

Behavioral and chemical ecology of ants (Hymenoptera, Formicidae) and their natural enemies in dynamic coffee agroecosystems.

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ABSTRACT

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Social insects rank among the most ubiquitous and ecologically dominant terrestrial animals on Earth. Complex communication and social organization are two defining features of social insect societies and ants, in particular, have evolved extensive systems of chemical communication. In both natural and agricultural systems, including coffee agroecosystems, ants are important predators and often have strong and complex effects on pest species. In this dissertation, I explore how chemical communication plays a role in dynamics between ants and their natural enemies within these coffee agroecosystems, to gain a better understanding of both how ants utilize their own chemical communication systems and how natural enemies of ants take advantage of these systems to exploit ants.

In the first dissertation chapter, I review the literature on chemical and visual cues that one natural enemy of the ant, the phorid fly, uses to successfully parasitize its host ants. Phorid fly parasitoids that use ants as hosts often require the use of multiple cues, ranging from general to highly specific, to home in on an ideal host. Here I outline the five common steps in which phorid flies use cues: (a) host habitat location, (b) host location, (c) host acceptance, (d) host discrimination and (e) host regulation. I then discuss our current understanding of how phorid flies use each of these steps to successfully parasitize ant hosts. Finally, I examine the wide variety of strategies and cues used by a multiple species of phorid flies within three separate genera (*Apocephalus*, *Pseudacteon*, and *Neodohrniphora*) and discuss future directions within this field of study.

In the second dissertation chapter, I investigate the role of *Azteca* ant cuticular hydrocarbon cues as a short-range cue for a suite of *Pseudacteon* spp. phorid fly parasitoids commonly found within coffee agroecosystems. Here I describe the hierarchy of cues *Pseudacteon* spp. phorid flies use to successfully identify *Azteca* host ants. I use behavioral observations in the field to show phorid flies are attracted to two cryptic *Azteca* taxa, but will only attack *Azteca sericeasur* (Hymenoptera: Formicidae: Dolichoderinae). To test whether the phorid flies are able to distinguish between the two *Azteca* taxa using their cuticular hydrocarbons, I first document and compare the cuticular hydrocarbons of two cryptic *Azteca* taxa using gas chromatography-mass spectrometry (GC/MS). Using cuticular hydrocarbon transfer experiments with live ants from both *Azteca* taxa, I identify the

cuticular hydrocarbons of *Azteca sericeasur* as a short-range host location cue used by *Pseudacteon lascinosus* (Diptera: Phoridae) to locate the ants.

In the third chapter, I describe two new species of *Myrmedonota* (Coleoptera: Staphylinidae) that are also natural enemies of *Azteca sericeasur* ants. Here I record this genus of beetle in Mexico for the first time, and describe *M. xipe* and *M. shimmerale* for the first time. This chapter also documents that both *Myrmedonota* species will aggregate towards agitated ants, to prey on *Azteca sericeasur* and these species will form mating swarms, either with no apparent landmark or in the vicinity of ants.

My fourth chapter examines the role of the beetle, *Myrmedonota xipe*, in *Azteca* ant-phorid fly interactions. Here I use pheromone bioassays to show that *M. xipe* is attracted to a component of *A. sericeasur* alarm pheromone. In the field experiments, I determine that the beetles are able to locate parasitized ants as prey items but not healthy ants. In choice tests in the lab, I also show that beetles will preferentially attack parasitized ants over healthy ants. Analysis of the choice tests also indicates that the aggression in parasitized ants is so reduced that beetles are essentially able to eat these ants alive without interruption. These results suggest that, although beetles are predators of the ants, by preying primarily on ants harboring phorid fly eggs, the beetles may also provide indirect positive effects for the ant colonies as a whole.

In my final dissertation chapter I examine the variation in cuticular hydrocarbon blends of three species of arboreal twig nesting ants, *Pseudomyrmex simplex*, *Pseudomyrmex ejectus* and another *Pseudomyrmex* sp. (referred to as PSW-53) commonly found within the coffee agroecosystems of Southern Mexico. In this study, I examined whether variation in the ecology and social structure of these species is reflected in the variation in their cuticular hydrocarbon profiles. I tested the hypothesis that the more abundant species with higher nest densities exhibit lower cuticular hydrocarbon variation. The results showed that worker ants of abundant *P. ejectus* and *P. simplex* from the study site exhibit significantly lower variation in cuticular hydrocarbon profiles than workers of the rare ant species *P. PSW-53*. Our study reinforces the idea that examining cuticular hydrocarbon profiles can provide insight into the colony structure of social insects.

