confirmed 165 gene and protein models with at least 2 peptides (SI Appendix, Chapter 10). It also resulted in the identification of proteins likely associated with nest defense (poison gland) and chemoperception (antenna).

Chromosomal coverage in the current draft assembly was assessed by the identification of telomeres. Most insects outside of the Diptera have telomeres consisting of TTAGG repeats. On the basis of karyotype data ($n = 16$), we expected 32 telomeres in *P. barbatus* (Taber *et al*. 1988). We searched the assembled genome and mate pair reads for TTAGG repeats and extended these where possible (Werren *et al*. 2010). In total, 27 of the expected 32 telomeres (88%) were found (SI Appendix, Chapter 11). These telomeres are even simpler than those of *A. mellifera* (Robertson & Gordon 2006). While most other insect telomeres commonly include retrotransposon insertions, these seem to be absent from the telomeres of *P. barbatus*.

Genome-wide analyses

The mean GC content of the *P. barbatus* genome is 36.5% and the mean ratio of observed to expected CpG [CpG(o/e)] is 1.57, both of which are within the ranges reported for other Hymenoptera (HBGSC 2006; Werren *et al*. 2010). We define compositional domains as sequence stretches of variable lengths that differ widely in their GC compositions. A comparison of GC compositional-domain lengths among

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insects shows that *P. barbatus* and *A. mellifera* have similar compositional domain-length

distributions (SI Appendix, Chapter 4). Among the compared insect genomes, the

hymenopterans have the smallest proportion (0.1-0.5%) of long compositional domains

(> 100 kb) as well as the widest range in GC compositional domains. Similar to the other

sequenced hymenopteran genomes, but in contrast to other insect orders, genes in

P. barbatus occur in the more GC-poor regions of the genome. Although the mean

CpG(o/e) values of hymenopteran genomes are among the highest observed, species-

specific patterns of CpG(o/e) within each genome are not consistent between the

hymenopterans studied (Figure 2.2). The distribution of CpG(o/e) in *P. barbatus* exons is

similar to insects without CpG methylation (although with greater variance) (Elango *et*

al. 2009) and suggests little germ line methylation despite the presence of a complete

methylation toolkit (see below and SI Appendix, Chapter 24). We used an indirect

method [single nucleotide polymorphisms (SNP) frequency: CpG – TpG] and direct

method [methylation-sensitive amplified fragment length polymorphism (AFLP) assay;

SI Appendix, Chapter 4) to determine the presence and frequency of active CpG

methylation in *P. barbatus*. We found that CpG/TpG (and vice versa) SNPs constitute

84% of all CpG-to-NpG polymorphisms. This is an indirect measure of CpG methylation

because it has been shown that a methylated cytosine in a CpG has a higher probability to

mutate into thymine (SI Appendix, Chapter 30). The more direct measure of CpG

methylation comes from an AFLP analysis that used methylation-sensitive and -

insensitive restriction enzymes. In a comparison of 209 individuals from every female

and developmental caste, 33% of all AFLP fragments showed the signature of

methylation (SI Appendix, Chapter 4). These findings suggest a role of DNA methylation

in genome regulation, but additional data are necessary to confirm these predictions and discern the biological role of DNA methylation in *P. barbatus*.

Gene ontology analyses detected significant enrichments in genes associated with sensory perception of smell, cognition, and neurological processes (SI Appendix, Chapter 5). These enrichments may reflect the heavy reliance on chemical communication in ants. Consistent with this and detailed analyses of chemosensory and cytochrome P450 gene families (see below), a gene orthology analysis including *Drosophila melanogaster*, *A. mellifera*, and *Nasonia vitripennis* found expansions of genes involved in responses to chemical stimuli and electron transport. The orthology analysis also found a small fraction of genes (3.2% of those in the analysis) common to both social insects studied (SI Appendix, Chapter 5); these genes may be important in processes related to the evolution or maintenance of sociality.

Repetitive DNA

Previous results for the *A. mellifera* (HBGSC 2006) and *N. vitripennis* (Werren *et al*. 2010) genomes illustrate two extreme cases of genomic repeat composition for Hymenoptera: *A. mellifera* is devoid of all except a few *mariner* (Robertson 1993) and rDNA-specific R2 (Kojima & Fujiwara 2005) transposable elements whereas *N. vitripennis* has an unusual abundance of repetitive DNA (Werren *et al*. 2010). The *P. barbatus* genome assembly contains 18.6 Mb (8% of genome) of interspersed elements (SI Appendix, Chapter 12). A total of 9,324 retroid element fragments and 13,068 DNA transposons were identified; however, the majority of interspersed elements (55,373, 8.8Mb, 3.75% of genome) could not be classified to a specific transposable element

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family. Gypsy/DIR1 and L2/CR1/Rex elements were the most abundant transposable elements; however, we discovered most families of known insect retrotransposable elements. Nearly 1% (269 loci/1 Mb) of the scaffolded genome is microsatellite DNA (SI Appendix, Chapter 13), greater than in most insects (Pannebakker *et al*. 2010), which are valuable markers for mapping and population genetic studies.

Chemoreceptor gene family expansions

One special focus of the manual annotation was proteins involved in chemoperception because of its important role in colony communication, a cornerstone of social living. Below we report insights from four gene families involved in odorant reception, the ionotropic receptors (IRs), gustatory receptors (Grs), odorant receptors (Ors), and cytochrome P450s.

The IR family in *P. barbatus* consists of 24 genes, compared with 10 in *A. mellifera* and 10 in *N. vitripennis* (Croset *et al*. 2010). Phylogenetic analysis and sequence comparison of IRs identified putative orthologs of conserved IRs that are present in other insect genomes and that are expressed in insect antennae (e.g., IR25a, IR8a, IR93a, IR76b) (Benton *et al*. 2009), but a number of ant-specific divergent IRs display no obvious orthology to other hymenopteran or insect receptors (SI Appendix, Chapter 14). Some of these IRs may fulfill contact chemosensory functions, by analogy to the gustatory neuron expression of species-specific IRs in *D. melanogaster* (Croset *et al*. 2010).

The *P. barbatus* Gr family contains 73 genes compared with just 11 in *A. mellifera* and 58 in *N. vitripennis*. Phylogenetic analysis of the Gr proteins (SI

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Appendix, Chapter 14) supports several conclusions about the evolution of this gene family. *A. mellifera* has lost multiple Gr lineages and failed to expand any of them (Robertson & Wanner 2006; Robertson *et al.* 2010), but gene losses are not restricted to *A. mellifera*, with some occurring in *N. vitripennis* and/or *P. barbatus*. The existence of at least 18 Gr lineages is inferred, with *A. mellifera* having lost function in 10 of them, *P. barbatus* 4, and *N. vitripennis* 5. *P. barbatus* has expanded two gene lineages independently of the two expansions seen in *N. vitripennis*. Expansion A is considered to be orthologous to the NvGr48-50 gene lineage and a large set of ≈50 highly degraded pseudogenes in *A. mellifera* (represented by AmGrX-Z), and expansion B is somewhat younger. We hypothesize that these are bitter taste receptors that lost function in *A. mellifera* at the time at which they transitioned to nectar feeding, ≈100 Mya, (Poinar & Danforth 2006). Bitter taste perception may be essential for *P. barbatus* to avoid unpalatable seeds (e.g., plant secondary chemicals).

The Or family also appears to be considerably expanded in *P. barbatus*, with 344 apparently functional genes among a total of 399 genes (the largest total known for any insect) compared with a total of 177 in *A. mellifera* and 225 in *N. vitripennis* (SI Dataset 2). We counted 365 ± 10 and 345 ± 10 glomeruli in 5 queens and 5 workers respectively (SI Appendix, Chapter 15), supporting an ≈1:1 relationship of Or genes to glomeruli resulting from convergence of the axons of all neurons expressing a particular Or on one glomerulus (Mombaerts 1999; Gao *et al.* 2000). A particularly large expansion of a 9-exon gene subfamily to 169 genes suggests that these genes might comprise the cuticular hydrocarbon receptors (SI Appendix, Chapter 14). Cuticular hydrocarbons have

gained many novel functions important in the context of social behavior, such as colony recognition and queen signaling (Endler *et al*. 2004; Hefetz 2007).

P. barbatus has 72 genes in the cytochrome P450 superfamily, compared with 46 in *A. mellifera* and 92 in *N. vitripennis* (HBGSC 2006; Werren *et al*. 2010). P450 subfamilies involved in detoxification of xenobiotics show some expansion, while those implicated in pheromone metabolism are enigmatically less expanded (SI Appendix, Chapter 16).

Evolutionary rate and pseudogene accumulation

An evolutionary rate analysis based on amino acid substitutions of the three hymenopteran species with a genome sequence, and *D. melanogaster* as an outgroup, showed that a significant part of the *P. barbatus* genome (4,774 orthologous genes conserved over approximately 350 million y) evolves at a similar rate as the *A. mellifera* genome, and the *A. mellifera* and *P. barbatus* genomes show slightly higher substitution rates than the *N. vitripennis* genome (Fig. 3, SI Appendix, Chapter 31). This analysis suggests that the slow evolutionary rate reported for *A. mellifera* may not be associated with sociality, but specific to the Hymenoptera.

A notable feature of *P. barbatus* chemosensory and P450 genes is that the pseudogenes commonly have multiple major mutations suggesting that they are mostly "middle-aged" pseudogenes. Normally a range of pseudogene ages can be inferred in the chemoreceptor gene families, from young pseudogenes with single mutations to gene fragments. We estimated the relative ages of the pseudogenes in Ors, Grs, and cytochrome P450s in *P. barbatus*, *A. mellifera*, and *N. vitripennis* by counting the number

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of obvious pseudogene-causing ("pseudogenizing") mutations per gene (stop codons,

intron boundary mutations, small frameshift insertions or deletions, or large insertions or

deletions). As shown in Figure 2.3, there is a contingent of considerably older

pseudogenes in these gene families in *P. barbatus*. The pattern in *P. barbatus* is in

contrast to *A. mellifera* and *N. vitripennis*, which have a greater number of young

pseudogenes. We hypothesize that the ant lineages that gave rise to *P. barbatus*

experienced a major change in chemical ecology ≈10-30 Mya, possibly as a consequence

of the increase in elevation of the Sierras and Andes to their present height (Poulsen *et al*.

2010; Cassel *et al*. 2009). These western mountain ranges created rain shadows on their

eastern sides and spawned the great American deserts. The North American members of

the genus *Pogonomyrmex* underwent a significant radiation adapting to these new

habitats (Taber 1998), so the gene expansions in the chemoreceptors and P450s might be

adaptations to novel seeds and plant families and their associated toxic components and

chemical signatures. Accumulated pseudogenes may therefore reflect a shift towards a

more specialized diet concurrent to the adaptive radiation of *Pogonomyrmex* spp.

(McBride 2007).

Innate immunity genes

Social insects live in dense groups with high connectivity, putting them at increased risk

for disease outbreaks, but they also have social immunity to minimize introduction and

spread of pathogens (Walker & Hughes 2009; Fefferman & Traniello 2008). Very

efficient social defenses (e.g., hygienic behaviors) or novel immune pathways were

hypotheses put forth to explain the presence of few (roughly half) innate immunity genes

in *A. mellifera* compared with *D. melanogaster* (and more recently the red flour beetle,

Tribolium castaneum) (HBGSC 2006; TGSC 2008). However, the more recently

sequenced genomes of *N. vitripennis* (Werren *et al.* 2010) and *Acyrthosiphon pisum* (pea

aphid) (IAGC 2010) also have "depauperate" complements of immune genes relative to

flies and beetles, which suggests that the gene complement of flies and beetles might be a

derived condition within insects. Indeed, the number of innate immune genes in

P. barbatus is more similar to the other hymenopterans (SI Appendix, Chapter 17).

Although all of the major signaling pathways are present in *P. barbatus* (IMD, Toll,

Jak/STAT, and JKN), only a few recognition proteins were identified, which suggests

either a highly focused immune system or an alternative unknown pathogen recognition

system. Interestingly, we found expansions of antimicrobial peptides relative to

A. mellifera. These expansions may correspond to a transition to living within the soil and

an increased exposure to bacterial and fungal pathogens.

Developmental networks and polyphenism

The production of alternative phenotypes during development may occur through the

regulation of several key nodes in specific networks during development (Davidson

2006; Abouheif & Wray 2002; Khila & Abouheif 2008). In ant colonies, queens and

workers fill divergent adaptive roles - dispersal and reproduction vs. colony maintenance-

and their functional differences are reflected in differences in morphology, physiology

and behavior, such as in wings and ovaries. *P. barbatus* workers are completely devoid of

wings at the adult stage and have ovaries a fraction of the size of the queen's. In analogy

to honey bees (Kucharski *et al.* 2008), we hypothesized that CpG DNA methylation may

play a role in the differential regulation of genes in wing and reproductive development networks of workers and queens. This hypothesis was computationally evaluated by examining the CpG dinucleotide content (Elango *et al*. 2009) of wing and reproductive developmental pathway genes relative to the genome (SI Appendix, Chapter 18). These developmental networks contain significantly fewer CpGs than random genes, suggesting that they are more methylated than most genes because methylated cytosines are more prone to deamination (Werren *et al*. 2010; Elango *et al*. 2009; Foret *et al*. 2009). These results are in contrast to data in *A. mellifera*, where housekeeping genes are the main targets of methylation (Elango *et al*. 2009; Foret *et al*. 2009) (which is also in contrast to vertebrates), and suggest a potentially divergent role of methylation in harvester ants compared with honeybees.

Gene regulation and reproductive division of labor

Various gene families/pathways were specifically targeted for manual annotation because of their known role in queen-worker caste determination (Smith *et al*. 2008). These families/pathways included the insulin/TOR-signaling pathway (SI Appendix, Chapter 19), yellow/major royal jelly genes (SI Appendix, Chapter 20), biogenic amine receptors (SI Appendix, Chapter 21), and hexamerin storage proteins (SI Appendix, Chapter 19). These candidate caste genes will be targeted for studying gene expression differences between castes using RNAi. The RNAi pathway is intact in *P. barbatus* (SI Appendix, Chapter 22) and RNAi has already been successfully implemented in another ant (Lu *et al*. 2009).

Similar to the other sequenced hymenopterans, *P. barbatus* has a full methylation toolkit (SI Appendix, Chapter 24). All three DNA methyltransferase genes (*Dnmt1-3*), and three methyl-binding proteins (*MBD*) are present in *P. barbatus*, but interestingly there is only a single copy of *Dnmt1* compared to two in *A. mellifera* and three in *N. vitripennis* (Werren *et al*. 2010). The loss of multiple copies of maintenance methyltransferase(s) in ants may have implications for the inheritance of epigenetic information.

We analyzed genes within 100 kb of four microsatellite markers diagnostic for the J-lineages (Schwander *et al*. 2007) with the hypothesis that some genes physically linked to the markers may cause the incompatibility between the lineages that leads to the loss of phenotypic plasticity and genetic caste determination (Cahan *et al*. 2004) (SI Appendix, Chapter 19). One interesting candidate from this analysis, *lozenge* (*lz*), has many described mutants in *D. melanogaster,* including sterility due to a loss of oogenesis and a spermathecum (Anderson 1945; Perrimon *et al*. 1986; Bloch *et al*. 2003; Khila & Abouheif 2010), two traits characteristic of worker ants.

Material and Methods

Genome sequencing and assembly

The genome and transcriptome of *P. barbatus* were sequenced entirely on the 454 XLR Titanium platform at SeqWright. Five runs were dedicated to unpaired shotgun reads on DNA isolated from a single haploid male ant, which generated over 6 million reads averaging 370 bp in length (after trimming). Two runs used 8-kb paired-end libraries based on DNA from four brothers of the previous male ant; this yielded a total of nearly

2.9 million reads, each averaging 262 bp in length (after trimming). The assembly

presented in this paper was created by CABOG 5.3 (Miller *et al*. 2008) open source

assembler. We substituted the OVL overlap module for the recommended MER

overlapper for performance reasons (see CABOG documentation at

http://sourceforge.net/apps/mediawiki/wgs-assembler).

The transcriptome was sequenced using a single 454 Titanium run, which

generated 10.4 Mb of sequence across 726,000 reads. The transcriptome was assembled

using the Newbler v2.3 assembly software (Roche).

The genome of *P. barbatus* was annotated with the automatic annotation pipeline

MAKER (Cantarel *et al*. 2008). The *ab initio* predictions of MAKER were further refined

to produce an official gene set used for computational analyses (SI Appendix, Chapter 2).

This set (OGS1.1) included all non-redundant ab initio predictions from all gene

predictors used by MAKER that were supported by an InterProScan domain (Quevillon

et al. 2005) and excluded any that were flagged as possible repeat elements. A second

official gene set (OGS1.2) was produced to include refined genes based on manual

annotation and has been submitted to NCBI. Manual annotations followed a standard

methodology described in the SI Appendix, Chapter 3. Detailed methods for specific

analyses are given in SI Appendix, Chapters 4-31.

Supplementary Material

Supporting information (SI) appendix Chapters 1-31 and datasets S01-S02 are available

at the *Proceedings of the National Academy of Sciences of the United States of America*

online (http://www.pnas.org/content/suppl/2011/01/24/1007901108.DCSupplemental).

Acknowledgements

Table 2.1. Comparison of metrics for recently sequenced insect genomes. Abbreviated author groups are listed as follows in the sources: HBGSC = Honey Bee Genome Sequencing Consortium, IAGC = International Aphid Genomics Consortium, and TGSC = *Tribolium* Genome Sequencing Consortium.

Species	Order/Name	Coverage (X)	N50 (kb)	# of Genes	Gene Set	Source
Pogonomyrmex barbatus	Hymenoptera (Harvester Ant)	12	793	17,177	OGS1	Smith, C.R. *et al.* 2011
Nasonia vitripennis	Hymenoptera (Jewel Wasp)	6.8	709	18,822	OGS1.2	Werren *et al.* 2010
Apis mellifera	Hymenoptera (Honey bee)	7.5	362	15,314 (10,157)	OGS3.2 (OGS1)	Elsik et al. 2014; (HBGSC 2006)
Acyrthosiphon pisum	Sternorrhyncha (Pea Aphid)	6.2	88.5	34,604	OGS1	IAGC 2010
Tribolium castaneum	Coleoptera (Red Flower Beetle)	7.3	990	16,404	Consensus set	TGSC 2008
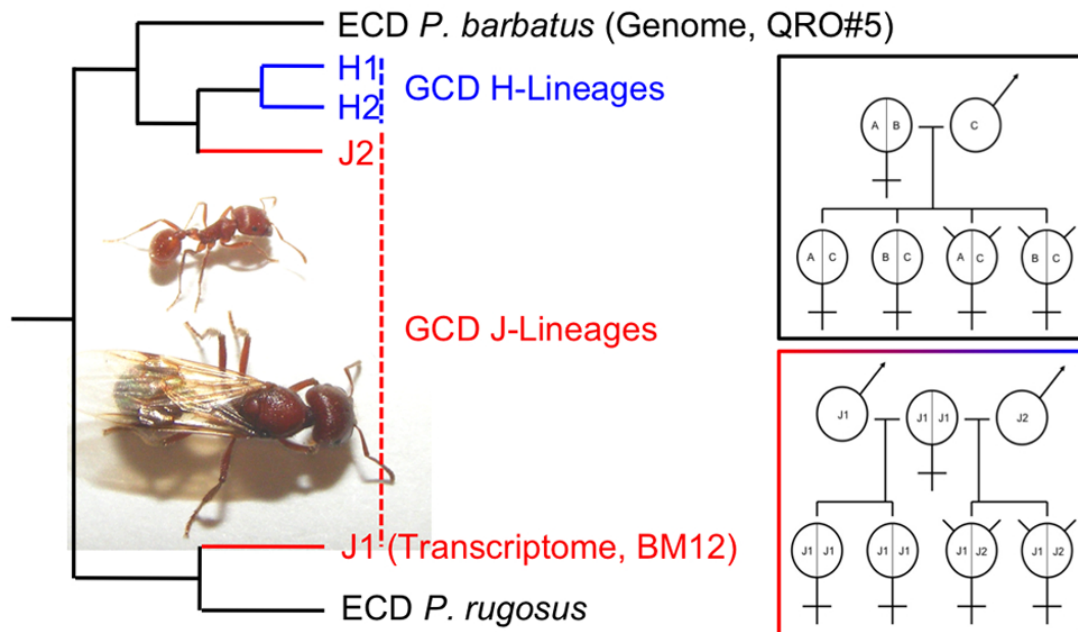
Figure 2.1. A pictorial description of the phylogenetic position of the samples used for the genome and transcriptome sequencing, with each put in the context of environmental and genetic caste determination (for a more complete phylogenetic tree, see SI Appendix, Chapter 1). The dependent lineages (H1/H2 or J1/J2) obligately co-occur because hybridization between them is necessary to produce workers, although within either J or H, the constituent lineages are reproductively isolated because interlineage hybrids cannot become queens (red/blue box). In the boxes to the right, workers are represented by "horned" female symbols. In all *P. barbatus*, the queen mates multiply; polyandry in genetic caste determining (GCD) colonies is obligate to produce both female castes (queens originate from intralineage matings and workers from interlineage matings). In environmental caste determination (ECD), alleles from any father have an equal chance to be in queens or workers (black box). Photo of gyne and worker *P. barbatus* by C.R. Smith.
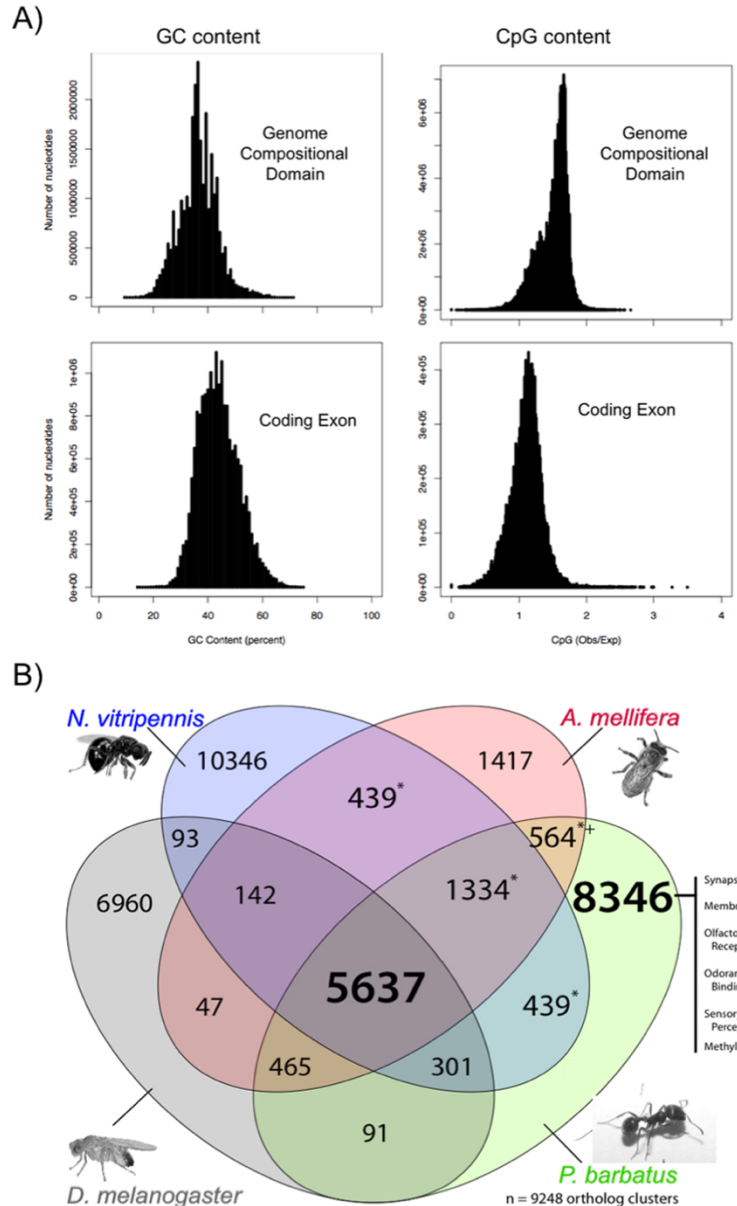
Figure 2.2. Genome-wide analyses on nucleotide and relative gene content. (A) Synopsis of GC and CpG(o/e) content of the *P. barbatus* genome. (Upper panels) Comparison of genome regions with the same GC composition. (Lower panels) Comparison of the same features for exons. These distributions are similar to those found in other hymenopterans, except that *P. barbatus* shows no evidence of bimodality in CpG(o/e) for either exons (like *A. mellifera*) or introns (like *N. vitripennis*) (for comparisons, see SI Appendix, Chapter 4). (B) A Venn diagram displaying overlap in orthologous genes in three hymenopteran and one dipteran insect (for a detailed description of the method, see SI Appendix, Chapter 5). A sub-set of gene ontology terms significantly enriched in *P. barbatus* are displayed to the right of the figure. (*) Hymenoptera-specific genes; (†) social Hymenoptera-specific genes.
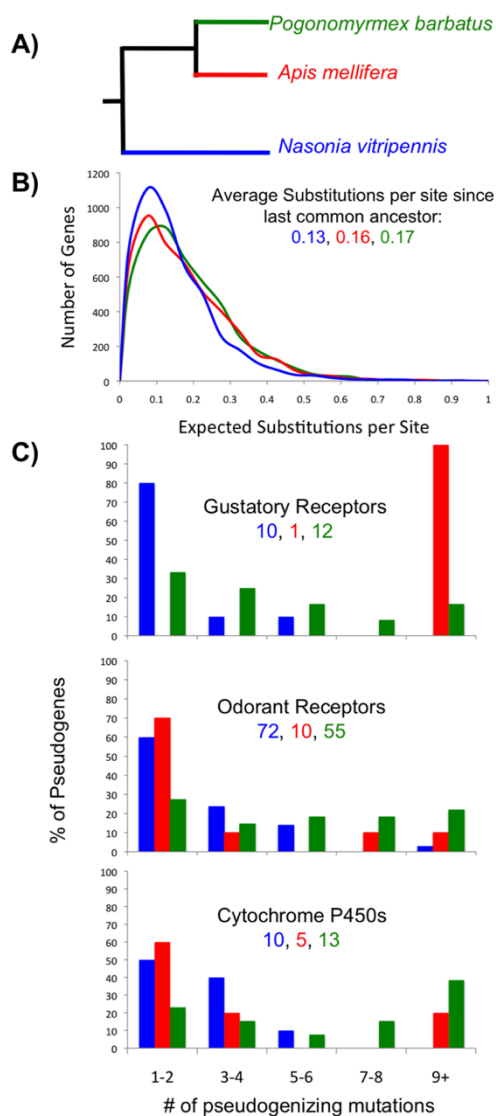
Figure 2.3. Evolutionary rate and the accumulation of pseudogene-causing ("pseudogenizing") mutations in three gene families in the ant *P. barbatus* (green), the honey bee *A. mellifera* (red), and the jewel wasp *N. vitripennis* (blue). (A) The relationships among analyzed taxa. (B) A comparison of the evolutionary rates based amino acid substitutions in a set of 4,774 orthologs shared among the three species and *D. melanogaster* (the outgroup). (C) The accumulation of pseudogenizing mutations in three ecologically relevant gene families (Gr, Or, and cytochrome P450s. The number of pseudogenes found in each species is written on each panel. Only one gene represents the Grs in *A. mellifera*; all other *A. mellifera* Gr pseudogenes had accrued a very high number of mutations and most are fragments. Of those analyzed here, the pseudogenes in *P. barbatus* tend to be much older than those in *A. mellifera* and *N. vitripennis* (ANOVA: $F_{2,156} = 4.7$, $P = 0.01$).