Overall, the results from my dissertation provide insight into the complex interactions between ants and their natural enemies, particularly shedding light on how these interactions are facilitated through the use of ant pheromones as cues and signals. The ants and their natural enemies that I focus on in my dissertation are also important players within the greater coffee agroecosystems in which they inhabit, and this body of work is thus critical for understanding how their network of interactions involving ants and their natural enemies may impact coffee pest control.

DEDICATION

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INTRODUCTION

Ants are diverse and abundant in tropical habitats, sometimes making up more than 85% of arthropod biomass in forest canopies (Holldobler and Wilson 1990, Davison 1997, Davidson et al. 2003). In both natural and agricultural systems, including coffee agroecosystems, ants are important predators and often have strong and complex effects on lower trophic levels (Way and Khoo 1992, Schmitz et al. 2000, Perfecto et al. 2014). Several ant species prey on coffee pests (Philpott and Armbrecht 2006), affect canopy arthropods in coffee plantations (Philpott et al. 2008), and protect coffee green scale (*Coccus viridis*) (Liere and Larsen 2010) creating a complex network of interactions that provide pest control within these agroecosystems. However, interactions between natural enemies of these ants can also strongly influence top down effects (Ives et al 2005, Perfecto et al. 2014) and these interactions are often mediated by pheromones. Complex communication is a defining feature of ant societies, and of the 12 functional categories of communication present within these societies, almost all are primarily or completely chemical in nature (Wilson and Holldobler 1990, 2009). Ants use of chemical signaling impacts colony social structure and interactions ants have with other species. Predators and parasitoids of ant species often eavesdrop on the chemical communication system of their host/prey in order to locate them and avoid aggressive interactions (Holldobler and Wilson 1990, Allan et al. 1996, Clark et al. 2000, Feener et al. 1996).

Two taxa of ants that have important impacts on coffee pests are the aggressive arboreal ant *Azteca sericeasur* and twig nesting *Pseudomyrmex* spp. ants (Gonthier et al 2013, Larsen and Philpott 2010). A great deal of work examines the role both taxa of ants play in the complex network of interactions within coffee agroecosystems yet relatively little is known about the chemical ecology of these ants or how their chemical communication systems mediate their interactions in within the larger coffee agroecosystem (Perfecto et al. 2014). Addressing these gaps in the literature will give us insights into the mechanisms by which social insect communication can mediate complex species interactions.

In the first dissertation chapter, I review the literature on chemical and visual cues that one natural enemy of the ant, phorid fly parasitoids, uses to successfully parasitized its host ants. Parasitoids have evolved effective and efficient tactics to successful target their hosts, several of which involve utilization of both chemical and visual cues. Many Dipteran parasitoids in the family Phoridae use social insects as hosts due to the reliability of their intraspecific chemical communication signals that make for effective host location cues (Brown and Feener 1991; Feener et al. 1996; Morehead and Feener 2000; Disney 1994). Phorid fly adults hover over insect hosts and dive down to insert an egg beneath the insect's exoskeleton. The larva hatches and moves through the insect's body, eating the contents, and then either pupates in the empty head capsule or leaves the insect's body to pupate in the soil. Later, the adult fly will emerge from the head of the dead ant to begin the cycle again (Cônsoi et al. 2001; Porter 1998; Disney 1994; Feener 1997). Phorid flies have direct parasitic effects on ants (i.e. cause ant mortality) and significantly change ant foraging behavior by limiting host resource acquisition behavior, modifying ant

competitive hierarchies, and dampening ant effects on herbivores (Feener 1981; Feener and Brown 1992; Orr et al. 1995, 2003; Vandermeer et al. 2002; Philpott 2005).

Host cue use varies based on individual phorid fly-ant relationships. Phorid fly parasitoids that use ants as hosts often require the use of multiple cues, ranging from general to highly specific, to home in on an ideal host. In this chapter, I outline the five common steps in which phorid flies use cues: (a) host habitat location, (b) host location, (c) host acceptance, (d) host discrimination and (e) host regulation. I then discuss our current understanding of how phorid flies use each of these steps to successfully parasitize ant hosts. Finally, I examine the wide variety of strategies and cues used by a multiple species of phorid flies within three separate genera (*Apocephalus*, *Pseudacteon*, and *Neodohrniphora*) and discuss future directions within this field of study.

In the second dissertation chapter, I investigate the role of *Azteca* ant cuticular hydrocarbons (CHCs) as a short-range cue for a suite of *Pseudacteon* spp. phorid fly parasitoids commonly found within coffee agroecosystems. Several species of *Pseudacteon* phorid fly parasitize *Azteca* ants, including *Pseudacteon lascinosus*, *Pseudacteon planidorsalis* and *Pseudacteon pseudocercus* that parasitize *Azteca sericeasur*. *P. lascinosus* is the largest of the three species and the most abundant at our field sites, *P. planidorsalis* is a smaller species and the second most abundant, while *P. pseudocercus* is the rarest of the phorid species (Reese and Philpott 2012). Presence of phorid fly parasitoids reduces the ant foraging rates by as much as 50% (Philpott et al. 2004, Philpott 2005), and also indirectly affects interactions between the ants and a wide range of competitors and mutualistic partners (Perfecto et al. 2014). Phorid flies are able to first locate and then hover over individual *A. sericeasur* workers by using the ant's alarm pheromone and movement as cues, however these cues alone are not enough to cause a phorid fly oviposit on an ant (Mathis et al. 2011). Additionally, *Azteca sericeasur* ant workers are notoriously cryptic and nearly morphologically identical to several other *Azteca* spp. (Longino 2007). In my study sites *Azteca sericeasur* co-occurs with another species of *Azteca* (currently unidentified but referred to hereafter as *Azteca* JTL020) whose workers appear almost identical to *Azteca sericeasur*. In this study I investigate how are phorid flies able to distinguish between these two species to correctly identify hosts by examining a three step hierarchy of cues phorid flies use to successfully identify host ants. First using behavioral observations, I show that phorid flies are attracted to and will hover over both *A. sericeasur* and *A. JTL020* workers. Then, I use chemical extract and subsequent analysis using Gas Chromatography/ Mass Spectrometry to document the cuticular hydrocarbons profiles of the two *Azteca* taxa. I tested whether two species of phorid fly, *Pseudacteon lascinosus* and *Pseudacteon planidorsalis*, use the cuticular hydrocarbons of *A. sericeasur* to discriminate between their host species and *Azteca* JTL020 shares other long-range visual and chemical cues with *A. sericeasur*. The results from this chapter identify that cuticular hydrocarbons are short-range host location cues used by at least one species of phorid fly to locate *Azteca sericeasur* ants and distinguish them from *A. JTL020*.

In the third chapter, I identify and describe two species of *Myrmedonota* (Coleoptera: Staphylinidae) as new natural enemies of *Azteca sericeasur*. The two new species of predatory beetle, *M. xipe* and *M. shimmerale* are the first recorded from this genus of beetle in Mexico. Ant societies attract a suite of symbiotic organisms that take advantage of a colony's abundant resources. While the life histories of most *Myrmedonota* beetles are poorly documented, several have been collected in the presence of either ants or

termites (Bourguignon and Roisin 2006; Maruyama et al. 2008). Beetles in the family Staphylinidae are common ant-associates, yet relatively little is known about the role of these beetles in their host colonies. Due to the formidable chemical and behavioral defenses of ants, beetles often possess complex strategies to safely interact with their ant symbionts (Kistner 1979, Stoeffer et al. 2011). Many beetles act as scavengers that hide in refuse piles, or as ant-mimicking social parasites within a host colony draining them of their resources, or occasionally as outright ant predators using their own arsenal of defensive chemicals to protect themselves against the ants. Other predators of the ants locate their prey by eavesdropping on the ants' communication system. (Holldobler and Wilson 1990). To describe these species, we provide bionomical information and illustrations of the prepared type specimens in addition to some initial behavioral description of these species.