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CHAPTER 3

EVOLUTION OF THE INSECT DESATURASE GENE FAMILY WITH AN

EMPHASIS ON SOCIAL HYMENOPTERA

Abstract

Desaturase genes are essential for biological processes, including lipid metabolism, cell signaling, and membrane fluidity regulation. Insect desaturases are particularly interesting for their role in chemical communication, and potential contribution to speciation, symbioses, and sociality. Here, we describe the acyl-CoA desaturase gene families of 15 insects, with a focus on social Hymenoptera. Phylogenetic reconstruction revealed that the insect desaturases represent an ancient gene family characterized by eight subfamilies that differ strongly in their degree of conservation and frequency of gene gain and loss. Analyses of genomic organization showed that five of these subfamilies are represented in a highly microsyntenic region conserved across holometabolous insect taxa, indicating an ancestral expansion during early insect evolution. In three subfamilies, ants exhibit particularly large expansions of genes. Despite these expansions, however, selection analyses showed that desaturase genes in all insect lineages are predominantly undergoing strong purifying selection. Finally, for three expanded subfamilies, we show that ants exhibit variation in gene expression between species, and more importantly, between sexes and castes within species. This suggests functional differentiation of these genes and a role in the regulation of reproductive division of labor in ants. The dynamic pattern of gene gain and loss of acyl-CoA desaturases in ants may reflect changes in response to ecological diversification and an increased demand for chemical signal variability. This may provide an example of how

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gene family expansions can contribute to lineage-specific adaptations through structural and regulatory changes acting in concert to produce new adaptive phenotypes.

Introduction

Gene families are sets of homologous genes generated by gene duplication events that often display functional similarity and frequently change in size along phylogenetic lineages (Tatusov *et al*. 1997). Expansion of gene families may occur due to gene duplication and subsequent divergence, whereas gene loss due to deletion or pseudogenization may lead to gene family contraction. The resulting turnover of genes has presumably been one of the driving forces behind the phenotypic differentiation between species (Olson 1999; Lynch & Conery 2000; Ranson *et al*. 2002; Robertson *et al*. 2003; Hahn *et al*. 2007). Both stochastic processes as well as natural selection influence the size of gene families (Hahn *et al*. 2005). Although there is dispute in the literature about the relative importance of structural versus regulatory changes (Hoekstra & Coyne 2007; Stern & Orgogozo 2008), both are likely important and may even act simultaneously, e.g., a recently duplicated gene can acquire a novel function, but at the same time the expression of this novel gene can vary significantly between species or castes (see below). Gene families that are involved in the perception or production of large varieties of semiochemicals (e.g., olfactory and gustatory receptors) or detoxification (e.g., cytochrome P450 monooxygenases and glutathione-S-transferases) tend to gain and lose genes rapidly, which seems to be correlated with changes in life history and ecology (Robertson *et al*. 2003; Emes *et al*. 2004; Feyereisen 2006; Després *et al*. 2007; McBride & Arguello 2007; Gardiner *et al*. 2008; Hansson & Stensmyr 2011).

In insects, chemical communication is a key attribute of recognition and communication, and a huge diversity of chemicals involved in this processes have been described (Greenfield 2002; Blomquist & Vogt 2003; Schulz 2004; Matthews & Matthews 2010. Semiochemicals help mediate interactions between organisms, and can be species-specific, sex-specific, and, in the case of eusocial insects, colony-, caste-, and task-specific. Insect recognition systems use semiochemicals that function both intraspecifically and interspecifically, and are categorized according to the species-relationship, behavior, and contextual environment in which they are emitted and received. Understanding the roles, developmental pathways, and evolution of insect chemical communication systems has been an exciting challenge to biologists for over five decades. Many initial studies of insect semiochemicals were focused on identifying the components of pheromones and how different compounds affected physiology and behavior. The first of these, bombykol, was identified as an important sex pheromone in the silkworm moth, *Bombyx mori* (Butenandt *et al.* 1959). Later, when its complete metabolic pathway was described, the desaturase gene *Bmpgdesat1* was found to be critically involved in both the production and perception of bombykol (Moto *et al.* 2004). In the years since, thousands of other insects and their semiochemicals have been investigated (catalogued in an online database, the Pherobase, http://www.pherobase.com), which have raised many new questions regarding their contribution to complex evolutionary processes including speciation, symbiosis, and sociality. With the ongoing advancement of chemical analysis and synthesis techniques, and the relatively recent advent of genomic and transcriptomic tools, new avenues of insight are now possible for our comprehension of these complex communication

systems in insects. Despite these advancements, relatively few genes involved in the synthesis of species-specific semiochemicals are actually known (Dallerac *et al*. 2000; Labeur *et al*. 2002; Roelofs *et al*. 2002; Moto *et al*. 2004; Chertemps *et al*. 2006; Niehuis *et al*. 2013).

The chemical components of insect communication systems have been studied extensively in Coleoptera, Diptera, Lepidoptera, and social Hymenoptera (Symonds and Elgar 2008). A majority of the compounds identified have been acetates, alcohols, aldehydes, ketones, and long chain hydrocarbons (10–30C), many of which contain carbon-carbon double bonds in varied positions and spatial formations (El-Sayed 2014). Since the 1980's, numerous studies have been conducted with the goal of understanding how insect semiochemicals are biosynthesized, i.e., *de novo* or from minimally modified, environmentally obtained chemical precursors, and furthermore, how specific modifications like hydrocarbon chain length alteration and carbon-carbon double bond introduction are made (Tillman *et al*. 1999). In the cockroach *Blattella germanica*, for example, the female contact sex pheromone – a dimethyl ketone hydrocarbon – is synthesized *de novo* through a series of steps following a fatty acid synthesis pathway (Chase *et al*. 1992). Similarly, in several species of dipterans and lepidopterans, a variety of semiochemicals are synthesized from fatty acid precursors such as myristic acid, palmitic acid, and oleic acid, which are then modified through subsequent biosynthetic steps (Bjostad & Roelofs 1981, 1983; Dillwith *et al*. 1981; Wicker-Thomas *et al*. 1997; Dallerac *et al*. 2000). Among the insects studied to date, a wide variety of semiochemical biosynthetic modification steps have been identified, e.g., acetylation, aromatization, decarboxylation, desaturation, elongation, hydrolysis, hydroxylation, methyl-branch

incorporation, oxidation, and reduction reactions (Tillman *et al*. 1999). Although all of

these steps are likely important for semiochemical biosynthesis generally, desaturation

appears to be especially important for the structural variation in semiochemicals (Knipple

et al. 2002; Roelofs & Rooney 2003; Fang *et al*. 2009). This is due to the fact that

desaturation occurs on a diverse range of substrates with both *cis* (Z) and *trans* (E)

stereoselectivities, which gives rise to unsaturated compounds with variation in chain

length, double-bond number, double-bond position, and double-bond orientation.

Because of this, a large number of insect semiochemical studies in Lepidoptera, and a

moderate number in Diptera, have focused on understanding the biochemical and genetic

diversity of desaturation reactions in recent years (Knipple *et al*. 2002; Roelofs & Rooney

2003; Hashimoto *et al*. 2008; Fang *et al*. 2009; Keays *et al*. 2011).

The primary group of proteins responsible for desaturation reactions are the

desaturases, which are specialized to introduce carbon-carbon double bonds into fatty

acyl chains, and are categorized into two main phylogenetic groups (Los and Murata

1998; Sperling *et al*. 2003). One of these two groups includes the soluble acyl-acyl carrier

protein (ACP) desaturases, which are largely found in the plastids of higher plants

(Sperling *et al*. 2003), and are involved in the conversion of saturated fatty acids to

monounsaturated fatty acids, e.g., oleic acid synthesis (Kachroo *et al*. 2007). The second

group is made up of membrane-bound acyl-lipid desaturases and membrane-bound acyl-

coenzyme A (CoA) desaturases. Evidence suggests that a subset of these proteins may be

distantly related to the acyl-ACP desaturases (Shanklin & Cahoon 1998; Sperling *et al*.

2003). Acyl-lipid desaturases appear to be primarily limited to plants and cyanobacteria

(Los & Murata 1998), whereas acyl-CoA desaturases are more ubiquitously found in

animals, yeast, and fungi, as well as many bacteria (Sperling *et al*. 2003). These membrane-bound desaturases are important for basic biological processes, including lipid metabolism, cell signaling, and maintaining fluidity in lipid membranes in response to changing temperature (Hazel & Williams 1990; Vigh *et al*. 1993; Tiku *et al*. 1996; Pyne & Pyne 2000; Miyazaki & Ntambi 2003). What is more, the biochemical functions of membrane-bound desaturases are quite diverse, in that they have been identified to catalyze at least 12 different regioselectivities (i.e., $\Delta 4 - \Delta 15$). However, regioselective function has been shown to inconsistently match with overall sequence similarity, which has made desaturase genes historically challenging to categorize (Ternes *et al*. 2002; Sperling *et al*. 2003; Tripodi *et al*. 2006).

In the past three decades, the functional characterization of desaturase genes has been carried out in a wide variety of organismal models. A key function is their role in the biosynthesis of mono- and poly-unsaturated fatty acids in a range of organisms, including cyanobacteria (Murata & Wada 1995), protists (Tripodi *et al*. 2006), fungi (Stukey *et al*. 1989), plants (Domergue *et al*. 2005; Smith *et al*. 2013), nematodes, (Watts and Browse 2000; Zhou *et al*. 2011), insects (Zhou *et al*. 2008), and mammals (de Antueno *et al*. 2001; Miyazaki *et al*. 2001). Furthermore, a number of acyl-CoA desaturase genes have been demonstrated to be crucially involved in semiochemical biosynthesis in many solitary insects, e.g., *Drosophila* fruit flies (Dallerac *et al*. 2000; Fang *et al*. 2002; Labeur *et al*. 2002), the silkworm *Bombyx mori* (Moto *et al*. 2004), and several additional lepidopteran species (Knipple *et al*. 2002). With this growing body of functional data, Hashimoto *et al*. (2008) found that membrane-bound desaturase genes could be subdivided into four subfamilies: 1) First Desaturases (primarily $\Delta 9$ and $\Delta 11$

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desaturases), which introduce the first double bond into the saturated acyl chain; 2)

Omega Desaturases ($\Delta12$ and $\Delta15$ desaturases), which introduce a double bond between

an existing double bond and the acyl end; 3) Front-End Desaturases ($\Delta4$, $\Delta5$, and $\Delta6$

desaturases), which introduce a double bond between an existing double bond and the

carboxyl end of an acyl chain; and 4) Sphingolipid Desaturases (sphingolipid $\Delta4$

desaturases), which introduce a double bond into sphingolipids at the $\Delta4$ position. In this

study we adopted Hashimoto's nomenclature.

Given the role of acyl-CoA desaturases in insect semiochemical production, these

genes have recently become an interesting family for study in social insects (Smith *et al*.

2011a; Smith *et al*. 2011b; Suen *et al*. 2011; Simola *et al*. 2013), and offer a promising

pathway to further our understanding of their recognition systems (Tsutsui 2013). In this

study, we determined the diversity of acyl CoA desaturase genes in 15 insects

emphasizing the variation in social Hymenoptera (ants and bees). These desaturases have

been demonstrated to be involved in the production of alkenes as part of the cuticular

hydrocarbon profile of *Drosophila* (Dallerac *et al*. 2000; Fang *et al*. 2002; Labeur *et al*.

2002), and are crucial for courtship behavior in this genus (Chertemps *et al*. 2006;

Bousquet *et al*. 2012). Furthermore, evidence suggests that changes in gene number and

expression of desaturases affect semiochemical diversity between closely related insect

species (Takahashi *et al*. 2001; Knipple *et al*. 2002; Roelofs and Rooney 2003;

Greenberg *et al*. 2006; Xue *et al*. 2007; Fang *et al*. 2009). Since social insects have a

highly developed communication system, largely based on cuticular hydrocarbons

(Hölldobler & Wilson 1990; Blomquist 2010), we expected an expansion of the

desaturase gene family in social Hymenoptera. The annotation of the complete acyl-CoA

desaturase repertoire allowed us to study the evolutionary history and mechanisms generating novel genes in these lineages. Furthermore, we present sex- and caste-specific gene expression patterns of three ant species, highlighting the importance of regulatory in addition to structural changes for the evolution of new phenotypes.

Results

Gene annotation and nomenclature

Searching the genome assemblies and predicted gene sets of 15 insect species allowed us to identify 218 putatively functional acyl-CoA desaturase genes characterized by a fatty acid desaturase type I domain (Table 3.1). We manually improved upon the annotation of many of these genes, with particular emphasis on the seven ant species represented in this study. Our comprehensive search also revealed 75 putative pseudogenes characterized by an interrupted reading frame, short length (less than 250 amino acids or two thirds of the average desaturase gene length in *D. melanogaster*), or the lack of a fatty acid desaturase type I domain. Although some of these genes may result from sequencing or genome assembly artifacts, most are likely remnants of once functional genes. Additionally, some genes may have been missed during annotation, yet their number is likely to be very small because we not only searched the genome assemblies for all 15 species, but also unassembled contigs for 8 of the 15 species (Supplementary Table S1). A small number of genes could not be equivocally identified as either functional or non-functional.

According to our similarity-based homology assessment and phylogenetic analyses (Figures 3.1 and 3.2; Supplementary Figures S1 and S2), insect acyl-CoA desaturases comprise eight orthologous groups. These subfamilies are referred to as Desat

A1 (*desat1, 2, F*), A2, B, C, D, E, Ifc (*ifc*) and Cyt-b5-r (*Cyt-b5-r*) in this study

(designated *D. melanogaster* genes in parentheses). Genes of subfamilies Desat A1

through E have previously been described as First Desaturases, predominantly stearoyl-

CoA $\Delta 9$ and $\Delta 11$ desaturases that introduce the first double bond at the 9th or 11th position

of a saturated acyl chain (Hashimoto *et al*. 2008). In contrast, Ifc genes are putatively

Sphingolipid Desaturases (Hashimoto *et al*. 2008) with $\Delta 4$ activity (Ternes *et al*. 2002),

and bear little sequence similarity to the remaining desaturase genes. The same is true for

Cyt-b5-r genes, whose molecular function is unknown. The latter two groups were

therefore treated separately in the phylogenetic analyses.

All subfamilies form highly supported monophyletic groups, with the exception

of Desat A1 and A2, whose monophyly and sister-group relationship is only weakly

supported in the main analysis (Figure 3.2). However, this relationship is corroborated by

the taxonomic distribution of these genes, indicating a deep split during insect evolution,

as well as the increase of confidence values for each clade after removing a small number

of divergent genes. The uncertain but likely sister-group relationship between Desat A1

and A2 is reflected in the names of these subfamilies. A possible sister-group relationship

could also be inferred for Desat B and C. Overall, the phylogenetic relationships between

the subfamilies could not be resolved confidently, though. Due to a lack of suitable

outgroups (high sequence divergence would have led to a loss of ingroup information

during sequence alignment and editing), the direction of evolution could also not be

determined in the main analysis, leaving the tree unrooted. A pruned dataset with

additional non-insect eukaryote desaturase genes was also found insufficient to resolve

this issue (Supplementary Figure S3).

Based on these phylogenetic results, we propose a new nomenclature for insect First Desaturase genes. Following a four-letter abbreviation of the species name (e.g., *Dmel* for *D. melanogaster*), this nomenclature incorporates the subfamily name as outlined above (e.g., *desatE* for subfamily Desat E) and a one-letter designation for each paralog copy. Paralog designation was chosen independently of phylogenetic position within each subfamily and includes putatively functional genes and putative pseudogenes. Well-supported subclades within subfamilies are thus not indicated in gene names, and only illustrated in Figure 3.1 (e.g., Desat B I, II and III). We chose not to adopt a naming scheme that fully reflects the homology relations as resolved by the phylogenetic analyses presented here (Van der Heijden *et al*. 2007) because of the lack of confidence in some parts of the gene tree. Subsequent phylogenetic analyses with improved resolution and accuracy might render such names obsolete. We also suggest retaining established gene names for functionally annotated genes of model organisms (e.g., *D. melanogaster desat1, 2* and *F*) to avoid confusion with already published results.

Gene copy variation

The insect acyl-CoA desaturase subfamilies differ considerably in the number of genes and the complexity of their evolutionary history. With an average number of genes per insect species of 0.9 and 1.1, subfamilies Desat D and E are mostly comprised of single-copy genes, although rare cases of lineage-specific gene duplication and loss can be observed as well. Desat C also represents a small, yet slightly more complex group. Besides limited expansions in *B. mori* and *N. vitripennis*, multiple independent cases of gene loss can be inferred from the gene tree, which results in an average number of genes

across insects of just 0.7. Multiple losses have particularly affected Hymenoptera, in which only the ants *H. saltator* and *C. floridanus* have retained a functional copy apart from *N. vitripennis*. Desat C is also the only one lacking a copy in *D. melanogaster*, exposing the risk of overly relying on homology assessment by the reciprocal BLAST method against a single model organism. All three subfamilies mentioned above have in common that evidence for once functional genes is missing in almost all insects, the only exception being two putative pseudogenes identified in *A. gambiae*. This suggests that the observed gene loss has occurred sufficiently in the past to eradicate all traces of former genes, and that little gene turnover (duplication followed by loss) has taken place more recently.

Similarly, the subfamilies Ifc and Cyt-b5-r also lean towards low copy numbers. However, while Ifc is almost exclusively composed of single-copy genes, most insect species studied here possess two Cyt-b5-r copies. Interestingly, these duplications have occurred independently in all species (Supplementary Figure S2).