My fourth chapter examines the role of the beetle, *Myrmedonota xipe*, in *Azteca* ant-phorid fly interactions. *M. xipe* beetles are often found near disturbed *A. sericeasur*, often mating or preying on *A. sericeasur* ants after the arrival of phorid flies. Here I test the hypothesis that if ants are parasitized by phorid flies, then ants become less aggressive, allowing beetles to gain access to ants, an otherwise non-accessible prey item. Furthermore, I hypothesize that this context dependent interaction is induced by the ant's alarm pheromone, which is also a phorid fly attractant. I use a combination of Solid phase micro-extraction (SPME) of *A. sericeasur* alarm pheromone, GC/MS analysis and subsequent pheromone bioassays to show that *M. xipe*, is attracted to, 2-heptanone, a component of *A. sericeasur* alarm pheromone. In the field experiments, I determine that the beetles are able to locate parasitized ants as prey items but not healthy or injured ants. Using choice tests in the lab, I also show that beetles will preferentially attack ants parasitized by *P. lascinosus* over healthy ants. Analysis of the choice tests also indicates that aggression, particularly mandible flaring, in parasitized ants is so reduced that beetles are essentially able to eat these ants alive without interruption. Together, these analyses indicate that although beetles are predators of the ants, by preying primarily on ants harboring phorid fly eggs, the beetles may also provide indirect positive effects for the ant colonies as a whole.

The final chapter of my dissertation (Chapter 5) explores the chemical communication system of the second taxa of ant predators within these coffee agroecosystems, *Pseudomyrmex* spp. Maintaining cooperation between nestmates while excluding intruders, social insects have well-developed nestmate recognition systems, which primarily rely on chemical signals (Howard and Blomquist 2005). Cuticular hydrocarbons often play an important role as signals in nestmate recognition (Gamboa et al. 1986, Krasnec and Breed 2013, Howard and Blomquist 2005). Cuticular hydrocarbon mixtures not only vary between species (Espelie et al. 1994, Gamboa, 2004), but their relative concentration ratios also vary between colonies, creating both species- and colony-specific profiles (Carlin and Holldobler 1983, Crosland 1989, Butts et al. 1993, Soroker et al. 1994, VanderMeer and Morel 1998, Krasnec and Breed 2013). Colony level variation in cuticular hydrocarbons plays an important role in nestmate recognition in most species of ants, and differences in cuticular hydrocarbon variation in non-invasive ant species may result in similar types of social organization (Holway 1998, Suarez et al. 2002, Sanders et al. 2003). Here I investigate the variation of CHC profiles in three species of twig-nesting ants; *Pseudomyrmex simplex*, *Pseudomyrmex ejectus* and *Pseudomyrmex* sp.

(hereafter PSW-53) that co-occur within coffee plantations in Chiapas, Mexico. These species were chosen because they act as predators on coffee pests and also because species differ in competitive success, relative abundance, and nest density. My aim in this study was to determine whether differences in the complexity of recognition cues (cuticular hydrocarbons) are associated with the variation in ecology and social structure of these ants. I collected chemical and spatial data for nests within my study sites to test two hypotheses: (1) that the three species of ant's cuticular hydrocarbon colony chemotypes are more variable between colonies than within colonies and (2) that the observed within species variation in CHC profile labels for each species correlates with their social structure and competitive dominance so that less competitive, more abundant, and densely populated ants exhibited reduced CHC variation and rare, sparsely populated, and more competitive ants exhibited increased CHC variation. Worker ants of abundant *P. ejectus* and *P. simplex* from the study site exhibit significantly lower variation in cuticular hydrocarbon profiles than workers of the rare ant species *P. PSW-53*. This study thus reinforces the idea that examining cuticular hydrocarbon profiles can provide insight into the colony structure of social insects.

In sum, the five chapters of my dissertation provide insight into the complex interactions between ants and also their natural enemies, particularly shedding light on how these interactions are facilitated through the use of ant pheromones as cues and signals. The ants and natural enemies that I focus on in my dissertation are also important players within the greater coffee agroecosystems they inhabit, and this body of work is thus critical for understanding how the network of interactions involving ants and their natural enemies may impact coffee pest control.

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CHAPTER 1

Current Understanding and Future Prospects of Host Selection, Acceptance, Discrimination
and Regulation of Phorid Fly Parasitoids that attack ants

Abstract

Phorid fly parasitoids (Diptera: Phoridae) have evolved a diverse array of cues used to successfully parasitize their ant hosts. Successful parasitism often involves (a) host habitat location, (b) host location, (c) host acceptance, (d) host discrimination and (e) host regulation. In this review we discuss our current understanding of how phorid flies use each of these steps to successfully parasitize ant hosts. We examine the wide variety of strategies and cues used by a multiple species of phorid flies within three separate genera (*Apocephalus*, *Pseudacteon*, and *Neodohrniphora*) and discuss future directions within this field of study.

Keywords: *Apocephalus*, cues, Formicidae, host selection, *Neodohrniphora*, parasitism, Phoridae, *Pseudacteon*

Introduction

Parasitoids have evolved effective and efficient methods of successful parasitism, many of which involve utilization of multimodal cues (Vinson 1976). Many dipteran parasitoids in the family Phoridae use social insects as hosts due to the reliability of their intraspecific chemical communication signals that make for effective host selection cues (Brown and Feener 1991, Disney 1994, Feener et al. 1996, Morehead and Feener 2000). Phorid fly adults parasitize ants by hovering over insect hosts and then diving down to insert an egg beneath the insect's exoskeleton (Disney 1994, Feener and Brown 1997, Porter 1998, Consoli et al. 2001). Phorid flies have direct parasitic effects on ants (i.e. cause ant mortality) and also significantly change ant foraging behavior by limiting host resource acquisition behavior, modifying ant competitive hierarchies, and dampening ant effects on herbivores (Feener 1981, Feener and Brown 1992, Orr et al. 1995, Orr et al. 2003, Vandermeer et al. 2002, Philpott 2005). There are phorids that attack ants from at least 22 genera across 5 subfamilies. Likewise, more than 20 genera of phorids attack ant hosts (Disney 1994). With such taxonomic diversification in ant-phorid relationships, the types of cues used by phorids to locate, select and successfully parasitize ant hosts are also quite diverse.

Successful parasitism requires a series of interactions between a parasitoid and its host. The process can be categorized into five general and sometimes overlapping steps: (a) host habitat location, (b) host location, (c) host acceptance, (d) host discrimination and (e) host regulation (Vinson 1976).

For phorid parasitoids, host location involves use of both habitat and host cues. Host habitat location is the use of environmental cues by the parasitoid to select areas to search for potential hosts. These cues may be directly related to the preferred environment of the host itself (volatiles from plants commonly used by hosts, for example) or related to the parasitoid's general habitat preferences (light, temperature, humidity conditions within a given area) (Vinson 1976). The host location process also requires that a parasitoid use long-range cues to be directed to its' host. However, unlike host habitat location cues, these cues come directly from the host itself. Ants communicate interspecifically by using complex pheromones. These pheromones often act as host location cues for parasitoids as they can be both reliable (with volatile pheromones highly conserved within a species or genus) and detectable (ants, being eusocial, live in relatively high densities and can produce large volumes of volatile pheromones) for the parasitoid (Vet and Papaj 1992).

Once a phorid parasitoid has located a potential host through long-range cues, the parasitoid requires host acceptance cues to trigger the parasitoid's oviposition behavior. Short-range cues such as movement, host size, and contact chemical cues have all been implicated in triggering phorid fly oviposition (Porter 1998, Wuellner et al. 2002, Mathis et al. 2011, Gazal et al. 2009, Silva et al. 2008, Orr et al. 1997, Gilbert and Morrison 1997, Morrison et al. 1997, Pesquero et al. 1996, Porter et al. 1995, Morrison and Gilbert 1998, Chen et al. 2009).

In addition to the cues that are required for overall host selection, host discrimination cues, used by parasitoids to detect and reject potential hosts that have been previously parasitized, can be present. While these cues are not necessary for parasitism, they can increase the likelihood of offspring success (Vinson 1976). Parasitoids can also increase the success rate of their offspring through host regulation, whereby parasitoids manipulate their hosts to promote the development of the next generation of parasitoids. Host regulation can involve altering the physiology of the host to facilitate growth and development of egg, larvae, or pupae of the parasitoid or altering host behavior to optimize nutrient intake or location within the external environment (Vinson and Iwantsch 1980).

This review focuses on our current understanding of the process by which phorid flies successfully parasitize ants. We examine the wide variety of strategies and cues used by multiple species of phorid flies within three separate genera (*Apocephalus*, *Pseudacteon*, and *Neodohniphora*) to successfully parasitize ant hosts.