In contrast, subfamilies Desat A1, A2 and B are characterized by a much higher number of genes and a more dynamic evolutionary history involving frequent episodes of gene gain and loss in multiple lineages. With 2.3 genes on average across insects, subfamily A1 features mostly single-copy genes and duplicates next to several significant lineage-specific expansions in *A. pisum*, *D. melanogaster* (*desat1*, *desat2* and *desatF*), *S. invicta* and most notably *B. mori* (8 genes). Ants are the only taxon in which we were able to find putative pseudogenes, including 18 in *S. invicta*, suggesting a high rate of gene turnover in this lineage. Similarly, 2.4 genes on average were found in subfamily A2 across insects. Large expansions here include *A. mellifera* and most notably

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T. castaneum (9 genes). The gene tree topology also suggests that the gene copies in ants can be traced back to one of two ancestral genes that originated in the Hymenopteran lineage before the divergence of ants (Figures 3.1 and Figure 3.2).

Finally, Desat B forms the single largest group of desaturase genes in insects with 4.3 genes per species on average. Apart from a substantial, recent expansion in *B. mori* (7 genes), the vast majority of these genes are found in Hymenoptera, where multiple episodes of gene family expansion and contraction can be inferred from the phylogenetic tree. The first of these episodes seems to have occurred near or after the evolutionary origin of Hymenoptera, but before the emergence of ants, giving rise to three subclades (I, II and III) represented in all ant and some of the non-ant Hymenopteran taxa (some genes have presumably been lost in *N. vitripennis* and the two bee species). This initial expansion was followed by more recent, lineage-specific expansions in most ant species and, to a lesser degree, *N. vitripennis*. Further evidence for considerable gene turnover regarding this subfamily in ants comes from a number of putative pseudogenes, all except one of which are found in ants. The highest number was observed in *L. humile*, whose genome harbors no less than 22 pseudogenes, more than any other lineage with regard to desaturases.

Genomic organization in insects

We studied the location, order and orientation of desaturase genes to shed light on the mechanisms that generated new genes and the evolutionary history of the gene family in insects. In five out of seven ants, the majority of functional desaturase genes are located on the same scaffold spanning a region of 100–150 kb, and are highly conserved with

respect to order and orientation (Figure 3.3, A–C). In *H. saltator* this cluster is broken up into two scaffolds, but the position of the genes on these scaffolds suggests they still map to the same chromosomal region. Only in *L. humile* most genes are found on separate scaffolds, a fact that might be partially explicable by the low degree of contiguity of the assembly. A notable exception to the microsynteny exhibited by most desaturase genes in ants is made by members of the Hymenoptera-specific Desat B subclade I (see Figure 3.1), which are consistently found on a different scaffold. The position of genes on the scaffolds and their sizes suggest that these genes and the desaturase core cluster are effectively unlinked, and most likely situated in different chromosomal regions. Many additional members of the highly expanded subfamilies Desat B and Desat A, both functional and pseudogenized, are located on their own scaffolds as well. Some form smaller, often tandemly arrayed clusters like Desat B genes in *L. humile* and *H. saltator*, while others are dispersed across many private scaffolds like Desat A1 genes in *S. invicta*. Finally, another exception is made by genes of the subfamily Desat C, which have only been retained by *H. saltator* and *C. floridanus*, and the ant orthologs of *ifc* and *Cyt-b5-r*, all of which are also found on separate scaffolds.

In the remaining Hymenoptera, the arrangement is similar (Figure 3.3, D). In both bee species, the desaturase core cluster spans about 80 kb (split across two scaffolds in *A. mellifera*, with additional Desat A2 genes occupying another). However, the single Desat B copy in bees is situated on separate scaffold at least Megabases away. The situation is slightly more complicated in the wasp *N. vitripennis*, where the core cluster is broken up into two groups located on different, unlinked scaffolds. As in ants, several Desat B paralogs are found in close proximity to the *desat1* ortholog, while others reside

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on their own scaffolds. Likewise, ifc and Cyt-b5-r genes occupy a different genomic region than all other desaturase genes.

Dipteran First Desaturase genes are found on a single chromosome arm – 3R in *D. melanogaster* (except *desatF*, which is on 3L) and 2R in *A. gambiae* – which displays a high degree of synteny between the species (Zdobnov *et al.* 2002). In both species, a tightly linked block of *CG9747*, *CG15331* and *CG9743* or their orthologs is separated from *desat1* and *CG8630* by several to many Megabases (only the relative position of *CG8630* differs between both species) (Figure 3.3, E).

This synteny is also displayed by *B. mori*, the closest relative of the Dipteran species in our taxon sampling. Here, members of all First Desaturase subfamilies are found in a 125 kb region, in an order reminiscent of that in *A. gambiae* (Figure 3.3, F). As seen in ants, genes produced by recent, lineage-specific expansions can be traced back to different genomic regions, and sometimes form smaller clusters (e.g., several Desat B paralogs). *T. castaneum* core genes occupy two smaller, unplaced scaffolds and may therefore be microsyntenic as well, while more recently generated Desat A2 paralogs comprise several independent clusters. In contrast to all other species studied, only *A. pisum* provides little evidence for synteny. With the exception of three genes from two subfamilies, Desat A1 and Desat E, all genes are scattered across different scaffolds. Like in Hymenoptera, ifc and Cyt-b5-r genes are physically unlinked from First Desaturase genes in all non-Hymenopteran species studied.

Selective forces acting on desaturase genes

The results of the signature of selection analysis provide little evidence for positive selection acting on desaturase genes in insects (Table 3.2). Under the most basic model M0, which assumes that the ratio of non-synonymous to synonymous substitutions is invariable among sites and branches, ω (= d_N/d_S) ranges from 0.08 to 0.21 among the desaturase subfamilies. More realistically allowing ω to vary among sites following a beta-distribution (models M7 and M8) results in a strongly L-shaped distribution in all subfamilies, indicating that most sites represent very small ω values and are thus under purifying selection. Adding another class for sites under positive selection (M8) results in a significantly better fit according to likelihood ratio tests (LRT) in two subfamilies, Desat A2 and Desat D. However, the estimated proportion of positively selected sites is either very small, or the ω values for this site class are very close to 1, attesting neutral rather than positive selection. Moreover, Bayes Empirical Bayes (BEB) analyses (Yang *et al*. 2005) fail to detect sites under positive selection with posterior probability > 95% in all subfamilies.

We then looked whether strongly expanded subclades of desaturase genes, and ant-specific genes in general, evolved under different selective pressures than the remaining (background) genes by assigning different ω values to foreground and background branches. Tested foreground branches included all ant-specific genes in each subfamily, as well as the *B. mori*-specific expansions in Desat A1 and Desat B, and the *T. castaneum*-specific expansion in Desat A2. In all cases, the branch-specific model (BA in Table 3.2, shown for ants only) proved to fit the data significantly better than the basic model M0. Foreground ω values with respect to ants were significantly higher than

background ω values in all subfamilies, with the biggest difference found in Desat B (0.27 and 0.11, respectively). In contrast, foreground ω values for *B. mori* and *T. castaneum* were significantly smaller than background ω values. Fixing ω for the foreground branches at 1 (model B0) did not lead to an improved fit over the unconstrained model BA because foreground ω values were estimated to be much smaller than 1.

Allowing ω to vary both among sites and branches using a branch-site model did not provide evidence for sites under positive selection in ants only (AA in Table 3.2). In fact, ω for sites allowing positive selection in ants only was estimated to be 1 in all subfamilies, confirming the role of purifying selection in desaturase genes across insect lineages. The branch-site test of positive selection was therefore non-significant.

Gene expression in ants

We found strong differences in the transcription levels of the First Desaturase genes across the sexes and castes in three ant species (Figure 3.4). The expression of three reference genes, however, did not vary strongly between sexes and castes within a species (coefficients of variation ≤ 0.38) but varied moderately between species (coefficients of variation ≤ 0.70), e.g., *P. barbatus* showed an overall lower expression level than the other two species. Hence, comparisons of expression patterns within a species reflect quantitative differences whereas interspecific comparisons can only be qualitative, i.e., presence or absence of clade specific expression, or expression ratios between clades.

In *H. saltator*, desaturase genes are generally expressed at very low levels (RPKM < 100) in workers relative to the reference genes. The expression profiles of adult, virgin queens and males are however dominated by the Desat E ortholog and several Desat B genes, some of which reach very high levels (RPKM > 1000) (Figure 3.4, A). This stands in contrast to *C. floridanus* (Figure 3.4, B), where the cumulative expression levels in minor workers, virgin queens and males are more balanced, and dominated by genes of Desat A1 and A2, whereas Desat B genes are barely represented. However, there is agreement between the two species in the moderate expression of Desat E and Desat D orthologs. *P. barbatus* expression profiles (Figure 3.4, C) resemble more closely those found in *H. saltator* in the fact that Desat B genes are more highly expressed than genes of any other subfamily. However, these genes hail from a different subclade within Desat B (I) than most that are overrepresented in *H. saltator* (II). Also in contrast, *P. barbatus* workers express First Desaturase genes more than virgin queens (data for adult males is not available). Overall expression levels, though similar or lower than in the other two species in absolute terms, seem to be higher relative to the reference genes. Consistent across all three species and most castes seems to be the proportionately moderate expression of the Desat E and Desat D orthologs. Lastly, several desaturase genes are strongly overrepresented in particular castes or sexes within a species. Most notably, *desatB_b* in queens and *desatB_a* in gamergates of *H. saltator*, and *desatA2_c* in *C. floridanus* workers were found to be expressed almost exclusively in these castes.

Discussion

Evolution of genomic organization

Studying the location, order and orientation of the acyl-CoA desaturase genes at the genomic level revealed a high degree of microsynteny within genomes, and strong organizational conservation across insect species. We found a cluster of genes including members of all First Desaturase subfamilies except Desat C in a wide range of species from ants and bees to *B. mori*, with only slight variations in gene order and orientation (Figure 3.3). Although in *D. melanogaster* and *N. vitripennis*, this cluster has apparently been broken up into smaller blocks by large-scale genomic rearrangements, it is still evident in microsynteny retained within those blocks. Conservation of the cluster-like organization of First Desaturase genes between species separated by millions of years of evolution suggests that these genes are ancestral to holometabolous insects. Hence, homologous recombination by unequal crossing-over seems to be the dominant mechanism generating these genes from a single ancestral gene. Since the aphid *A. pisum* possesses a full complement of acyl-CoA desaturase genes, these events must have preceded the split between hemipterans and holometabolous insects, even though genomic rearrangements have obliterated most traces of ancient microsynteny in this species. Phylogenetic analyses further suggest that the ancestral expansion of the First Desaturase genes occurred presumably along the insect lineage, as orthologs found in non-insect arthropods, nematodes and vertebrates seem to be derived from duplication events succeeding the divergence between these lineages and insects (Hashimoto *et al.* 2008; Supplementary Figure S3).

The genes making up this ancestral cluster are thus the source from which new, lineage-specific desaturase genes were generated in insects. The genomic organization of these novel lineage-specific genes suggests two mechanisms shaping the evolution of desaturase genes in insects, homologous recombination and chromosomal mutations. Closely linked or even tandemly arrayed gene copies provide evidence that homologous recombination gave rise to many novel genes, both within and outside of the original cluster. For instance, the expansion of Desat B genes in the Attines, *A. cephalotes* and *A. echinatior*, can be traced back to such events (Figure 3.3, A). The duplications of the ancestral genes which generated the ant-specific subclades Desat B II and III, and Desat A2 I and II (Figures 3.1 and 3.2) seem to have involved homologous recombination as well, because extant copies are found in close proximity to each other in most ant species (Figure 3.3, A–C). Finally, most recent, species-specific expansions have resulted in tandem arrays of novel genes, e.g., the expansion of Desat B subclade II genes in *H. saltator*, Desat A2 genes in *T. castaneum* and most likely Desat B genes in *B. mori* (Figure 3.3, F).

However, the presence of unlinked desaturase genes in many insect species, most notably the members of the ant-specific Desat B subclade I, indicates another mechanism generating novel genes. Since functional genes of this category closely resemble gene copies located in the core cluster in terms of intron-exon structure and thus contain intronic sequence, retrotransposition seems not to be involved in the process (Long 2001). Instead, chromosomal mutations like segmental duplication followed by chromosome rearrangement could be responsible, an ill-understood process frequently observed in other animal lineages (Samonte & Eichler 2002). Segmental duplication

results in longer stretches of identical sequence, including non-coding intergenic DNA. We did not find evidence for high sequence similarity between regions adjacent to genes located in the core cluster and unlinked genes, although since little selection pressure is expected to act on intergenic DNA, mutations accumulated over time might have erased these traces.

Evolution of gene repertoire

The acyl-CoA desaturase gene family is characterized by a highly dynamic evolutionary history in insects. Careful annotation and phylogenetic analysis of both functional and pseudogenized genes allowed us to infer eight subfamilies which differ strongly in their degree of conservation and frequency of gene gain and loss (Figures 3.1 and 3.2; Supplementary Figures S1 and S2). Three of the eight subfamilies are characterized by frequent expansions and higher rates of gene turnover, which also do not affect insect lineages equally. While the First Desaturase subfamilies Desat C, D and E as well as the Ifc and Cyt-b5-r subfamilies are largely comprised of single-copy genes, subfamilies Desat A1, A2 and B harbor a much higher number of genes (Figures 3.1 and 3.2). Some cases of gene family expansion, most notably the episode of gene gain that gave rise to the ant-specific Desat B subclades I, II and III, can be traced back to deeper splits in the insect phylogeny. However, the vast majority of expansions seem to have occurred more recently. Taxa that are disproportionally affected by such lineage-specific expansions include *B. mori*, *T. castaneum*, *N. vitripennis* and most ant species, particularly *H. saltator*, *L. humile*, *C. floridanus* and *S. invicta* (Table 3.1). Similarly, the majority of pseudogenes are found in only a few species, most notably the two invasive ant species

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L. humile and *S. invicta*, indicating a particularly high rate of gene turnover in these lineages. In contrast, Dipteran (*D. melanogaster*, *A. gambiae*) and bee (*A. mellifera*, *B. terrestris*) genomes contain a lower number of functional and non-functional desaturase genes on average than most other insects, and do not show extensive lineage-specific expansions (Table 3.1). It is worth noting that the unequal taxonomic distribution of the lineages represented here may mask more ancient expansions in some cases, though. For instance, the expansion of Desat A1 in *B. mori* might have occurred early during Lepidopteran evolution, in line with the long branches characterizing this group of genes in our study (Figure 3.1). This is further supported by the finding of Knipple *et al*. (2002), which found orthologous copies of these genes in multiple Lepidopteran species. As genome data of more species is becoming available to fill taxonomic gaps, the pattern of gene gain and loss in other lineages might turn out to more closely resemble that observed in ants, with multiple independent episodes of expansion and contraction along their evolutionary trajectories.

The consistently low copy-number and lack of pseudogenes in the subfamilies Desat C, D and E as well as the subfamilies including *ifc* and *Cyt-b5-r* (Figure 3.1; Supplementary Figures S1–S2) suggest that these genes are housekeeping genes, possibly serving a basic function in lipid metabolism pathways. Unsaturated fatty acids, the products of the enzymatic activity of desaturases, are essential for many basic cellular functions, including energy storage, cell signaling and the regulation of membrane fluidity (Hazel & Williams 1990; Vigh *et al*. 1993; Tiku *et al*. 1996; Pyne & Pyne 2000; Miyazaki & Ntambi 2003). In contrast, high gene turnover rates in the other subfamilies are more indicative of lineage specific adaptations and potentially play an important role

in the phenotypical differentiation between clades (Olson 1999; Lynch & Conery 2000; Ranson *et al*. 2002; Robertson *et al*. 2003; Hahn *et al*. 2007; Khalturin *et al*. 2008; Colbourne *et al*. 2011; Voolstra *et al*. 2011). While both stochastic processes as well as selection influence the size of gene families, particularly large differences in gene family size between genomes may be attributed to adaptation (Hahn *et al*. 2005). We have previously shown that size variation of the acyl-CoA desaturase gene family along the insect phylogeny differs significantly from expectation due to neutral mutation and genetic drift (Simola *et al*. 2013). Such significant differences in gene family size between clades can be indicative of selection.

Gene duplication is thought to free one copy from the selective pressures operating on the ancestral gene; therefore we expected to find signatures of relaxed selective constraint or positive selection in the genes of the expanded desaturase subfamilies (Ohno 1970). Unexpectedly, the results of our signature of selection analysis provided little evidence for either, and instead revealed generally strong purifying selection acting on all desaturase genes in insects (Table 3.2). The ratio of non-synonymous to synonymous substitutions ω proved to be slightly higher in the expanded subfamilies Desat A2 and B than in the more conserved subfamilies, but still remains very low ($\omega < 0.25$). Desat A1 did not display an elevated ratio ω, despite harboring expansions in multiple lineages. Results from the more accurate site model analyses (Yang *et al*. 2000) revealed that the low average values of ω are due to an abundance of sites under purifying selection in contrast to only a small number of sites under neutral or weak positive selection. Since even recent gene duplicates from the same species differ by at least 10% on the amino acid sequence level, the low ratios of non-synonymous to

synonymous substitutions cannot be attributed to a lack of sequence variation. We also found no indication of strong differences in the selective pressure acting on lineages defined by large gene expansions in individual subfamilies. Although we were able to detect significantly higher values of ω in ant genes in comparison to genes from other taxa, the differences were small and remained indicative of strong purifying selection. The slight relaxation of selective pressure relative to other insects seems more likely to be intrinsic to ants than related to differences in gene turnover and gene family size because both more conserved (Desat C, D, and E) and more variable (Desat A1, A2, and B) subfamilies were affected equally. A reason for this might be the decreased efficiency of selection in ants due to the smaller effective population sizes typical for social insects (Crozier & Pamilo 1996; Gadau *et al*. 2012). The lack of positive selection in the single-copy desaturase subfamilies is in line with the hypothesis that these genes constitute housekeeping genes performing essential metabolic functions. The strength of purifying selection remains puzzling in the larger subfamilies, though, where redundant copies are expected to evolve more freely and acquire novel, lineage-specific functions. However, it is consistent with small ω ratios reported previously from desaturases in *D. melanogaster* (Keays *et al*. 2011) and *B. mori* (Knipple *et al*. 2002). These studies included *desat1* and *Bmpgdesat1*, two genes that have been shown to be involved in pheromone production in these two species, and members of the expanded subfamily Desat A1. This observation may indicate that although duplicated desaturase genes can change function rapidly it may not be due to fundamental changes in the enzymatic function and coding sequence of the gene, which would remain under strong purifying selection, but rather in the differential expression of these new copies in time and space.

Functional differentiation in ants

The comparison of the First Desaturase gene expression levels in three ant species provided evidence for both highly conserved as well as species-, sex- and caste-specific expression patterns depending on desaturase subfamily (Figure 3.4). Genes from subfamilies Desat D and E are expressed consistently across all categories, supporting the notion that they represent housekeeping genes involved in basic lipid metabolic processes. Desat C genes, which are present only in *H. saltator* and *C. floridanus*, are barely expressed in any category. This may indicate that these genes, which have been lost in many insect species including the bee and most ant species represented in this study, no longer fulfill an essential function in most insects.

In contrast to the single-copy genes mentioned above, genes of the expanded subfamilies Desat A1, A2 and B display variation in gene expression between species, and between sexes and castes within species. Notably, we see no consistent sex-, and caste-specific use of orthologous genes between ant species as has been observed for other genes expanded in ants like *vitellogenin* (Corona *et al.* 2013). Instead, the expression pattern in each species is largely dominated by either Desat A1 and A2 (*C. floridanus*), or Desat B genes (*H. saltator*, *P. barbatus*), but never both. Moreover, only Desat B genes of subclade II are expressed at high cumulative levels in *H. saltator* (with the exception of *desatB_b* of subclade I in virgin queens), whereas *P. barbatus* exhibits predominantly subclade I gene expression. As these data were compiled from several studies, the differences across species observed here might have been influenced by different laboratory conditions and RNA sequencing methodologies. However, *C. floridanus* and *H. saltator*, which exhibit some of the most pronounced gene

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expression differences discussed above, were raised in the same laboratory space at Arizona State University under highly similar conditions, and their RNA was sequenced at the same time by the same research group (Bonasio *et al*. 2010). Additionally, the expression levels of the reference genes within each species show only moderate differences (coefficients of variation ≤ 0.38), whereas the differences between species are somewhat more pronounced (coefficients of variation ≤ 0.70), thus preventing detailed quantitative comparisons. Notwithstanding, we can still compare expression patterns between species in terms of differences in the relative frequency of individual clades. For example, one notable pattern includes the Desat A1 subfamily, which is relatively frequent in *C. floridanus* and *H. saltator,* but almost undetectable in *P. barbatus* (Figure 3.4). Another, even more convincing example is the expression differences in Desat B Subclade I and Desat B subclade II between *P. barbatus* and *H. saltator* in all castes and sexes with the exception of *H. saltator* workers which have an overall reduced expression of all desaturase genes (Figure 3.4). In *P. barbatus*, Desat B subclade I is very prominently expressed whereas Desat B subclade II shows very little expression. In contrast, *H. saltator* shows the opposite pattern, which cannot be explained by generally lower expression in *P. barbatus*. In contrast to the previous two species, *C. floridanus* has very low expression of both Desat B subclades I and II (Figure 3.4). Hence, the caste and sex specific expression pattern between these three species representing three ant subfamilies (diverged approximately 100 mya) demonstrates that each lineage has radically modified the expression patterns of First Desaturase subclades.

The intraspecific expression differences between castes and sexes are not as extreme as the interspecific differences; however, there are significant and possibly

functionally relevant caste and sex differences (Figure 3.4). For instance, *desatB_b* is expressed almost exclusively in *H. saltator* virgin queens, but not workers or males, and only very lowly in gamergates (Figure 3.4). On the other hand, *desatB_a* expression is specific to *H. saltator* gamergates, whereas *desatB_d* is strongly overrepresented in virgin queen and males, which in turn express much more *desatB_f* than gamergates and virgin queens (Figure 3.4). Another example includes the worker-specific *C. floridanus* gene *desatA2_c*. These cases of genes originating from recent lineage-specific expansions with differential intraspecific expression patterns are suggestive of gene duplication followed by neofunctionalization (Ohno 1970). Another noteworthy observation regarding First Desaturase gene expression in ants is that these genes do not seem to fulfill metabolically essential roles, as workers in *H. saltator* display very low expression levels, particularly of Desat A1, A2 and B. This pattern of functional differentiation among lineages and within species, and the fact that genes stemming from expanded subfamilies do not seem to be essential for survival, fit the expectation of genes involved in the production of semiochemicals, as described for several desaturase genes in *D. melanogaster* (*desat 1*, *2*, *F*; Dallerac *et al*. 2000; Fang *et al*. 2002; Labeur *et al*. 2002) and *B. mori* (*Bmpgdesat1*; Moto *et al*. 2004).

Changes in the expression and number of acyl-CoA desaturase genes have been shown to affect the diversity of semiochemicals between closely related insect species (Takahashi *et al*. 2001; Knipple *et al*. 2002; Roelofs & Rooney 2003; Greenberg *et al*. 2006; Xue *et al*. 2007; Fang *et al*. 2009). In ants, the use of cuticular hydrocarbons in chemical communication is widespread (Hölldobler & Wilson 1990; Blomquist 2010), and unsaturated compounds like alkenes have been suspected of providing sufficient

diversity to act as key discriminatory compounds (Martin & Drijfhout 2009). Indeed, cuticular hydrocarbon profiles differ strongly between species, sexes, castes and developmental stages in ants, including *H. saltator*, *C. floridanus* and *P. barbatus* (Wagner *et al*. 1998; Liebig *et al*. 2000; Endler *et al*. 2004). For instance, cuticular hydrocarbon profiles, including unsaturated compounds, have been shown to undergo a shift when individuals transition from non-reproductive to reproductive status in *H. saltator* (Liebig *et al*. 2000), a change that may be correlated with the caste-specific gene expression pattern described above. Differences in the relative proportions of various alkenes have also been found between workers and queens of *P. barbatus* (Cash EI, unpublished data). As the evolution of castes in ants added another layer of complexity to the chemical communication system employed by insects, its genetic regulation might have been facilitated by novel genes with variable expression patterns. This case may thus provide an example for both structural and regulatory changes acting in concert to produce a new phenotype.

The production of cuticular hydrocarbons is not the only function of desaturase genes in insects. Desaturases have also been shown to be involved in the synthesis of bombykol (Moto *et al*. 2004) and other unsaturated compounds serving as volatile pheromones (Roelofs & Rooney 2003). Moreover, many components of insect chemical communication systems that have been studied in Coleoptera, Diptera, Lepidoptera, and social Hymenoptera include carbon-carbon double bonds (El-Sayed 2014), and thus presumably also require the activity of desaturases for synthesis. Many more may still await discovery, including a significant portion of the abundance of recruitment and alarm pheromones and other glandular secretions employed by ants, only a fraction of

which have been described (Hölldobler & Wilson 1990). Desaturase gene expansions in solitary species like *B. mori*, *N. vitripennis* and *T. castaneum* that rival those seen in ants may be involved in the production of such undiscovered compounds.

Furthermore, differences in the repertoire of insect desaturase genes may also be due to differences in diet and climatic conditions requiring changes in lipid metabolic pathways. Again, the large number and frequent turnover of desaturase genes in many insect lineages may reflect their enormous ecological success and diversity. This seems to be especially plausible for beetles (*T. castaneum*) and ants, two groups that have colonized nearly every terrestrial habitat, and are also extremely diverse in terms of their diet. For instance, ants include generalists (e.g., *C. floridanus*) and specialists (e.g., the leaf-cutters), herbivores, detritivores, omnivores (e.g., *C. floridanus*) and predators (e.g., *H. saltator*), and residents of diverse ecosystems ranging from deserts (e.g., *P. barbatus*) to tropical rainforests (e.g., the leaf-cutters), and thus rely on very different diets and are exposed to very different climatic conditions. The same is true for beetles, one of the most speciose, ecologically diverse, and successful insect lineages. Thus, changes in the desaturase gene repertoire may reflect changes in both ecological niches and chemical communication needs which arose during the evolution of various insect lineages. Indeed, novel genes have been shown to underlie lineage-specific adaptations including responses to changing environmental stimuli (Colbourne *et al*. 2011; Voolstra *et al*. 2011). Patterns of repeated size changes are also found in other gene families in ants, like P450 cytochromes and olfactory receptors, possibly for similar reasons (i.e., the need to detoxify or perceive compounds encountered in new environments, respectively; Simola *et al*. 2013).

Social organization alone may not require an expanded repertoire of desaturase genes, as demonstrated by the fact that the two bee species represented in this study revealed the smallest number of genes. As opposed to bees, however, ants display a much wider range of social complexity in terms of colony size, number of queens and queen-worker differentiation, and possess a more elaborate chemical communication system (Hölldobler & Wilson 1990). Combined with their ecological diversity, this may explain why the number of desaturase genes is so much higher in ants than in bees. The particularly high number of both functional and non-functional genes in *L. humile* and *S. invicta* may even be a testimony to these lineages' ability to quickly adapt to new ecological niches and changes in social organization in the past, features which more recently became instrumental in their success as invasive species.

Conclusions

Genomic organization and phylogeny testify that acyl-CoA desaturases represent an ancient gene family characterized by multiple episodes of expansion and contraction during evolution of insects. Subfamilies differ strongly in their degree of conservation and frequency of gene gain and loss, which also do not affect insect lineages equally. Ants display particularly large expansions in three First Desaturase subfamilies, in stark contrast to bees. Hence, eusociality itself cannot explain this pattern of disproportionate gene gain. As the number of genes in *N. vitripennis*, a solitary hymenopteran, rivals that of ants in some parts of the desaturase tree, the richness of desaturase genes in ants seems to be a part of the Hymenopteran heritage that has been lost in bees. What are the driving forces promoting and maintaining this rich gene repertoire in ants? Multiple causes

appear to provide plausible explanations: Variation in gene expression between ant species, and more importantly, between sexes and castes within species, suggest functional differentiation of these genes and a role in the regulation of reproductive division of labor in ants. Since First Desaturase genes found in these subfamilies are involved in the production of mating signals in *D. melanogaster* and *B. mori*, we hypothesize that the homologous genes in ants serve a role in the elaborate chemical communication system of ants. The expansions observed in ant First Desaturase genes may therefore have provided genetic raw material facilitating social evolution in ants as the evolution of castes and social organization added another layer of complexity to this system. On the other hand, ants also vary considerably in life history traits and the environment they live in, leading to very different diets and exposure to different climatic conditions. Desaturases could therefore have contributed to lineage-specific adaptations with regard to these differences, requiring changes in lipid metabolic pathways. Finally, the dynamic evolution of acyl-CoA desaturases may reflect changes in both ecology and chemical communication systems, responding to ecological diversification and an increased demand for chemical signal variability during ant evolution. This may provide an example for how gene family expansions can contribute to lineage-specific adaptations and how structural and regulatory changes act in concert to produce new adaptive phenotypes. Further studies elucidating the molecular function of acyl-CoA desaturases, and members of the expanded subfamilies in particular, are required to discern their significance for the ecology, chemical communication, and social evolution in ants.

Materials and Methods

Identification of insect desaturase genes

Genome assemblies and predicted gene sets of 15 insect species, including seven ant species (*Acromyrmex echinatior*, *Atta cephalotes*, *Camponotus floridanus*, *Harpegnathos saltator*, *Linepithema humile*, *Pogonomyrmex barbatus* and *Solenopsis invicta*), three non-ant Hymenoptera (*Apis mellifera, Bombus terrestris* and *Nasonia vitripennis*), and representatives of Diptera (*Drosophila melanogaster* and *Anopheles gambiae*), Lepidoptera (*Bombyx mori*), Coleoptera (*Tribolium castaneum*), and non-holometabolous insects (*Acyrthosiphon pisum*) were obtained from their respective community databases (Supplementary Table S1).

We chose all ten acyl-CoA desaturase genes characterized by a fatty acid desaturase type I domain in *D. melanogaster* (Keays *et al.* 2011), namely CG15531, CG9743, CG9747, CG8630, *desat1*, *desat2*, *desatF*, *ifc* (*infertile crescent*), CG17928, and *Cyt-b5-r* (*Cytochrome b5-related*) as queries to find homologous sequences in the 14 other species. First, predicted gene sets were searched with BlastP (Altschul *et al.* 1997) using an e-value cut-off of 0.0001 to obtain the majority of putatively functional genes as identified by automatic annotation pipelines. In the case of *A. cephalotes*, *L. humile* and *P. barbatus*, manually annotated desaturase gene repertoires were already available based on previous work (Suen *et al.* 2011; Smith *et al.* 2011a; Smith *et al.* 2011b). To identify genes and gene fragments not represented in the predicted gene sets, we also searched the genome assemblies of all 15 species, and unassembled contigs for 8 of 15 species (Supplementary Table S1) with TBlastN using an e-value cut-off of 0.001. Genomic regions surrounding hits that were found to be lacking an existing gene model were then

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subjected to a GeneWise (Birney *et al*. 2004) analysis to predict the gene structure. The same strategy was used to guide manual editing of existing gene models that did not align well with the *D. melanogaster* query. Information about all genes used in this study, including their genomic location, is compiled in the supplementary material (Supplementary Table S2). Nucleotide and amino acid sequences are available from the authors upon request.

Genes were categorized as functional if they contained an open reading frame of at least 250 amino acids (approximately two thirds of the average desaturase gene length in *D. melanogaster*) and a fatty acid desaturase type I domain (IPR005804) according to InterPro (Hunter *et al*. 2009). Shorter genes or genes lacking this domain were classified as pseudogenes unless the truncation resulted from unresolved or misassembled sequence in the genome assembly. In rare cases, genes could not be assigned to either category, for example if a substantial part of the gene was masked by unresolved sequence. Fragments on very short scaffolds which were identical in sequence to parts of full-length desaturase genes were assumed to be assembly artifacts and excluded from the analyses.

Phylogenetic reconstruction

Preliminary phylogenetic analyses revealed that the *D. melanogaster* genes *ifc* and *Cyt-b5-r* bear little sequence similarity to the remaining desaturases and each other. To improve the quality of the alignment, we therefore excluded all *ifc* and *Cyt-b5-r* orthologs, as well as all pseudogenes and some ambiguous genes from the main phylogenetic analysis. The resulting amino acid dataset contained 170 genes classified as First Desaturases by Hashimoto *et al*. (2008) from 15 species, and was aligned with the

L-INS-i algorithm implemented in MAFFT version 7 (Katoh *et al*. 2002; Katoh & Toh 2008). To remove divergent and poorly aligned positions, we used Gblocks version 0.91b (Castresana 2000) on the lowest stringency settings, resulting in a final alignment of 218 amino acid positions. According to the Akaike Information Criterion corrected for small sample size, ProtTest version 2.4 (Abascal *et al*. 2005) revealed LG with four discrete gamma rate categories (Le & Gascuel 2008; Yang 1996) as the model of molecular evolution with the best fit to the data (models combining gamma rates and a proportion of invariable sites, G+I, were omitted from the analysis following the argument of redundancy put forward by A. Stamatakis, RAxML version 7.0.4 manual, page 20). Based on this model, a maximum likelihood tree was reconstructed with RAxML version 7.2.6 (Stamatakis 2006), and nodal confidence values obtained with 1000 rapid bootstrap replicates (Stamatakis *et al*. 2008).

Phylogenetic trees were computed as outlined above for Ifc (314 amino acid positions derived from 17 putatively functional genes) and Cyt-b5-r (384 amino acid positions derived from 25 putatively functional genes). All alignments are available from the authors upon request.

Signature of selection analysis

To evaluate the role of natural selection during the evolution of the desaturase gene family in insects, the ratios of non-synonymous (d_N) and synonymous (d_S) substitution rates were determined using codon substitution models implemented in the software package PAML version 4.4 (Yang 2007). For each First Desaturase subfamily identified in the main phylogenetic analysis (Desat A1 through E), we computed and trimmed an

amino acid alignment using MAFFT and Gblocks as described above, and converted it into a codon alignment with PAL2NAL version 14 (Suyama *et al.* 2006). Maximum likelihood trees were calculated for each subclade using RAxML based on the LG model as outlined above, and used to inform the PAML analyses alongside the codon alignments. Genes of each subfamily were then analyzed using the following site-, branch- and branch-site-specific models of codon substitution:

The basic model M0 assumes that the ratio $\omega = d_N/d_S$ is invariable among sites and branches (Goldman & Yang 1994). In contrast, the site-specific models M7 and M8 are based on the more realistic assumption that ω varies among sites, but not branches (Yang *et al.* 2000). As M8 allows for a fraction of sites to be under positive selection ($\omega > 1$), but M7 does not, this pair forms a likelihood ratio test (LRT) of positive selection with degrees of freedom (df) = 2. We also investigated whether selection acted differently on desaturases in ants, *B. mori* and *T. castaneum*, because these taxa are characterized by especially large gene expansions. To this end, we first applied an LRT with df = 1 comparing M0 and a branch-specific model estimating ω separately for branches specified *a priori* (foreground branches, i.e., all branches leading to and within ants, or *B. mori* and *T. castaneum*, respectively) and the background branches. The latter model, which allows for positive selection, was also tested (df = 1) against a null model which fixes ω at 1 to determine whether ω is significantly higher than 1 along the foreground branches (Yang 1998). Finally, we investigated whether only some sites are under positive selection along the foreground branches by applying the branch-site test of positive selection. This test (df = 1) is based on a branch-site model, which allows ω to

vary both among sites and branches, and a null model which caps ω at 1 (Yang *et al*. 2005). Substitution rates were not studied in Ifc and Cyt-b5-r genes.

Gene expression analysis

Sex- and caste-specific desaturase gene expression levels were gathered from RNA-seq data of three ant species, *H. saltator*, *C. floridanus* and *P. barbatus* and. RNA-seq data of *H. saltator* and *C. floridanus* was obtained from Bonasio *et al*. (2010) and are described there in more detail. Briefly, pools of non-reproductive (*H. saltator*) or minor (*C. floridanus*) workers of various ages, queens and males were used to construct cDNA libraries from poly-A RNA and sequenced on a Illumina 1G Genome Analyzer (Illumina, San Diego, CA) with a paired-end module. Reads were aligned to the most recent genome assemblies using TopHat (Kim *et al*. 2013), and read counts expressed as RPKM values to account for differences in gene length and total number of reads (Mortazavi *et al*. 2008).

P. barbatus RNA-seq data was acquired individually from two adult workers and two virgin queens. cDNA libraries were constructed from poly-A RNA using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA) and sequenced from both ends on the HiSeq 2000 Sequencing System (Illumina, San Diego, CA) with current v3 chemistry. Reads were aligned to the *P. barbatus* genome assembly v1.0 using TopHat v2.0.8 (Kim *et al*. 2013) and mapped to the Official Gene Set v1.2 using Cufflinks v2.0.2 (Trapnell *et al*. 2010). RPKM values were calculated from read counts as above, and averaged across worker and queen datasets, respectively.

Whereas the *H. saltator* and *C. floridanus* datasets were obtained from specimens raised in the same laboratory and by using the same sequencing protocol, the *P. barbatus* data were acquired under different conditions. In order to control for these differences, we determined gene expression levels (as RPKM values) and variance of seven housekeeping genes commonly used to normalize gene expression across samples (Scharlaken *et al*. 2008; Cheng *et al*. 2013). Four of these, including actin and GAPDH, proved to be highly variable within species, which is in line with previous studies advising against their use (reviewed in Bustin 2000). The remaining housekeeping genes, EF1-beta, RPL18 and RPL13A, showed consistency within species (coefficients of variation ≤ 0.38), and to a lower extent, between species (coefficients of variation ≤ 0.70), and were thus used as reference genes allowing the comparison of First Desaturase gene expression levels both within and between species.

Supplementary Material

Supplementary figures S1–S3 and supplementary tables S1–S2 are available at *Molecular Biology and Evolution* online (http://mbe.oxfordjournals.org/content/suppl/2014/12/03 /msu315.DC1).

Acknowledgments

Table 3.1. Number of putatively functional and pseudogenized (in parentheses) acyl-CoA desaturase genes in insects.

	Desat A1	Desat A2	Desat B	Desat C	Desat D	Desat E	Ifc	Cyt-b5-r	**Total**
Acyrthosiphon pisum	4	2	2	1	0	1	1	2 (1[b])	13 (1[b])
Tribolium castaneum	2	9 (1)	1	1	1	1	1	2	18 (1)
Bombyx mori	8	1	7	2 (1)	1	1	1	2	23 (1)
Drosophila melanogaster	3	1	1	0	1	1	1	2	10 (0)
Anopheles gambiae	2	1	3 (1)	1 (1)	1	1 (1)	2	2	13 (3)
Nasonia vitripennis	2	1	8	4	0	1	1	2	19 (2[a])
Apis mellifera	1	4 (1)	1	0	1	2	1	2	12 (1)
Bombus terrestris	1	1	1	0	1	1	1	1	7 (1[a])
Harpegnathos saltator	2	2	8	1	1	1	1	1	17 (0)
Camponotus floridanus	1	3	6	1	1	1	1	3 (2[b])	17 (4[ab])
Linepithema humile	1 (1)	1 (5[b])	7 (22)	0	1	1	1	1	13 (28[b])
Pogonomyrmex barbatus	1 (3)	3 (2)	4 (2)	0	1	1	1	1	12 (7)
Solenopsis invicta	5 (18)	3	2 (1)	0	1	1	2	2 (3)	16 (22)
Atta cephalotes	1	2	6 (2)	0	1	1	1	1	13 (2)
Acromyrmex echinatior	1	2	8	0	1	1	1	1	15 (0)
Total	35 (22)	36 (10[b])	65 (28)	11 (2)	13 (0)	16 (1)	17 (0)	25 (6[b])	

Note — [a] includes pseudogenes that could not be assigned unambiguously to a particular desaturase subfamily, [b] includes partially sequenced or otherwise ambiguous genes which may be either functional or pseudogenized.

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Table 3.2. Signatures of selection acting on First Desaturase genes in insects. Select parameter estimates and likelihood ratio test (LRT) results are shown for each of the six subfamilies.

Model – parameters	Desat A1 (*desat1*)	Desat A2 (CG8630)	Desat B (CG9747)	Desat C –	Desat D (CG9743)	Desat E (CG15531)
Basic/site models						
M0: ω	0.09	0.13	0.21	0.08	0.09	0.14
M7: p, q	0.65, 5.09	0.51, 2.50	0.62, 1.81	0.78, 5.23	0.33, 2.18	1.15, 6.03
M8: p_1, ω	0.00, 3.74	0.02, 1.08	0.00, 1.00	0.01, 1.18	0.06, 1.00	0.01, 1.00
Branch models (ants)						
B0: ω_0 ($\omega_1 = 1$)	0.01	0.09	0.08	0.05	0.04	0.10
BA: ω_0, ω_1	0.05, 0.14	0.10, 0.16	0.11, 0.27	0.06, 0.15	0.05, 0.12	0.11, 0.16
Branch-site models (ants)						
A0: p_{2a} ($\omega_2 = 1$)	0.07	0.10	0.08	0.16	0.03	0.12
AA: p_{2a}, ω_2	0.07, 1.00	0.10, 1.00	0.08, 1.00	0.16, 1.00	0.03, 1.00	0.12, 0.09
LRT, P						
M7 vs. M8	1	**0.037**	1	0.678	**0.001**	0.698
M0 vs. BA	**0.010**	**<0.001**	**0.001**	**<0.001**	**<0.001**	**<0.001**
B0 vs. BA	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
A0 vs. AA	1	1	1	1	1	1

Note — ω: ratio of non-synonymous to synonymous substitution rates, p, q: beta distribution shape parameters (M7), p_1: proportion of sites under positive selection (M8), ω_0, ω_1: background and foreground ω values, respectively, P: likelihood ratio test P-value (significant results in bold).

Figure 3.1. Reconstruction (part 1) of the phylogeny of insect First Desaturase genes, illustrating the division of the gene family into six subfamilies. Given its large size, the tree was separated into two parts for better viewing. The dotted line indicates where this separation was made. See part 2 (Figure 3.2) for the remaining clades: Desat A2 (CG8630) and Desat A1 (*desat1, 2, F*). The unrooted maximum likelihood tree was obtained from 170 genes of 15 species, with confidence values at the edges derived from 1000 rapid bootstrap replicates. Gene names follow the updated nomenclature proposed in this study, except for genes that have previously been characterized in the literature (in bold). Species are indicated by four-letter prefixes as follows: Aech = *Acromyrmex echinatior*, Acep = *Atta cephalotes*, Cflo = *Camponotus floridanus*, Hsal = *Harpegnathos saltator*, Lhum = *Linepithema humile*, Pbar = *Pogonomyrmex barbatus* and Sinv = *Solenopsis invicta* (all ants, in color), and Acpi = *Acyrthosiphon pisum*, Amel = *Apis mellifera,* Agam = *Anopheles gambiae*, Bmor = *Bombyx mori*, Bter = *Bombus terrestris*, Dmel = *Drosophila melanogaster*, Nvit = *Nasonia vitripennis* and Tcas = *Tribolium castaneum*.

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Figure 3.2. Reconstruction (part 2) of the phylogeny of insect First Desaturase genes, illustrating the division of the gene family into six subfamilies. See part 1 (Figure 3.1) for the remaining clades: Desat E (CG15531), Desat D (CG9743), Desat C (no Dmel ortholog), and Desat B (CG9747). The unrooted maximum likelihood tree was obtained from 170 genes of 15 species, with confidence values at the edges derived from 1000 rapid bootstrap replicates. Gene names follow the updated nomenclature proposed in this study, except for genes that have previously been characterized in the literature (in bold). Species are indicated by four-letter prefixes as follows: Aech = *Acromyrmex echinatior*, Acep = *Atta cephalotes*, Cflo = *Camponotus floridanus*, Hsal = *Harpegnathos saltator*, Lhum = *Linepithema humile*, Pbar = *Pogonomyrmex barbatus* and Sinv = *Solenopsis invicta* (all ants, in color), and Acpi = *Acyrthosiphon pisum*, Amel = *Apis mellifera,* Agam = *Anopheles gambiae*, Bmor = *Bombyx mori*, Bter = *Bombus terrestris*, Dmel = *Drosophila melanogaster*, Nvit = *Nasonia vitripennis* and Tcas = *Tribolium castaneum*.

Figure 3.3. Genomic organization of First Desaturase genes in insects: *Acromyrmex echinatior* (A), *Solenopsis invicta* (B), *Camponotus floridanus* (C), *Bombus terrestris* (D), *Drosophila melanogaster* (E) and *Bombyx mori* (F). Scaffold names refer to the genome assemblies listed in supplementary table S1, Supplementary Material Online. Closed scaffold symbols indicate scaffold ends, while open symbols indicate that the illustrated region is located away from scaffold ends. Colors and lower case letters are used to identify genes as presented in the phylogeny (Figures 3.1 and 3.2).
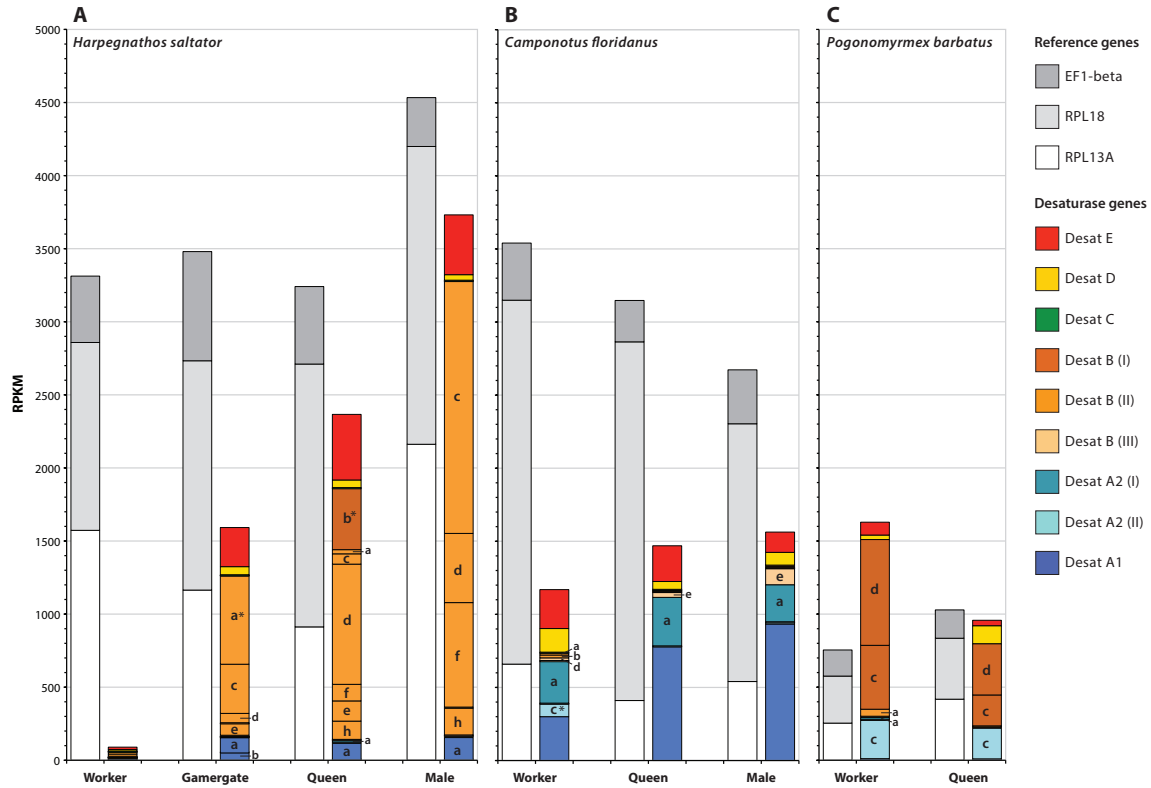
Figure 3.4. Gene expression levels of reference genes and First Desaturase genes in different sexes and castes of three ant species: *Harpegnathos saltator* (A), *Camponotus floridanus* (B), and *Pogonomyrmex barbatus* (C). Data is shown in reads per exon kilobase per million (RPKM), and were obtained from independent RNA-seq experiments. Medium grey, light grey, and white bars represent reference genes (EF1-beta, RPL18, and RPL13A respectively), while colored bars and lower case letters are used to identify desaturase genes as presented in the phylogeny (Figures 3.1 and 3.2). Asterisks indicate desaturase genes showing strong differential expression in a particular caste within a species. Also note that *C. floridanus* workers were of the 'minor' worker caste, queens of all three species were virgin (unmated) queens, and data was not available for *P. barbatus* males.

CHAPTER 4

GENETIC, DEVELOPMENTAL, AND SOCIAL INFLUENCES ON CUTICULAR

HYDROCARBON VARIATION IN THE RED HARVESTER ANT,

POGONOMYRMEX BARBATUS

Introduction

Communication among ants is predominantly olfactory in nature (Hölldobler, 1999), and

nestmate recognition typically occurs through chemical cues called cuticular

hydrocarbons (CHCs; Lahav *et al*. 1999; Wagner *et al*. 2000; Akino *et al*. 2004; Ozaki *et

al*. 2005; Martin *et al.* 2008). CHCs are believed to have evolved for the primary function

of maintaining water balance and preventing desiccation (Gibbs, 1998), but were later

coopted for insect recognition systems. In ants, although CHCs are primarily associated

with nestmate recognition, they are also important for reproductive and mate signals

(reviewed by Martin and Drijfhout, 2009). Nestmate recognition cues are complex

mixtures of the intrinsic chemical profiles of all workers (Hölldobler and Michener,

1980; van Zweden *et al*. 2009), the queen (Carlin and Hölldobler, 1986; Liebig *et al*.,

2000), and the environment (Liang and Silverman, 2000; Tissot *et al*., 2001, Wagner *et

al*., 2001), which results in a "gestalt" odor unique to each colony (Crozier and Dix,

1979). Although much is known about ant nestmate recognition systems, many questions

still remain about the relative contributions of genetics, development, and environment,

to the individual and colony-level CHC profile of many species. Furthermore, the

availability of numerous ant genomes in the past 5 years provide new exciting tools for

genetic studies of CHCs (Tsutsui 2013). In order to explore these genetic effects,

however, it is first necessary to disentangle the effects of other factors of CHC variation

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such as caste, population, development, and environment. With this in mind, and with the goal of performing future, functional genetic studies of CHCs in *Pogonomyrmex barbatus*, I carried out a descriptive analysis of two genetic components, and one developmental/environmental component of CHC trait variation in *P. barbatus*.

An important step to understanding the genetic architecture and evolution of nestmate recognition cues was through a collaboration with the *P. barbatus* genome project (Smith *et al*. 2011b) as well as three other ant genome projects (Smith *et al*. 2011a; Suen *et al*., 2011; Simola *et al*., 2013). In these studies, I used manual annotation and phylogenetic analyses to identify 170 first desaturase genes across seven ant species as well as eight other insect species (Chapter 3; Helmkampf *et al*. 2014). Among these, I identified ten candidate desaturase genes potentially involved in CHC alkene biosynthesis and recognition cues in *P. barbatus,* which includes *Pbar_desat1*: a gene that is orthologous to known *Drosophila* desaturase genes involved in cuticular alkene biosynthesis (Dallerac *et al*. 2000; Fang *et al*. 2002; Labeur *et al*. 2002). Differences in the relative proportions of various cuticular alkenes have also been found between workers and queens of *P. barbatus*. Given these findings, I tested for the relationship between desaturase gene expression and CHC alkene abundance among *P. barbatus* queens and workers.

Another interesting, potential source of genetic influence on CHC profile variation in *P. barbatus* is the presence of genetically distinguishable castes, i.e., a genetic caste determination (GCD) system, wherein workers feature a genotype distinct from the queens (Helms Cahan *et al*. 2002; Julian *et al*. 2002; Volny & Gordon 2002). GCD populations are selectively disbursed throughout *P. barbatus* populations with

environmental caste determination (ECD), and are believed to have evolved from complex hybridization events between *P. barbatus* and its 'sister species' *P. rugosus* (Helms Cahan & Keller 2003). As a result, the *P. barbatus* system consists of a 'pure species' ECD lineage, and two *P. barbatus*/*P. rugosus* hybrid GCD lineages (J1 and J2). Since the discovery of these hybrid genetic lineages within *P. barbatus*, new questions have arisen about lineage-related differences leading to CHC variation. One important feature currently known about the genetics of these three lineages is that J1 and J2 (GCD) lineages are dependent on each other in order to produce queens and workers (but not males, since they develop from haploid, unfertilized eggs). Given this, selection should act on gynes such that they successfully mate with males from each lineage so that they can achieve a fully functional colony (i.e., production of workers, new gynes, and males). On the other hand, males should be selected to mate only with gynes of the same lineage, since interlineage matings result in an evolutionary dead-end (i.e., workers).

Investigating the role of genetic lineages in CHC variation will be a significant aspect of understanding the genetic bases of CHCs and recognition systems in *P. barbatus*. To date, only one study has investigated the role of genetic lineage in CHC variation in *P. barbatus*, which was restricted to a small sample (n = 13) of only males (Volny *et al*. 2006). Here, I expand on previous findings of lineage differences in males, and explore CHC variation in a larger sample consisting of males and gynes of both lineages (J1/J2). Furthermore, given that *P. barbatus* workers from GCD lineages are J1/J2 hybrids, it will be important to determine what, if any, contribution maternal lineage makes to worker CHC variation. Field studies in the sister species, *P. rugosus,* have shown no significant differences in the worker interaction between GCD and ECD

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lineages or within GCD lineages (Julian & Helms Cahan 2006), but the influence of lineage on CHC variation is not yet known for GCD *Pogonomyrmex* workers.

Finally, in addition to genetic components, CHCs may also be affected by a variety of developmental and environmental factors. To date, several studies have looked at CHC variation in *P. barbatus,* and identified effects such as: worker task-group (Wagner *et al*. 1998; Sturgis & Gordon 2013), worker colony membership (Wagner *et al*. 1998; Sturgis & Gordon 2013), worker task-related environment (Wagner *et al*. 2001), and colony rearing conditions (Tissot *et al*. 2001). Missing from these studies, however, is a basic understanding of how *P. barbatus* ontogeny affects CHC variation. Initial studies of CHC variation in *P. barbatus* (Wagner *et al*. 1998) found that field-collected workers performing tasks outside the nest (i.e., foragers and patrollers) tend to have a relatively lower proportion of CHC methylalkanes and alkenes when compared with inside-nest workers (i.e., nest maintenance workers). In a follow-up experiment Wagner *et al*. (2001) showed that temperature and humidity can significantly contribute to this variation, such that hotter/drier conditions increase the abundance of cuticular *n*-alkanes, therefore reducing the relative proportions of cuticular methylalkanes and alkenes. These findings were necessary for understanding how environmental differences affect CHC proportions within a colony among task-groups. Since then, however, little has been done to show how other factors like ontogeny and social environment contribute to CHC variation in *P. barbatus*. Previous studies of ant CHC ontogeny in *Aphaenogaster senilis* have shown that quantities of CHCs can increase with individual age (Ichinose & Lenoir 2009), and that proportions of CHCs in *Camponotus aethiops* vary depending on genes and environment (van Zweden *et al*. 2009). In order to gain an understanding of the role

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of specific genes in CHC biosynthesis, it was first necessary to determine the development of the recognition cue phenotype, i.e., the CHC profile. Doing so allowed me to identify the relative contributions of worker age and social environment to CHC profile variation, which were important for future studies of gene function and CHC biosynthesis.

Materials and Methods

Cuticular alkene and desaturase gene expression analysis

To study the relationship between desaturase gene expression and cuticular alkene abundance, I collected foundress queens the morning after a mating flight which occurred in August 2014 at Scottsdale Community College (SCC; Latitude: 33.516804, Longitude: -111.879994). Newly mated *P. barbatus* foundresses were identified and collected as they actively dug nests in the soil. To avoid injuring the foundresses, I waited for queens to emerge naturally during the digging process, carefully picked each up with featherweight forceps, and placed them separately into collection tubes. Upon returning to the lab, foundresses were then transferred to glass tubes half-filled with water and stoppered with cotton balls, and reared in complete darkness at 30°C for five weeks until CHC profiles and desaturase gene mRNA levels could be sampled. An additional sample of workers were collected from two-year-old, lab-reared colonies, each containing one queen, brood, and ≈ 500 workers (rearing conditions described in detail below).

Measurements of CHC (alkene) abundance were acquired with solid-phase micro extraction (SPME), by gently rubbing a fiber (SUPELCO, coated with a 30μm polydimethylsiloxane film) for 5 min on the dorsal gaster of each queen (n = 31)) and

worker (n = 12). This non-fatal technique was preferable over solvent techniques to ensure that mRNA levels were not significantly degraded during CHC sampling. CHC coated fibers were then inserted into the injection port of a gas chromatograph (GC) equipped with a flame ionization detector (FID) to determine the abundance and proportions of cuticular alkenes present. The integrated CHC peaks areas were measured with Enhanced ChemStation (Agilent Technologies 2005), and the peaks of cuticular alkenes were identified based on Kovats indices. The abundances of cuticular alkenes were them summed together based on double-bond position (i.e., Z5, Z7, or Z9) for each peak, and used in subsequent desaturase gene correlation analyses. Double bond positions in cuticular alkenes were determined from a separate sample of 10 gynes using a dimethyl disulfide (DMDS) protocol previously described by Liebig *et al*. (2009). The abundances of cuticular alkenes were them summed together based on double-bond position (i.e., Z5, Z7, or Z9) for each peak, and were statistically analyzed with the statistical package R (version 3.2.2) using MANOVA to determine the effects of caste on levels of alkenes between queens and workers. Finally, *post hoc* one-way ANOVAs (corrected for multiple comparisons) were used to determine which alkene classes showed significant differences in the abundance between *P. barbatus* queens and workers.

Immediately after CHCs were collected, ants were placed separately in micro centrifuge tubes and snap frozen in liquid nitrogen to preserve mRNA levels. Expression of three internal reference genes and 9 desaturase genes were measured by quantitative reverse transcriptase PCR (qRT–PCR), using the oligonucleotide primers listed in Table 4.1. Desaturase gene expression was normalized to the three internal reference genes

(*ef1beta*, *RPL13A*, and *RPL18*; Table 4.1), and expression differences between queens and workers were statistically analyzed using the ΔΔCT method.

CHC sampling and genetic lineage (J1/J2) analysis

To assess the role of genetic lineage (J1/J2) on CHC profile variation, I collected live *P. barbatus* alates (reproductive ants), and foraging workers for CHC analyses. This occurred during the summer mating swarm (July 2013), also located at Scottsdale Community College (Latitude: 33.516804, Longitude: -111.879994). Alate gynes (virgin queens) and males were allowed to fly to the swarm and immediately collected (n ≈ 100 each sex) as individuals dropped to the ground. Two hours after sampling was complete, ants were then frozen and stored (-80°C) for later CHC and genetic lineage analyses. Because the lineages of alate gynes and males were unknown upon collection, they were selected randomly from stored samples during CHC collection and lineage analysis (n = 21 per per sex, total n = 42).

To collect CHC profiles, each ant (i.e., gyne or male) was placed in a glass vial containing 200 µL hexane and allowed to soak for 10 min. Ants were gently swirled in the vial for 10 sec at the beginning and end of the soak period. Hexane extracts containing dissolved CHCs were transferred directly to glass, microvolume inserts, and dried under a stream of high-purity nitrogen gas. CHC samples were finally redissolved in 20 µL of hexane, and 1 µL of the final sample was analyzed.

All CHC profiles were analyzed on an Agilent 6980N series gas chromatograph (GC) equipped with a DB1-MS (Agilent J&W) non-polar capillary column (length, 30 m; ID, 0.25 mm; film thickness, 0.25 µm) and connected to an Agilent 5975 mass selective

detector (MSD; −70 eV, electron impact ionization; Transfer Line 300°C; Quad 150°C; Source 230°C). Samples were injected using an automatic liquid sampler (ALS) in the splitless mode through the GC injection port set at 260°C. Helium was used as the carrier gas at 1 ml min−1. The column temperature was initially held at 60°C for 2 min before increasing to 200°C at a rate of 20°min−1, and then to 320°C at a rate of 5°C min−1, where it was finally held for 5 min. Hydrocarbons were characterized using a combination of diagnostic ions, Kovats indices, and comparisons to already published *P. barbatus* CHC profiles (Tissot *et al*. 2001). To determine the effect of genetic lineage (J1 and J2) on CHC variation, I measured the peak areas of the 43 most abundant CHCs in Enhanced ChemStation (Agilent Technologies 2005), and analyzed the results through non-metric multidimensional scaling analysis (NMDS) of Euclidean distances calculated from the relative proportions of CHCs for each sample.

Genetic lineage was determined by isolating genomic DNA from males' gasters and queens' thoraces using the Chelex isolation method (Gadau 2009). DNA samples were then used in a standard PCR with primers ("LCO" and "HCO") that target amplification of a segment of the mitochondrial cytochrome oxidase 1 (*cox1*) gene. Partial *cox1* fragment amplifications were checked on a 1% agarose gel for expected fragment size (630-bp), and then sequenced. Resultant sequence chromatograms were checked for quality, and aligned with previously generated *Pogonomyrmex cox1* sequences (Anderson *et al*., 2006a) using the L-INS-i algorithm implemented in MAFFT v7 (Katoh, 2002). Sample sequences were manually trimmed to match the reference dataset, and a neighbor joining tree was reconstructed with MAFFT v7. Nodal support values were obtained by a rapid bootstrap analysis of 1000 replicates. The final *cox1*

phylogeny allowed me to identify lineage relationships between my samples and ones previously classified (Anderson *et al.*, 2006a).

Colony rearing and ontogeny experiment

For the study of development and social environment, workers form lab-reared colonies of *P. barbatus* were used. The study was conducted in the summer of 2014 with two-year-old colonies each containing one queen, brood, and ≈ 500 workers. Queens were acquired similar to the methods described for collection of desaturase gene foundresses, differing only in the collection date, July 2012, and the specific location of the swarm at SCC (Latitude: 33.516775, Longitude: -111.882810). Colonies were maintained in the lab at 30°C in stacked plastic containers (5 levels, each level 4 in × 4 in × 2 in high) and connected interiorly with plastic tubing (ID: 1/2 in). Each container/level was lined on the inside walls with Fluon ® to prevent escape of ants, and stocked with 2–4 water-filled tubes to maintain hydration. All three bottom levels were coated exteriorly with mat-black spray paint to minimize light infiltration, and the top two levels were left clear to allow for light. In all colonies, the queen and brood naturally stayed into the lower (i.e., darker) levels, and the workers moved throughout all levels as needed. Colonies were fed *ad libitum* with seeds (a 50:50 mixture of Kentucky bluegrass and Nyjer), and supplemented once a week with artificial ant diet (Bhatkar & Whitcomb 1970).

Six *P. barbatus* lab-reared colonies were selected based on similar worker numbers (≈500 per colony, with representatives of both GCD lineages (J1 and J2; n = 3 each). Genetic lineage had been previously determined by sampling workers (n = 2) from each colony using the same methods described earlier. At the start of the experiment,

newly eclosed adult workers (callows, n = 10) were collected and marked with paint dots

and wire belts tied around the petiole (paint mark: Sharpie ® oil-based paint markers;

wire belts: 34 gauge, silver plated, colored wire) to keep track of individuals and their

respective ages. After the initial CHC sample was collected (T = 0 days; SPME method

described below), half of the marked workers from each colony were then placed in a

social isolation treatment (n = 30), and the other half were placed back into their natal

colony (n = 30). Isolation nest conditions were similar to full-colony nests, except for the

number of levels provided (i.e., one black level on the bottom and one clear level on top),

and the complete lack of queen, nestmates, and brood. Feeding and watering regimes

were kept identical. To test for the effect of social interacts on CHC profile development,

each isolated worker was reared alone into an isolation nest for the duration of the

experiment, only being removed briefly from treatment for CHC sampling (7 samples,

< 10 min each). Using non-fatal, solid-phase micro extraction (SPME; described above),

I sampled each marked worker's CHCs at eight time points throughout treatment (T = 0,

1, 3, 5, 7, 10, 20, and 30 days), and determined the abundance and proportions of CHCs

present using a gas chromatograph equipped with a flame ionization detector (GC-FID).

The integrated CHC peaks areas were measured with Enhanced ChemStation

(Agilent Technologies 2005), and the 46 most abundant CHC peaks were used to

calculate the total abundance, proportional abundances, and Euclidean distances of CHCs

for each worker/time point. Average CHC abundances (i.e., the total profile) and relative

abundances of different compound classes (i.e., *n*-alkanes, alkenes, and methyl-branched

alkanes) were then compared between the treatment types. Repeated measures ANOVAs

(corrected for multiple comparisons) were used to determine the effects of age (i.e., post

eclosion developmental time point), social environment, and genetic lineage on levels of CHC compound classes. *Post hoc* Bonferroni tests were used to determine which groups showed significant differences in the amount of CHCs between *P. barbatus* workers. Finally, nonmetric multidimensional scaling (NMDS) analyses were used to look for overall patterns of CHC profile differences in younger (day 0) and older (day 30) workers.

Results and Discussion

Alkene abundance and desaturase gene expression

Study of cuticular alkene variation revealed that queens and workers have qualitatively similar alkene profiles, but differ in alkene abundance ($F_{1,45} = 22.458$, $P < 0.0001$; Wilk's $\Lambda = 0.38958$). *Post hoc* one-way ANOVA tests show that queens have a significantly higher abundance of Z7 alkenes compared with workers ($F_{1,45} = 14.661$ $P < 0.001$), but no significant differences were found between queens and workers for Z9 alkenes ($F_{1,45} = 4.0405$ $P = 0.0504$) or Z5 alkenes ($F_{1,45} = 2.2414$ $P = 0.1413$; Figure 4.1, A). After identifying variation in cuticular alkene abundance, I then examined variation in desaturase gene expression for the same set of samples. Despite the higher abundance of Z7 alkenes found in queens, only one of nine desaturase genes, i.e., *Pbar_desatA1*, showed higher expression in queens compared with workers, and minimally so (0.10 fold higher; Figure 4.1, B). The remaining eight desaturase genes showed higher expression in workers ranging from 0.10 fold to 2.62 fold more than queens (Figure 4.1, B).

Genetic lineage (J1/J2) effects on CHCs

Phylogenetic analysis of the mitochondrial *cox1* gene sequence revealed that 6 of 21 gynes (29%) and 7 of 21 males (33%) were of the J1 lineage, with the remaining gynes (15/21; 71%) and males (14/21; 67%) coming from the J2 lineage. A similar skew between the two lineages has been identified at this site before (Anderson *et al.* 2006b). NMDS analyses of alate CHCs show differences in the cuticular hydrocarbon profiles among the four groups tested (Figure 4.2); however, separation based on genetic lineage (J1/J2) only appears to occur among the males, with little to no lineage separation occurring in the gynes.

The CHC differences found in males could potentially serve as a mechanism for mating decisions in this GCD population of *P. barbatus* where queens are dependent on mating with both lineages. On the other hand, the lack of lineage differences found in gyne CHCs suggests that selection may be favoring a more diverse female signal in GCD populations. The GCD interlineage dependency likely serves as a selective force acting on mating behavior, and may allow gynes to discriminate between potential male mates. Recent studies of *P. barbatus* mating behavior in another GCD population showed that males transfer sperm at a significantly higher rate during intralineage matings compared with interlineage matings, and intralineage matings are significantly shorter than interlineage crosses (Herrmann & Helms Cahan 2014). These findings are indicative of antagonistic sexual coevolution, in which both males and gynes are capable of adjusting their mating behavior according to the type of cross (intra- versus inter-lineage), and suggests that each sex/lineage are using cues to discriminate between mates. In my study population, I found GCD lineage differences in CHCs between *P. barbatus* males, but not

in gynes. This suggests that CHCs may only be useful for *P. barbatus* gynes in making

mating decisions - at least for the GCD population studied here. Alternatively, the lack of

lineage-related differences found among gynes may be due to the small number of J1

gynes sampled (because of the strong lineage skew). Future work would need to be

carried out that directly investigate the potential use of CHCs in mating behavior in GCD

P. barbatus populations. Despite their function remaining unknown, this study shows that

GCD lineage affects CHC profile variation among *P. barbatus* males.

Ontogeny study

Analysis of CHC ontogeny shows that CHCs of *P. barbatus* are significantly more

abundant in older (20 -30 day) adult workers regardless of rearing treatment (Figure 4.3,

A), and that all four classes of CHC compounds (i.e., *n*-alkanes, alkenes, monomethyl-

alkanes, and dimethyl-alkanes) are significantly affected by worker age (Table 4.2). In

contrast, social environment only significantly affected worker alkenes and dimethyl-

alkanes. NMDS analysis of CHCs at T = 0 (i.e., 0 days post eclosion) show that workers

of both treatment groups initially showed high variation and overlap (i.e., no clear

separation) between the colonies and lineages tested (Figure 4.3, B). On day 30 (i.e., T =

30 days post eclosion), however, NMDS analysis shows that the treatment groups (i.e.,

colony versus isolation) had a clear effect on CHC variation, with separation occurring

between the two treatments (Figure 4.3, B). These results indicate that both ontogeny and

social environment both contribute to the "gestalt" nestmate recognition cues of

P. barbatus workers, whereas genetic lineage does not appear to have a significant

contribution to worker CHC variation. Future investigations of the genetic bases of cuticular hydrocarbon variation should be careful to control for these factors.

Conclusions

Given the established importance of CHCs in social insect communication, and specifically recognition systems, it was important to begin with an examination of *P. barbatus* hydrocarbons themselves, i.e., their production and composition. My investigation of the genetic, developmental, and social environmental factors affecting variation in CHCs within *P. barbatus* colonies showed that genetic differences related to CHC production are likely occurring at multiple levels including population (i.e., J1/J2 lineage differences), sex (i.e., gyne versus male differences), and caste (i.e., queen versus worker differences). Furthermore, differences between queen and worker desaturase gene expression suggest that these genes may be important for recognition systems (i.e., queen reproductive signals and/or nestmate recognition cues), with *Pbar_desatA1* being of particular interest for future functional studies. Future investigations of desaturase gene function in *P. barbatus* may be best focused on their role in alate (gyne) and queen CHC production, since these castes show the highest abundance and variation in cuticular alkenes. Alternatively, functional studies of desaturase genes might also be successful in targeting alkene production in either late pupal or late-stage callow workers. Given the significant effects of ontogeny on CHC abundance (Figure 4.3, A), targeting desaturase genes in early developmental stages might show greater effects on alkene abundance if this development is disrupted. Finally, manipulation of the social environment must be carefully considered in the context of CHCs and recognition systems, as both may be

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affected by such a change. Similar findings have been reported in *Aphaenogatser senilis* where postpharyngeal gland (PPG) hydrocarbons significantly increased in social groups compared with isolated workers, but cuticular hydrocarbons did not (Ichinose & Lenoir 2009). My findings additionally support the role of the colony (i.e., the social environment) in the "gestalt" model of nestmate cues (i.e., CHCs), but also suggest the importance of innate components (i.e., genetics and development) in ant CHCs.

The genetics of recognition systems in social insects is largely unexplored. This poses a gap not only in eusocial insect biology, but also in studies of complex adaptive systems such as chemical communication. My findings concerning the genetic, developmental, and social basis of recognition cues provide insight into the major drivers of the evolution of colony recognition and social insect communication, while laying the necessary foundational work for future genetic examinations. Functional genetic studies (e.g., using techniques such as RNA interference) hold considerable promise for understanding the genetic bases of social insect nestmate recognition systems, and have been successfully implemented in studies of *Drosophila* CHCs (Dallerac *et al*. 2000; Fang *et al*. 2002; Labeur *et al*. 2002). Using these tools to further the advancement of molecular studies of CHC production, e.g., acyl-CoA desaturases, in social insects offer exciting opportunities to discern their significance for ecology, chemical communication, and social evolution in ants.

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Table 4.1. Desaturase gene and mitochondrial *cox1* oligonucleotide sequences, target fragment lengths, and qPCR metrics for gene expression and genetic lineage studies.

Target Gene	Oligo Sequence	Fragment Length (bp)	qPCR Efficiency (%)	R^2
Pbar_desatA1	Forward: CCAGAAGTCAGGGAGAAGGG Reverse: TTCGTCCATGTCTCGTTCCA	120	98	0.99797
Pbar_desatA2_a	Forward: AAACCCATGTACGCCTGGTA Reverse: TATAGCTGTCGGTGGAGCTG	134	102	0.99946
Pbar_desatA2_b	Forward: CCATATAATGCACGCCGAGG Reverse: ATCGCTGCAATCAATCGTGG	105	108	0.99789
Pbar_desatA2_c	Forward: TGGGGCGAAAGTTTCTGGTA Reverse: AGACTGCAGCATTGTTGACG	95	103	0.99964
Pbar_desatB_b	Forward: ACTTGGCTTGTACCGTCTCA Reverse: AGATCGGGGTTTGGTGTCTT	96	106	0.99530
Pbar_desatB_c	Forward: CTGGTTGTAACTGGCGAAGG Reverse: CGATAAAGAACGCCGAGTGA	109	102	0.99812
Pbar_desatB_d	Forward: TTGCGATACTGATGCCGATC Reverse: TTCATCATCACCCAGCCACA	75	101	0.99717
Pbar_desatD	Forward: AGATGATGAGCCAGCGATCA Reverse: TCGCTTTGTACGCCTTATGC	137	102	0.99812
Pbar_desatE	Forward: CTGCACGCCTGATGATAACA Reverse: CGTCAACCGGGAATTTGTCT	96	95	0.99896
Pbar_ef1beta	Forward: CTCAACACCTACCTCGCTGA Reverse: TTCGCCGGGAAGTGACTTAA	168	104	0.99851
Pbar_RPL13A	Forward: ACTTGGACGATTGGCTGCTA Reverse: GGACCACGTGCAGGATTTAC	165	105	0.99947
Pbar_RPL18	Forward: CGATGACGCCAGGATCTTTG Reverse: ACCACCAGCCTTCAAAATGC	94	103	0.99185
CO1	LCO: GGTCAACAAATCATAAAAGATATTGG HCO: TAAACTTCAGGGTGACCAAAAAATCA	630	NA	NA

Table 4.2. Repeated measures ANOVA effects of ontogeny, social environment, and colony of origin on CHC abundance in *P. barbatus* workers. Significant effects are highlighted in **bold**.

Treatment	CHC class	*df*	*F*	*P*
Ontogeny (age)	*n*-alkanes	7	50.24	**<0.001**
	alkenes	7	36.33	**<0.001**
	monomethyl-alkanes	7	25.35	**<0.001**
	dimethyl-alkanes	7	12.54	**<0.001**
Social Environment	*n*-alkanes	1	2.72	0.100
	alkenes	1	5.65	**0.018**
	monomethyl-alkanes	1	1.91	0.168
	dimethyl-alkanes	1	4.03	**0.045**
Colony of Origin	*n*-alkanes	4	5.01	**0.001**
	alkenes	4	2.20	0.068
	monomethyl-alkanes	4	1.57	0.181
	dimethyl-alkanes	4	1.26	0.285
Error		385		
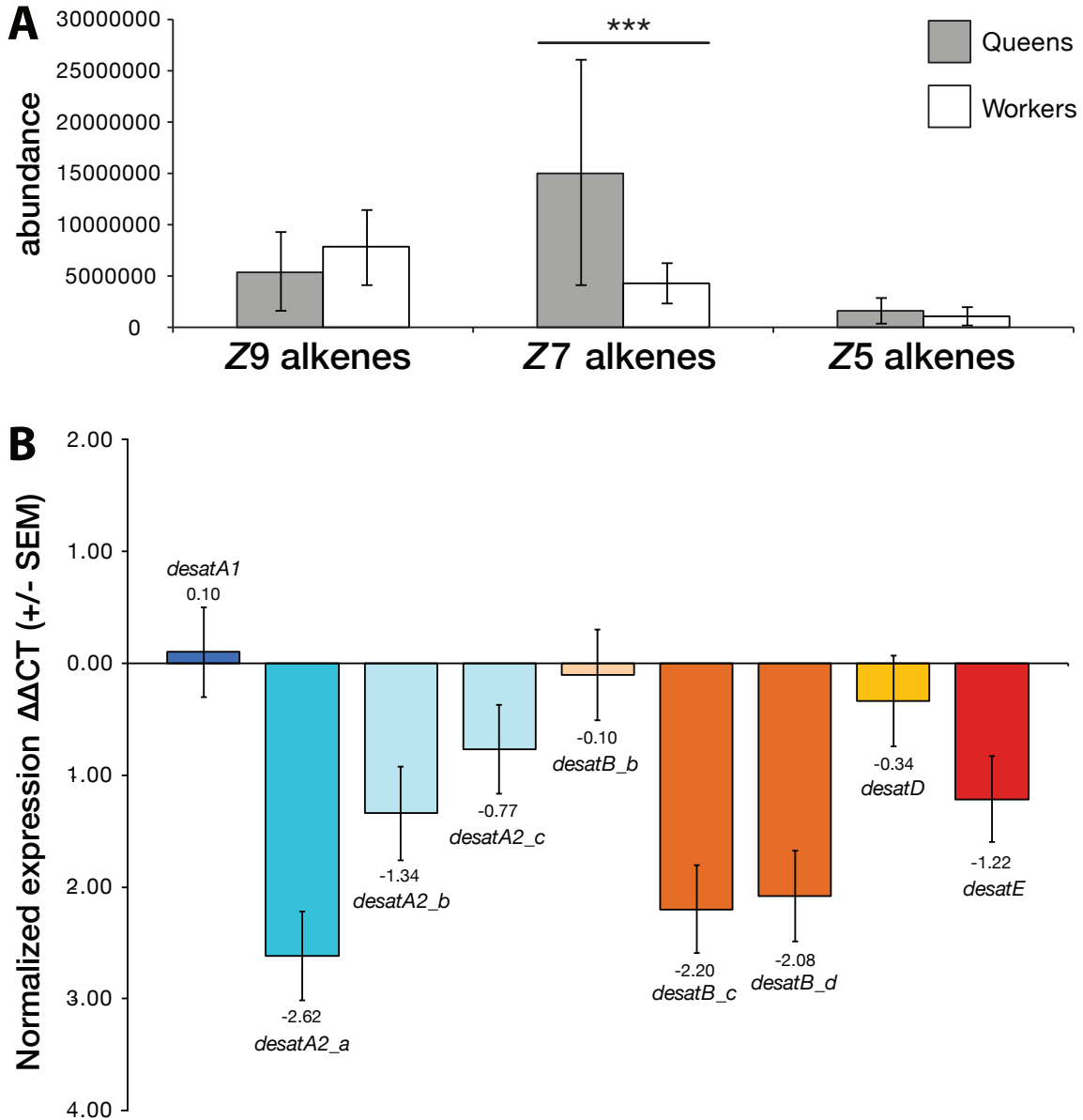
Figure 4.1. Cuticular alkene abundance and desaturase gene expression differences in *P. barbatus* queens and workers. (A) Mean cuticular alkene abundance (+/- SEM) in queens (gray bars) and workers (white bars). (B) Bar graph of normalized desaturase gene expression (+/- SEM) representing fold differences between queens and workers. Positive values indicate normalized expression that was higher in queens, whereas negative values indicate normalized expression that was higher in workers. Bars are color coded according to desaturase genes and their respective clades identified in Chapter 3 (Figures 3.1 and 3.2; Helmkampf *et al*. 2014)
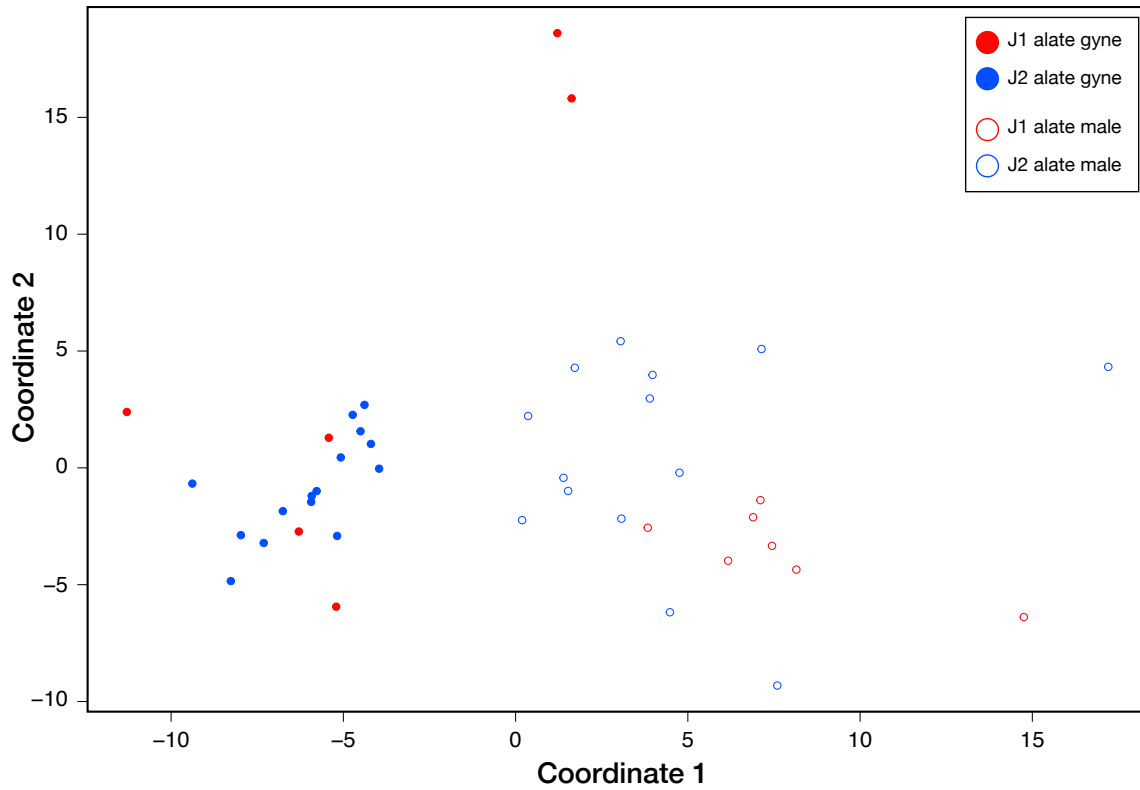
Figure 4.2. Non-metric multidimensional scaling analyses of CHC variation among alate gynes and males of two *P. barbatus* lineages (J1/J2). Gynes (filled circles) and males (open circles) are color coded according to their genetic lineage (J1 lineage = red; J2 lineage = blue).
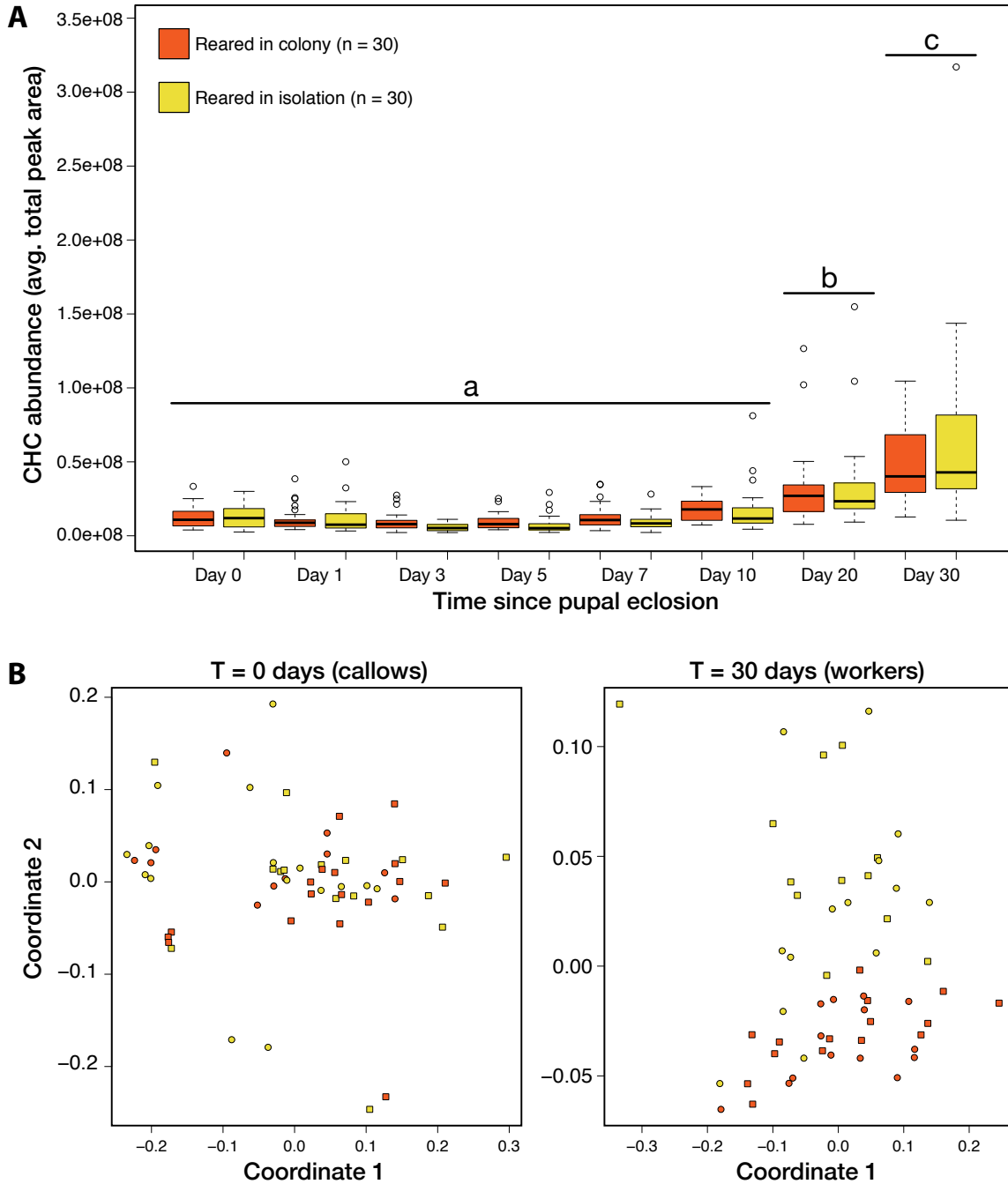
Figure 4.3. Effects of ontogeny and social environment on cuticular hydrocarbon variation in *P. barbatus* workers. (A) Boxplots (average, upper and lower quartiles, 95% confidence intervals, and outliers) representing the abundance of CHCs on *P. barbatus* workers across eight timepoints (days) after pupal eclosion. Workers were placed in one of two treatment groups: those reared in their natal colony (orange boxes), or those reared in isolation (yellow boxes). (B) NMDS plots of 46 most abundant peaks in *P. barbatus* worker CHC profiles at two timepoints (T = 0 days and T = 30 days) for both treatments (Colony/J1 = orange dots; Colony/J2 = orange squares; Isolated/J1 = yellow dots; Isolated/J2 = yellow squares).

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CHAPTER 5

CONTEXTUALIZING COMBAT: THE EFFECTS OF PRIOR EXPERIENCE,

CUTICULAR HYDROCARBONS, AND SEASONALITY ON NESTMATE

RECOGNITION BEHAVIOR IN THE RED HARVESTER ANT,

POGONOMYRMEX BARBATUS

Abstract

Territorial behavior, including aggressive interactions with neighbors, is adaptive when the benefits of securing resources outweigh the costs of defending a territory. Costs may come from conspecific neighbors encroaching on a territory or from floaters passing through. To reduce the costs of territoriality, organisms may restrict aggression to those who pose the greatest threat. A growing body of evidence shows that some group-living animals (including ants and termites) exhibit the "nasty neighbor" phenomenon, i.e., groups behave more aggressively towards individuals from neighboring groups than towards individuals from more distant groups. In ants, neighboring colonies may pose the greater threat, because recruitment and invasions can be achieved more easily, given their relatively close proximity. The nasty neighbor phenomenon has strong implications for the nestmate recognition systems of social insects that exhibit these territorial differences in behavior. In this study, I explored the effects of prior experience, cuticular hydrocarbons (CHCs), and seasonality on territorial aggressive behavior in the harvester ant *Pogonomyrmex barbatus*. I tested these three factors using two types of behavioral assays across three seasons. The first, a pair-wise aggression assay, tested the amount of aggression between worker-worker interactions of known experience and neighboring relations in *P. barbatus*. The second, a chemically coated "dummy" assay, used CHC

coated glass beads to determine their specific effects on the amount of aggressive behaviors exhibited by *P. barbatus*. My results show that *P. barbatus* worker ants are significantly more aggressive towards non-nestmate workers from neighboring colonies than non-nestmates from more distant colonies, and both prior experience with non-nestmates and familiarity with non-nestmates have significant effects on nestmate recognition behavior. Furthermore, when presented with glass beads coated with different CHC profiles alone, worker ants exhibited significantly more aggression towards neighboring colony non-nestmate CHC profiles than distant colony non-nestmate profiles, thus corroborating the findings of the paired, worker-worker behavior assays. Notably, however, these effects were strongly dependent on the season in which the workers were tested, suggesting that aggression is limited to seasons when colony growth and reproduction are important, and thus resource competition is high. These results indicate that *P. barbatus* shows qualities of the "nasty neighbor" phenomenon, with evidence for seasonal plasticity, and that variation in this territoriality aggression is due, in part, to associative learning of neighboring colony recognition cues (i.e., CHCs) with territorial experience.

Introduction

Territoriality is a ubiquitous phenomenon observed across a diverse range of animals. Territorial behaviors in ants may consist of inter/intraspecific signaling, monitoring, and aggression; they can be instinctive or learned, and primarily exist as a way of accessing and protecting valuable resources (Wilson 1975). Depending on the environmental conditions in which a population or species has evolved, the spatiotemporal distribution

of resources including: nutritional needs, nesting/dwelling materials and space, access to mates, and safety of offspring, among others, can effect territorial strategies (Hölldobler & Lumsden 1980; Kaufmann 1983). Given the complexity of these dynamics, territorial behaviors can vary widely across taxa, yet some notable patterns have emerged. In particular, two territoriality phenomena, one named the "dear enemy" effect (Fischer 1954) and the other named the "nasty neighbor" effect (Temeles 1990), involve the intraspecific relationships between neighboring biological entities, e.g., individuals, breeding pairs, and colonies.

The "dear enemy" effect describes a relationship between neighbors whereby each entity tends to direct significantly less aggression and territorial behavior towards their nearest neighbors than towards more distant neighbors or unsettled passersby (Fischer 1954). This phenomenon has largely been observed in solitary species (Temeles 1994), but also in several eusocial, ant species (Heinze *et al*. 1996; Beye *et al*. 1998; Langen *et al*. 2000; Pirk *et al*. 2001; Dimarco *et al*. 2010). Two hypotheses commonly cited when explaining the "dear enemy" phenomenon are the threat-level hypothesis and the familiarity hypothesis. The threat-level hypothesis argues that neighbors and strangers may be competing for different resources, and therefore represent different threats to an established territory holder. In this scenario, strangers may represent 'floaters' trying to find a new territory, and are thus perceived as a higher threat (Temeles 1994). The familiarity hypothesis, on the other hand, argues that relationships between neighbors become established as a result of habituation to the familiar neighbor, which leads to reduced aggression (Wilson 1975).

Conversely, the "nasty neighbor" effect, involves entities that direct significantly more intraspecific aggression towards nearest neighbors than more distant ones (Temeles 1994; Müller & Manser 2007). This phenomenon has been observed primarily in group-living taxa, but also some solitary species, including: termites (Dunn & Messier 1999), ants (Gordon 1989; Sanada-Morimura *et al*. 2003; van Wilgenburg *et al*. 2007; Thomas *et al*. 2007; Newey *et al*. 2010), mammals (Herbinger *et al*. 2007; Müller & Manser 2007; Schradin *et al*. 2010), and birds (Temeles 1990; Olendorf *et al*. 2004; Brunton *et al*. 2008; Yoon *et al*. 2012; Gentry & Jawor 2015). Several studies have argued that group-living likely contributes to this phenomenon, because groups compete for more resources than individuals and pairs, and, as group-size increases, resource demands increase (Müller & Manser 2007; Newey *et al*. 2010). In such a context, a conspecific "outsider" individual may represent a real threat, because additional members of the other group could be recruited to that territory if it is not aggressively defended. This is especially relevant for neighboring groups, where recruitment and invasions can be achieved more easily, because of their relatively close proximity. Given these findings, neighboring relationships may have significant effects on nestmate recognition in ants, because neighboring colonies may pose greater threats than distant colonies in environments where there is colony overlap.

In recent years, several authors have demonstrated notable intraspecific and intercolonial variation in territorial relationships, including plasticity in the direction of aggressive behaviors, e.g., variation between monodomous and polydomous populations of *Formica pratensis* ants (Benedek & Kóbori 2014), and higher levels of aggression between colonies of intermediate distances, e.g., in *Crematogaster scutellaris* ants, likely

due to polydomy (Frizzi *et al.* 2014; Frizzi *et al.* 2015). Furthermore, territorial

aggression in ants has been shown to vary based on context (Buczkowski & Silverman

2005; Tanner & Adler 2009), task-group (Newey *et al.* 2010; Sturgis & Gordon 2013),

and experience (van Wilgenburg *et al.* 2010a). Findings like these indicate that, even

within a species, territorial relationships may differ due to a variety of genetic,

developmental, and environmental conditions (reviewed in Sturgis & Gordon 2012).

Establishing complex territorial relationships such as these necessitates the ability

of organisms to learn cues from their neighbors. In ants, (non)nestmate recognition cues

are largely communicated by a class of chemicals known as cuticular hydrocarbons

(CHCs; Lahav *et al.* 1999; Wagner *et al.* 2000; Akino *et al.* 2004; Ozaki *et al.* 2005;

Martin *et al.* 2008), but the relative contributions of genetic and environmental factors to

nestmate recognition is still poorly understood in many species (d'Ettorre & Lenoir

2010). Studies of the unicolonial Argentine ant, *Linepithema humile*, for example, show

strong effects from exogenous cues such as environmental conditions and diet (Chen &

Nonacs 2000; Liang & Silverman 2000), which may be capable of overriding heritable

effects in populations introduced in California (Buczkowski *et al.* 2005) where genetic

variability is low due to recent invasion by a small population (Tsutsui & Case 2001).

Argentine ants introduced to Europe, on the other hand, do not show evidence of an

overall genetic bottleneck, and instead have been argued to be fixed for different

recognition alleles (Giraud *et al.* 2002). Moreover, in several other ant species,

recognition cues are strongly affected by genetic components. For example, comparisons

between colonies with high genetic relatedness (e.g., monogynous/monoandrous) show

more similar CHC patterns than comparisons between colonies with low relatedness (e.g.,

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monogynous/polyandrous and polygynous) (Vander Meer & Morel 1998; Vasquez *et al*. 2009; van Zweden *et al*. 2011). Notably, in addition to CHC variation eliciting intraspecific aggression among ant colonies, previous territorial experience has also been shown to affect the propensity to fight (van Wilgenburg *et al*. 2010). Therefore, having sufficient nestmate recognition cue variation, as well as opportunities to learn these cues through prior experience with intraspecific non-nestmates, may be essential to maintaining territorial relationships.

In this study, I investigated territorial relations in the red harvester ant, *Pogonomyrmex barbatus*. Two previous studies bear relevance to the question of the complexity of neighboring relationships, and thus nestmate recognition in this species. The first showed that *P. barbatus* distinguishes between members of near and distant colonies by exhibiting a stronger reduction in foraging in response to the former than the latter (Gordon 1989). By contrast, a more recent study found that *P. barbatus* does not significantly differentiate near from distant neighbors when tested in the context of intercolony aggression (Sturgis & Gordon 2013). To address this ambiguity, I conducted a series of behavior assays measuring responses between *P. barbatus* colonies representing three types of colony experience/relationships: isolated colonies (i.e., inexperienced with intraspecific non-nestmates), distant colonies (i.e., experienced with non-nestmates, but not familiar with those tested against), and neighboring colonies (i.e., experienced with non-nestmates, and familiar with those tested against). Doing so allowed me to reassess the effect of colony (in)experience and spatial relationships on territorial aggression, and thus, nestmate recognition in *P. barbatus*. Furthermore, a growing body of evidence shows that territorial relationships may fluctuate, especially

when the level of resource competition varies across seasons. Previous examples of seasonal effects on territoriality have been found in ants (Mabelis 1979; Ichinose 1991; Katzerke *et al*. 2001), birds (Golabek *et al*. 2012), and mammals (Schradin *et al*. 2010). Therefore, testing for the effects of seasonal variation in *P. barbatus* aggression may help explain the disagreement of previous results, and provide important contextual information for interpreting how territorial behavior and relationships, as well as nestmate recognition cues, change over a colony's annual life cycle. Better understanding how experiential and seasonal factors affect territoriality will be beneficial to advancing theoretical models of nestmate recognition in social insects, and help explain contradictory results in the literature (e.g., Gordon 1989, and Sturgis & Gordon 2013).

Materials and Methods

Colony distribution and sampling

The study was carried out in the greater Phoenix, AZ area from June 2013 to April 2016. Worker ants from 31 colonies of *Pogonomyrmex barbatus* were collected at two sites within the Phoenix metropolitan area over the course of the experiments. I sampled eleven colonies from site "UPR," located along a Union Pacific railroad line in Tempe, AZ (33.330985°, -111.951833°), and the remaining fourteen colonies were sampled at site "SCC," located around Scottsdale Community College in Scottsdale, AZ on undeveloped land surrounding the campus (33.513481°, -111.879575°). For each site, colonies were mapped within the sampled range, and intercolony distances (m) were recorded to determine average distance between all isolated colonies, distant colonies, and neighboring colonies tested (Figure 5.1).

Paired-worker behavior assays

To test for significant differences in aggressive/territorial behavior between colonies, I collected *P. barbatus* workers from field colonies at two sites in the Phoenix metropolitan area: Scottsdale Community College (SCC; Figure 5.1, A) and along the Union Pacific railroad in Tempe, AZ (UPR; Figure 5.1, B). Live workers were sampled in the morning between 7:00 am and 9:00 am, a two-hour period of time overlapping with the onset of colony foraging activity (Gordon 1986). Focal workers were observed on the nest mound (i.e., within 50 cm from the colony entrance), and collected only if they were both (a) carrying no objects (i.e., not foraging for resources or performing nest maintenance tasks), and (b) walking around the mound antennating other workers (≥ 2 interactions of 1-3 sec each). Previous studies have described similar behavioral castes, but termed them differently, i.e., "guards" in honey bees (Butler & Free 1952; Seeley 1985) and "patrollers" in ants (Gordon 1987). Our collection criteria differed slightly from these descriptions, so, to minimize confusion, we avoided theses terms and simply refer to these ants as "workers" for this assay. Field-collected workers were then brought directly from the sites to the lab, and immediately paint marked with a unique three-color combination code, i.e., one color on the head, and two colors on the thorax, using Sharpie ® oil-based paint markers. After paint-marking, ants were regrouped by colony, placed in clear plastic containers (4 in $\times$ 4 in $\times$ 2 in high) lined with Fluon ® to prevent escape, and provided with water-filled tubes to avoid dehydration. Workers were given a minimum of 30 min to acclimate, in order to reduce aggression due to handling and manipulation, before being used in a 3 min video-recorded, one-on-one aggression assay.

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All ants were used in behavior assays on the same day that they were collected in order to minimize changes in behavior due to lab conditions.

For each trial, two workers were placed separately in open-ended, Fluon ® lined glass tubes (3/4 in diameter $\times$ 1 in high), which were previously placed within the test arena - a Fluon ® lined, white porcelain dish (2 in diameter $\times$ 1.5 in high). Glass tubes were used to keep ants separate while they acclimated for 1 min to the test arena, and the arena floor was previously lined with lightly dampened (water) filter paper (Whatman ® No. 1). During the experiments, glass tubes (30 total) and arena dishes (14 total) were continually rotated throughout the trials, filter paper liners were discarded and replaced after each trial to avoid chemical contaminant effects on the ants' behavior, and each was lightly cleaned with ethanol (200 proof) soaked cotton balls prior to reuse.

Four main treatments were used to test for aggressive/territorial behavior between paired-worker introductions: (a) two nestmates (n = 34 colonies; negative control), (b) two non-nestmates from isolated colonies with no nearby neighbors (n = 7 colonies; "inexperienced" treatment), (c) two non-nestmates from distant colonies, but with other neighboring colonies nearby (n = 27 colonies; "experienced" but unfamiliar treatment), and (d) two non-nestmates from neighboring colonies (n = 27 colonies; "experienced" and familiar treatment). Colonies were considered neighbors if they were < 40 m apart, and isolated if their nearest "neighbor" was > 40 m apart. Mean (±SD) neighbor colony distance was 18.08 ± 10.41 m, mean (±SD) distant colony distance was 285.77 ± 151.79 m, and mean (±SD) isolated colony distance from its nearest "neighbor" was 68.53 ± 27.05 m. Each individual worker ant was used only once in a bioassay, and treatments were replicated such that all possible colony pairings were evenly distributed within a

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treatment type. These experiments were repeated over two time periods, summer (June –

July) and fall (September – October), to test for seasonal differences in territorial

behavior. A total of 240 trials were conducted over the course of the experiment

(summer: 120 trials; fall: 120 trials). Each trial was video recorded (Canon EOS 6D

DLSR camera body; Canon EF 100mm F2.8 L IS USM macro lens; 60 fps) for a

minimum of 3 min after contact was initiated between the ants.

The duration (sec) of two behavioral categories, i.e., (i) antennation and (ii)

biting/grappling, were recorded by an observer who was 'blind' to the treatment type to

avoid confirmation bias (van Wilgenburg & Elgar 2013). Video observations were

performed at $0.5\times$ playback speed to ensure accuracy of scoring, and were scored twice -

focusing each time on only one of two ants per assay. We documented ethogram scores

with the assistance of the computerized event recording software, ETHOM (Shih & Mok

2000). The behavioral results were then averaged for each pair and were statistically

analyzed with the statistical package R (version 3.2.2) using one-way ANOVA (corrected

for multiple comparisons) to determine the effects of treatment and seasonality on levels

of aggression between workers. Finally, post-hoc Tukey's HSD tests were used to

determine which treatment types and seasons showed significant differences in the

amount of antennation and aggressive behavior between *P. barbatus* workers.

Chemical analysis

For both seasons, i.e., summer and fall, we collected 36 live foraging ants each

from eight neighbor colony pairs used in subsequent CHC "dummy" assays. Foragers

were collected if they were observed on the nest mound carrying a seed in the direction of

the colony entrance. We chose to focus on this behavioral caste for study of CHCs, because previous work has shown that the highest levels of aggression are exhibited towards *P. barbatus* foragers (Sturgis & Gordon 2013). Ants were brought to the lab and freeze-killed (-80°C) prior to cuticular hydrocarbon extraction. Four ants were arbitrarily selected from each colony sample to test for individual-level CHC quantity and percent composition. The remaining 32 ants were reserved for extractions used in CHC "dummy" assays (described below). Each forager was placed in a glass vial containing 200 μL hexane and allowed to soak for 10 min. Ants were gently swirled in the vial for 10 sec at the beginning and end of the soak period, to ensure contact between the solvent and cuticular surface. We chose to forego an additional sample fractionation step (e.g., using silica gel filled minicolumns), because previous analyses showed that non-hydrocarbon chemicals and contaminants were undetectable. Therefore, 200 μL hexane extracts containing dissolved CHCs were transferred directly to glass, microvolume inserts, and dried under a stream of high-purity nitrogen gas. CHC samples were redissolved in 20 μL of hexane containing an internal standard (C19, nonadecane) of known concentration, and 1 μL of the final sample was analyzed.

All CHC profiles were analyzed on an Agilent 6980N series gas chromatograph (GC) equipped with a DB1-MS (Agilent J&W) non-polar capillary column (length, 30 m; ID, 0.25 mm; film thickness, 0.25 μm) and connected to an Agilent 5975 mass selective detector (MSD; −70 eV, electron impact ionization; Transfer Line 300°C; Quad 150°C; Source 230°C). Samples were injected using an automatic liquid sampler (ALS) in the splitless mode through the GC injection port set at 260°C. Helium was used as the carrier gas at 1 ml min−1. The column temperature was initially held at 60°C for 2 min before

increasing to 200°C at a rate of 20°min−1, and then to 320°C at a rate of 5°C min−1, where it was finally held for 5 min. Hydrocarbons were characterized using a combination of diagnostic ions, Kovats indices, and comparisons to already published *P. barbatus* CHC profiles (Tissot *et al.* 2001).

To determine the effect of intercolony CHC variation, we measured the peak areas of the 21 most abundant CHCs (Figure 5.3, A) in Enhanced ChemStation (Agilent Technologies 2005). The internal standard (C19) was also measured for each sample and used to calculate the total abundance (ng) of CHCs present. Additionally, the relative proportions of CHCs were calculated for each sample to determine overall intercolony CHC variation, as well as the relationship between intercolony CHC distance, spatial distance, and aggression. Overall CHC variation was determined through non-metric multidimensional scaling analysis (NMDS) of Euclidean distances that were calculated from the relative proportions of CHCs for each sample.

Cuticular hydrocarbon "dummy" assays

To understand the role of CHCs in maintaining territorial relationships between *P. barbatus* colonies, we carried out a series of assays to test how workers behave towards CHC coated glass beads (i.e., "dummies"). CHC dummies were prepared by placing two pools of 16 foragers from each colony, previously reserved from CHC analyses, into two glass vials (3 mL, 16 ants each) and soaking them in 1600 μL of hexane for 10 min. As with individual extractions, the pools of ants were gently swirled in the vial for 10 sec at the beginning and end of the soak period, to ensure contact between the solvent and cuticular surface. While ants soaked, we evenly distributed 16

clean, glass beads (4mm diameter; previously washed with hexane) across four additional

vials (3 mL). When the soaking period was complete, the two pools of CHCs in hexane

(~3200 µL total) were combined in a single glass vial and dried to approximately 2×

concentration (1600 µL). From this CHC pool, aliquots (400 µL) of the 2× CHC-hexane

extract were transferred to each of the vials containing 4 beads. CHC-bead vials were

then dried under a stream of N(g), sealed, and kept at 25°C until their use in confirmation

tests or "dummy" assays.

Successful transfer of CHCs to glass beads was confirmed by randomly collecting

1 CHC-bead from each vial preparation and re-extracting their CHCs. Each bead was

treated as an individual ant and extracted and analyzed with the same methods described

in the chemical analysis steps above. Total CHC abundance per bead (ng) was calculated

from an internal standard (C19), and the relative proportions were calculated from the

sum of peak areas. The abundance and relative proportion values were then compared

with representative samples of forager ants for each colony to ensure that CHC

abundances and proportions were not significantly different between CHC-beads and

individual ants (Figure 5.3, C).

"Dummy" assays were conducted similarly to paired-worker behavior assays,

with two modifications: first, one of the workers was replaced with a glass bead (aka a

"dummy") from one of four possible treatments; second, assays were video recorded for

only 2 min after initial contact (preliminary studies indicated that ants rarely interact with

beads after 2 min, regardless of treatment). Each worker ant was tested only once, and

placed with one of four treatments: (a) blank bead (negative control; i.e., hexane washed,

dry glass bead), (b) nestmate bead (positive control; glass bead coated with forager CHCs

from the same colony), (c) distant bead (distant non-nestmate treatment; glass bead coated with forager CHCs from a distant colony, i.e., > 40m between the focal colonies tested), or (d) neighbor bead (neighboring non-nestmate treatment; glass bead coated with forager CHCs from a neighboring colony, i.e., < 40m between the focal colonies tested). A total of 16 trails per treatment per season were run for CHC "dummy" assays (n = 128 total trials). As with paired-worker assays, all worker ants were tested in the lab on the same day of collection from the field, and all CHC-beads were used within 5 days of preparation. Video recordings were made for each trial, and observations were made at $0.5\times$ playback speed by an observer who was "blind" to the treatment. For each trail the duration (sec) of antennation and biting + grappling were recorded. Behavioral results were then analyzed for differences between treatments and seasons using ANOVA and *post hoc* Tukey's HSD tests.

Results

Paired-worker aggression

We found that *P. barbatus* workers spend significantly more time antennating and biting non-nestmate workers from neighboring colonies, and that this behavior is significantly affected by season (Figure 5.2). Territorial behaviors between non-nestmates is high in the summer season (pre-mating flight, June-July), and drops to low levels in the fall season (September-October). ANOVA test results indicate that both treatment and season have significant effects on the amount of antennation (treatment: $F_{3, 239} = 26.362$, $P < 0.001$; season: $F_{1, 239} = 44.119$, $P < 0.001$) and biting/grappling (treatment: $F_{3, 239} = 623.475$, $P < 0.001$; season: $F_{1, 239} = 12.972$, $P < 0.001$) behaviors exhibited in pairwise

aggression assays between *P. barbatus* non-nestmate workers. Furthermore, pairwise *post hoc* Tukey's HSD results indicate that non-nestmate workers exhibit significantly more antennation and biting/grappling when tested with workers from neighboring colonies than workers from distant colonies, but this only holds true for the summer season (adjusted $P = 0.0001$; Figure 5.2). Finally, *post hoc* Tukey's HSD comparisons between nestmates and non-nestmate workers from isolated colonies show lower levels of these behaviors with no significant differences between the two (adjusted $P > 0.05$).

Cuticular hydrocarbon profile and variation

P. barbatus foragers from different colonies have qualitatively similar CHC profiles, and therefore only differed quantitatively based on the relative and total abundance of CHC peaks present (Figure 5.3, A and B). We found that non-parametric, multidimensional scaling analysis of the 20 most abundant CHC peaks did not fully distinguish workers based on the colony from which they were collected, and instead showed numerous instances of CHC profile overlap between colonies (Figure 5.3, B). Additionally, variation in the spread of CHC colony profiles appears high, with some colonies showing a lot of spread (e.g., colony UPR002, brown diamonds) and others showing much less spread (e.g., colony UPR005, yellow diamonds) (Figure 5.3, B). Finally, we confirmed that CHC-coated glass bead "dummies" were not quantitatively different (ANOVA, $F_{1, 59} = 3.142$, $P = 0.08$) from representative foragers of the same colony (Figure 5.3, C). Notably, however, *post hoc* comparisons using the Tukey's HSD test indicated significant quantitative differences in CHC abundance between foragers collected in different seasons (adjusted $P = 0.02$), i.e., summer-collected foragers had significantly

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higher quantities of CHCs (Mean ± SD = 9001.23 ± 6541.41 ng) than fall-collected

foragers (Mean ± SD = 5505.46 ± 2181.11 ng; Figure 5.3, C).

CHC-elicited aggression

In the CHC "dummy" assays, we found that *P. barbatus* workers spend significantly

more time antennating and biting non-nestmate coated CHC dummies from neighboring

colonies. However, as with the paired-worker aggression assays, this behavior is

significantly affected by season (Figure 5.4). Similarly, CHC-elicited aggression is high

in the summer and reduces to low levels in the fall. ANOVA test results indicate that

colony spatial relationships have significant effects on the amount of aggressive

behaviors exhibited in dummy assays by *P. barbatus* workers. *P. barbatus* workers show

significant differences in antennation of CHC coated dummies based on treatment ($F_{3, 127}$

= 6.176, $P < 0.001$) and season ($F_{1, 127}$ = 16.756, $P < 0.001$). The same pattern was found

for biting/grappling behaviors (treatment: $F_{3, 127}$ = 6.185, $P < 0.001$; season: $F_{1, 127}$ =

4.573, $P < 0.05$). *Post hoc* Tukey's HSD results indicate that non-nestmate workers only

exhibit significant differences in antennation when comparing blank beads (the negative

control) with neighbor coated CHC-beads in the summer season (adjusted $P = 0.002$;

Figure 5.4, A); all other comparisons were not significantly different within a given

season. Finally, for biting + grappling behaviors, only summer-collected neighboring

workers exhibited significantly more aggression (adjusted $P < 0.05$; Figure 5.4, B).

Discussion

Territoriality has evolved as a mechanism of securing resources among biological entities (Wilson 1975). The challenge is to moderate the costs of territorial behavior, e.g., time/energy expenditure, loss of resources, injury, and -in more extreme cases- death, so that the benefits outweigh them. (Super)organisms, e.g., ant colonies, may minimize costs by limiting aggression to foreign conspecifics posing the greatest threat (Temeles 1994). One way to achieve this is to establish territorial relationships, e.g., dear enemies or nasty neighbors, with nearby conspecifics (Temeles 1994). In *P. barbatus*, territorial relationships have previously been demonstrated to effect workers' foraging behavior, such that colonies react more strongly to neighbors than distant colonies (Gordon 1989). As a result, Gordon (1989) hypothesized that workers may be capable of learning cues from neighboring colonies (i.e., a significant conspecific threat), and use this information, in part, to better direct aggression towards individuals that pose the greatest threat. More recently, however, a follow-up study found that *P. barbatus* workers do not significantly discriminate between different non-nestmates when tested in the context of intercolony aggression (Sturgis & Gordon 2013). In their discussion Sturgis and Gordon (2013) argued that the lack of support for differences in aggression may have been due, in part, to low statistical power.

In this study, I reviewed aggressive behavior in a large sample size of *P. barbatus* workers/colonies, and tested additional factors that may account for variation in territorial responses including seasonality, experience, and cuticular hydrocarbons. I found that *P. barbatus* worker ants are significantly more aggressive towards non-nestmate workers from neighboring colonies than non-nestmates from more distant colonies, thus providing

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additional support for a "nasty neighbor" relationship in *P. barbatus*. Importantly, however, seasonal comparisons of *P. barbatus* aggression showed that the nasty neighbor effect only held true for workers measured in the summer season (i.e., the months leading up to the mating flight). Furthermore, inexperienced workers (i.e., those tested in assays where workers came from isolated colonies) showed no significant differences in aggression compared with workers tested in nestmate (negative control) assays. Taken together, these results indicate that seasonal factors likely contribute significantly to variation in aggressive behavior, and both prior experience with non-nestmates and familiarity with non-nestmates likely also have significant effects on territoriality behavior in *P. barbatus*. Finally, *P. barbatus* workers also showed a summer-specific, nasty neighbor response to CHC "dummies," indicating that these chemical cues are likely a key component of their non-nestmate recognition system. These findings lend further support for the hypothesis that *P. barbatus* workers are capable of distinguishing different types/classes of non-nestmates - a conclusion that has been implicated for numerous other ants including: *Leptothorax nylanderi* (Heinze *et al*. 1996), *Formica pratensis* (Beye *et al*. 1998; Pirk *et al*. 2001; Benedek & Kóbori 2014), *Pheidole* (Langen *et al*. 2000), *Pristomyrmex pungens* (Sanada-Morimura *et al*. 2003), *Iridomyrmex purpureus* (van Wilgenburg *et al*. 2007), *Linepithema humile* (Thomas *et al*. 2007); *Acromyrmex lobicornis* (Dimarco *et al*. 2010), *Oecophylla smargdina* (Newey *et al*. 2010), and *Crematogaster scutellaris* (Frizzi *et al*. 2015).

Understanding the context and selective pressures under which ants learn non-nestmate cues has been of particular interest for the past 20 years. Because ants are more likely to encounter conspecific non-nestmates from nearby colonies than more distant

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colonies, opportunities for associative learning of non-nestmate recognition cues (e.g.,

CHCs) paired with aggressive territoriality experience occur primarily through

neighboring colony interactions. Interestingly, my analysis of the 20 most abundant

CHCs from the CHC profiles of *P. barbatus* foragers showed varying degrees of overlap

between colonies, with only a single colony being completely separated in chemical

spatial distance (Figure 5.3, B). Despite the low intercolony CHC distance, however,

workers exhibited significantly more aggression towards neighbor non-nestmate CHCs

than distant non-nestmate CHCs during the summer season (Figure 5.4, B). This suggests

that some of the lower abundance CHCs not included in the NMDS analysis may also be

important for associative learning and nestmate discrimination. Indeed, many of the

lower level CHCs found on *P. barbatus* workers include mono- and di-methylalkanes as

well as alkenes - compound classes that have been implicated as being important for

chemical recognition systems given their structural variability (Akino *et al*. 2004; Lucas

et al. 2005; Martin *et al*. 2008; Martin & Drijfhout 2009; van Wilgenburg *et al*. 2010b).

Therefore, the potential importance of less abundant CHCs in *P. barbatus* nestmate

recognition should not be discarded without testing them first. Additionally, more

detailed studies of the relationship between chemical (CHC profile) distance, neighbor

relationships, and aggression in *P. barbatus* may reveal that the CHC profile overlap

among colonies correlates with variation in aggressive behavior. For example, non-

nestmate workers with more similar CHC profiles may show lower aggression than non-

nestmates with greater CHC differences, regardless of their spatial relationship (e.g., see

Martin *et al*. 2012). Teasing apart the role(s) of different CHCs in *P. barbatus* nestmate

recognition remains a challenge, in part, because of the number and variety of CHC

compounds present on workers' cuticles, but also because different colonies may use different parts of the CHC profile to discriminate between non-nestmates. Furthermore, access to relevant synthetic and/or purified methyl-branched and unsaturated hydrocarbons is limited, and would need to be improved in order to proceed with future experimental studies.

Here, I present evidence that *P. barbatus* shows seasonal differences in non-nestmate aggression, with a nasty neighbor relationship being detected in the summer season. This adds to the growing body of evidence for variation in territorial aggression among ants due to neighboring colony relationships. Similar to these findings, several studies of territorial aggression in ants have shown behavioral variation across seasons (Mabelis 1979; Ichinose 1991; Katzerke *et al*. 2001) and experience levels (van Wilgenburg *et al*. 2010a). Additional spatiotemporal factors of territorial aggression and neighboring relationships may include population differences (Tsutsui *et al*. 2000; Benedek & Kóbori 2014), breeding season (Hölldobler 1975; Rissing & Pollock 1987), resource competition (Temeles 1990; Parr & Gibb 2010; Golabek *et al*. 2012), and physiological state (e.g., hormone levels, Kostowski *et al*. 1975; macronutrient levels, Grover *et al*. 2007; body size, Nowbahari *et al*. 1999; etc.). Future work on nestmate recognition and territorial relationships in *P. barbatus* would benefit from explicitly testing these factors as well as the specific role(s) of CHCs and CHC profile distances.
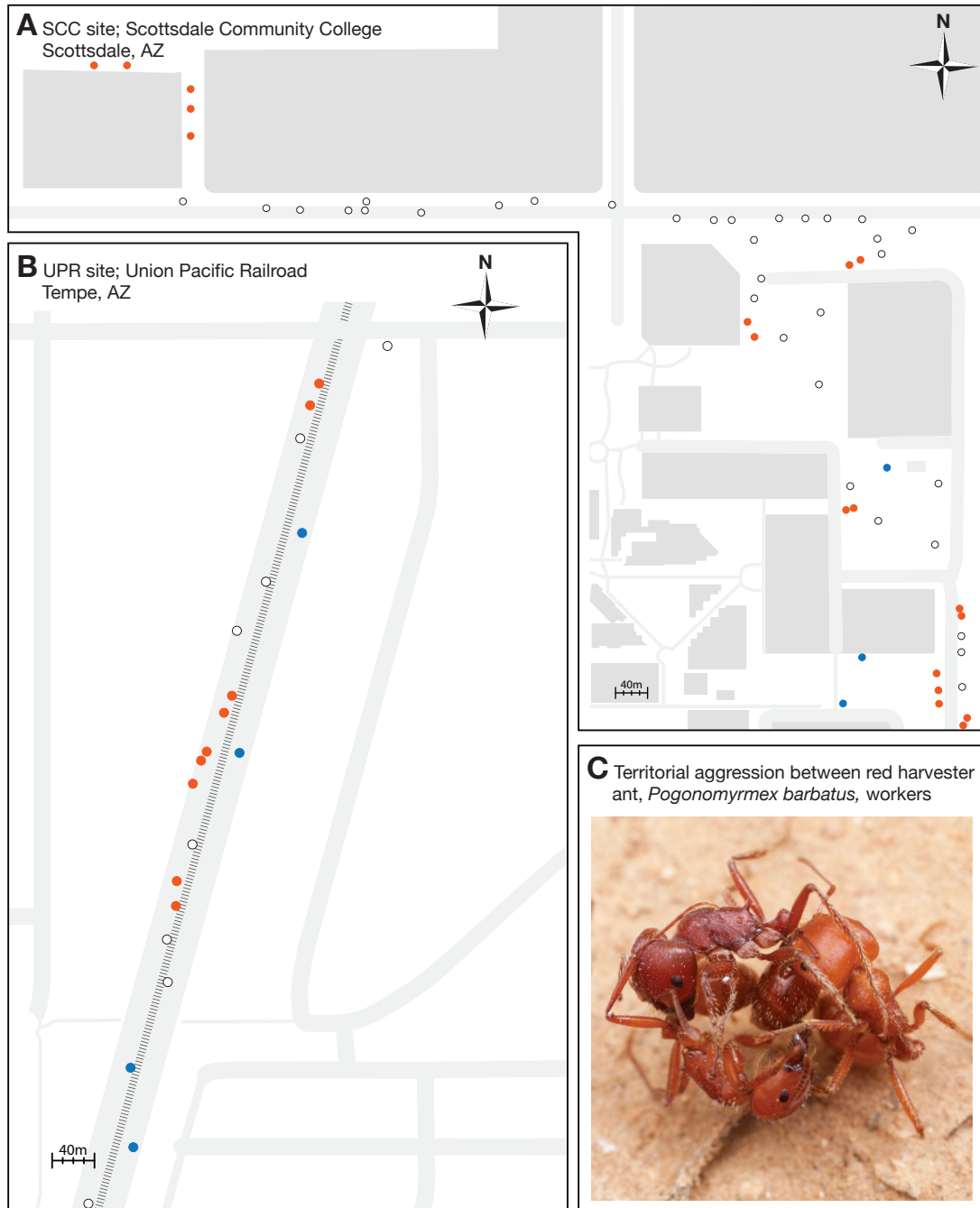
Acknowledgements

Figure 5.1. Map of *P. barbatus* colonies at study sites (A) Scottsdale Community College (SCC; Scottsdale, AZ), and (B) Union Pacific Railroad (UPR; Tempe, AZ), located in the Phoenix metropolitan area. Red dots represent colonies used in neighbor (experienced/familiar colony) and distant (experienced/unfamiliar colony) behavioral assays, whereas blue dots indicate colonies used in isolated (i.e., nearest "neighbor" distance > 40 m; inexperienced/unfamiliar colony) behavioral assays. White dots signify other *P. barbatus* colonies known to be present but not used in assays. (C) Pictured is a representative example of territorial aggression between neighboring *P. barbatus* workers in the field.

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Figure 5.2. Box and whisker plots representing the summary of results (average, upper and lower quartiles, 95% confidence intervals, and outliers) for pairwise, worker-worker aggression assays. Boxes are color coded according to treatment (see color legend within figure), and separated by behavior, (A) antennation and (B) biting + grappling, for the three seasons, spring, summer, and fall, in which they were measured. Significant differences are indicated above upper most error bars with lower case letters (ANOVA associated *post hoc* Tukey's HSD test, adjusted $P < 0.05$).
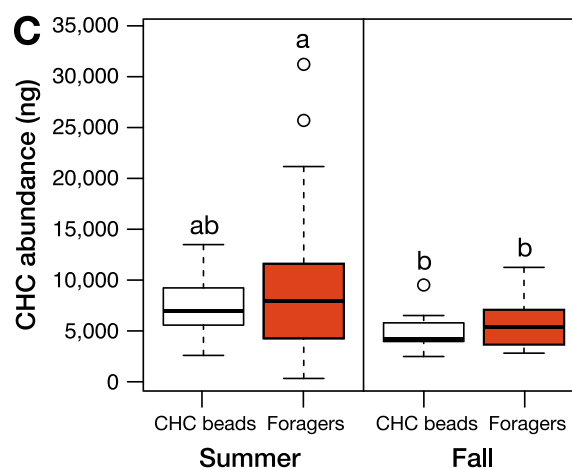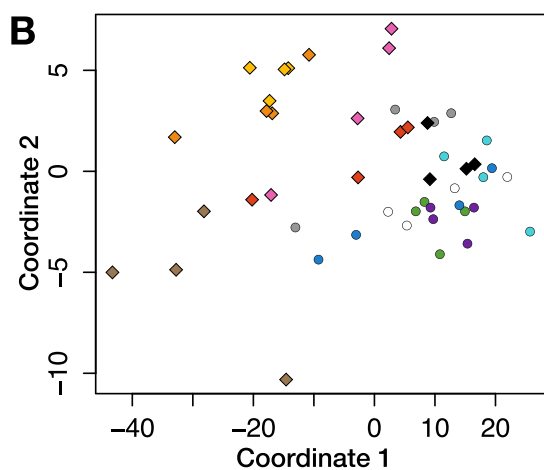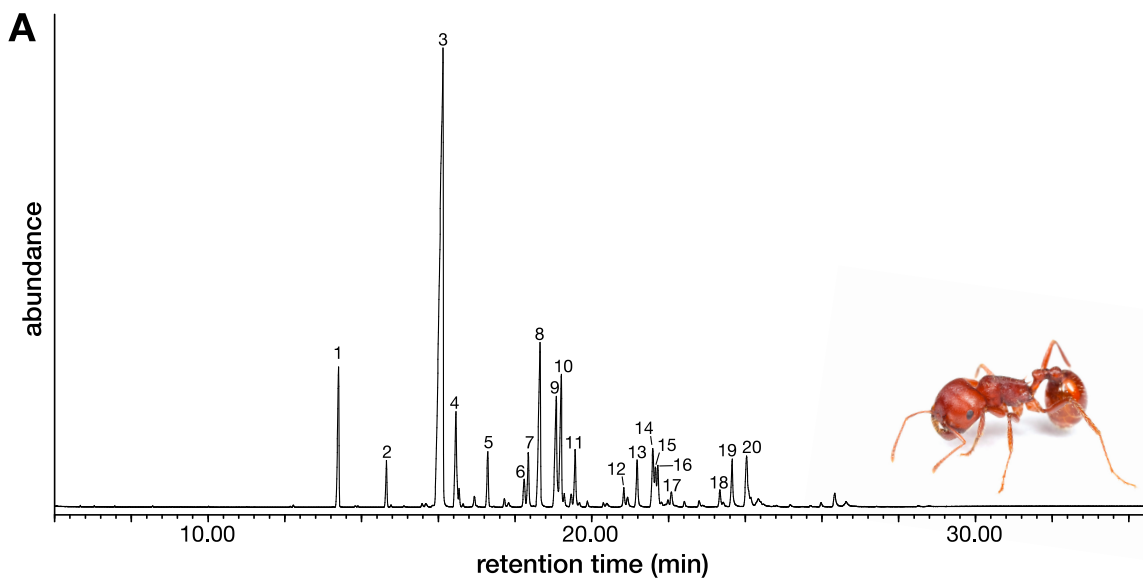
Figure 5.3. Cuticular hydrocarbon variation among *P. barbatus* workers and CHC "dummies." (A) Representative gas chromatogram of a *P. barbatus* worker cuticular hydrocarbon profile. Numbered peaks indicate the 20 most abundant peaks used in comparative analyses of relative CHC abundances (1 = C23; 2 = C24; 3 = C25; 4 = xMe-C25; 5 = C26; 6 = *Z9*-C27:1; 7 = *Z7*-C27:1; 8 = C27; 9 = xMe-C27; 10 = xMe-C27; 11 = x,xDiMe-C27; 12 = *Z9*-C29:1; 13 = C29; 14 = xMe-C29; 15 = xMe-C29; 16 = xMe-C29; 17 = x,xDiMe-C29; 18 = *Z9*-C31:1; 19 = C31; 20 = xMe-C31). (B) Nonmetric multidimensional scaling (NMDS) plot of chemical distances for 4 representative workers from 12 *P. barbatus* colonies collected in the summer season. Individual ants are represented by colored diamonds (U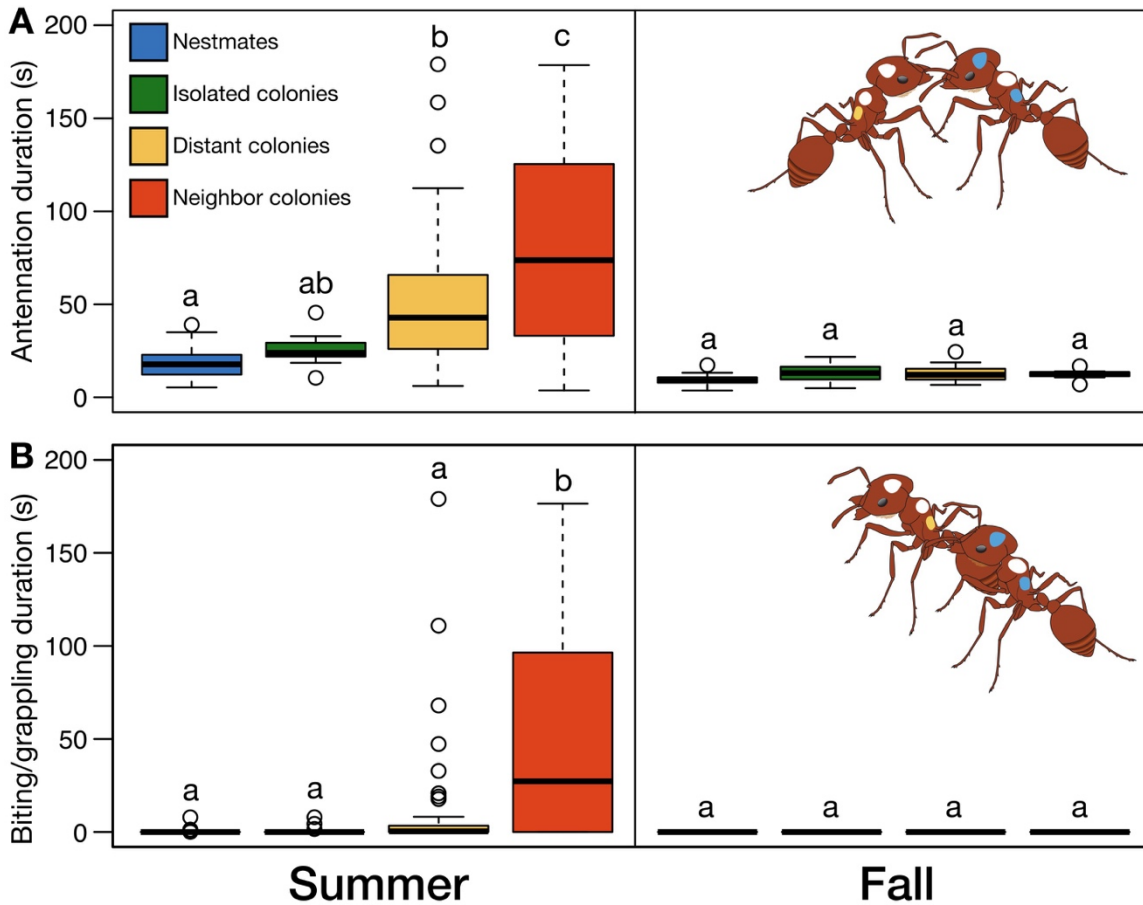PR site) or circles (SCC site) according to their colony of origin. (C) Box and whisker plots representing the summary of results (average, upper and lower quartiles, 95% confidence intervals, and outliers) for CHC quantities (ng) of CHC "dummies" (white boxes) and *P. barbatus* foragers (red boxes) measured in two seasons (summer and fall). Significant differences are indicated above upper most error bars with lower case letters (ANOVA associated *post hoc* Tukey's HSD test, adjusted *P* < 0.05).

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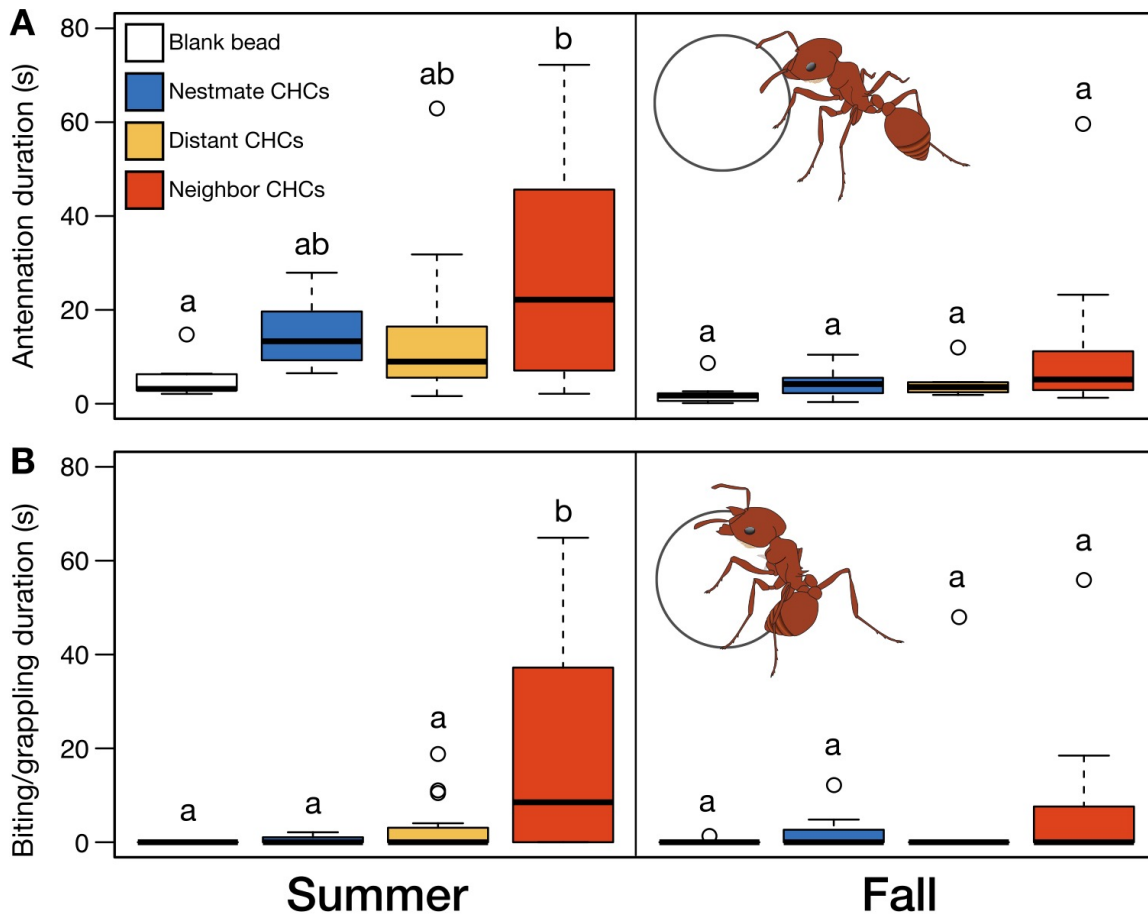
Figure 5.4. Box and whisker plots representing the summary of results (average, upper and lower quartiles, 95% confidence intervals, and outliers) for cuticular hydrocarbon coated glass bead assays (i.e., CHC "dummies"). Boxes are color coded according to treatment (see color legend within figure), and separated by behavior, (A) antennation and (B) biting + grappling, for the two seasons, summer and fall, in which they were measured. Significant differences are indicated above upper most error bars with lower case letters (ANOVA associated *post hoc* Tukey's HSD test, adjusted *P* < 0.05).

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APPENDIX A

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