Host Habitat Location

Parasitoid habitat preference is a major factor that determines where parasitoids will search for hosts and therefore which hosts will be successfully parasitized. Some hosts are selected not because they have a greater degree of inherent suitability but because they happen to be in an environment where parasitoid abundance is greater or where parasitoids are better able to detect cues released by their hosts (Vinson 1976). Light levels affect attack rates of several species of phorid flies. For example, *Neodohniphora tonhascai* and *Neodohniphora elongate* both attack *Atta sexdens* at significantly higher rates when in high light level laboratory conditions (Braganca et al. 2008). Field experiments with *Pseudacteon litoralis* and *Pseudacteon tricuspis* which attack ants in the *Solenopsis saevissima* complex, show these species prefer lower light levels (i.e. just after sunrise and before sunset) and higher light levels (mid-day sun) respectively (Pesquero et al. 1996). Analogously, lab experiments with *Pseudacteon curvatus* show that the flies attack *Solenopsis* spp. ants on darker backgrounds at greater rates than ants on white or light backgrounds [29]. *Pseudacteon* spp. phorids that attack the *Solenopsis saevissima* also display habitat preferences based on environmental factors such as temperature, rainfall, photoperiod length, sugar availability, wind, humidity and number of days with frosts (Folgarait et al. 2003, Chen et al. 2005, LeBrun et al. 2008).

Habitat complexity also affects phorid fly attack rates. Two species of phorid flies, *Apocephalus* sp. 8 and *Apocephalus* sp. 25 attack their host ants (*Pheidole diversiphilosa* and *Pheidole bicarinata* respectively) at higher rates when leaf litter is less complex, most likely because the ants are able to take better refuge in more complex leaf litter (Wilkinson and Feener 2007). Further, *Pseudacteon* spp. attack rate on *Azteca instabilis* is higher in coffee plantations with lower shade tree canopy complexity although the exact set of habitat

variables that create a preference for lower shade complexity remain unclear (Pardee and Philpott 2011).

Host Location

The long-range cues used by phorid flies to home in on potential hosts have been examined in several phorid-ant relationships. Some phorids travel at least 10-20 m to reach hosts and possibly up to 50 m, thus host location cues are likely generally volatile compounds, which can be detected by parasitoids well beyond the visual range of their hosts (Porter 1998b). While sound cues have the potential to be long range and have been documented in some non-phorid parasitoid-insect interactions, to date no phorid flies have been recorded to use sound as a cue in ant host location (Disney 1994, Zuk and Kolluru 1998). Paralleling the rich diversity of volatile ant pheromones, chemical host location cues used by phorid flies can vary widely in structure, glandular origin and purpose in ant-phorid relationship (Table 1). Long-range cues for phorids derive from several glands (mandibular, pygidial, etc.) and represent a wide array of pheromone types (trail, alarm, etc.). Several specific examples of these cues for different ant-phorid relationships follow.

The first set of host location cues documented for phorids were in the “giant tropical ant” *Paraponera clavata* attacked by the phorid, *Apocephalus paraponerae*. Parasitism of *P. clavata* by *A. paraponerae* was first observed in 1958 by C. W. Rettenmeyer on Barro Colorado Island, Panama. Rettenmeyer originally suspected that the flies were attracted to audible stridulations made by *P. clavata* individuals when alarmed. However, field observations showed that *A. paraponerae* were attracted to mandibular gland extracts of *P. clavata* that contain alarm pheromone (Brown and Feener 1991, Feener et al. 1996). The two major products of the mandibular glands of *P. clavata*, 4-methyl-3-heptanol and 4-methyl-3-heptanone (characterized in Hermann et al. 1984) were tested individually and both attract *A. paraponerae* (Feener et al. 1996).

Another species of phorid fly that utilizes the alarm pheromones of its host is, *Pseudacteon brevicauda*. Studies show that these phorid flies are attracted to mandibular gland extracts of their host, *Myrmica rubra* (Witte et al. 2010). Within these glands are 3-octanone, 3-nonanone, and 3-octanol (Cammaerts et al. 1981). The two ketones were found to attract *P. brevicauda* from a distance. While the alcohol, 3-octanol, did not attract flies from long distances, it was found to increase the “alertness” of the flies at a closer range, possibly indicating its synergistic role in host location or a possible role in host acceptance, however, further observations are needed to confirm the role of this compound (Witte et al. 2010).

Formic acid, a relatively common alarm and defense compound from the venom glands of ants, is the primary host location cue attracting *Pseudacteon formicarum* to the ants *Lasius niger* and *Lasius emarginatus* (Maschwitz et al. 2008). The use of formic acid is relatively common in ants and previously *P. formicarum* was thought to be one of the only phorid flies with multiple hosts because these flies frequently arrive to areas where a wide variety of ants using formic acid are aggregated. However, it was recently discovered that *P. formicarum* is specific to ants in the genus *Lasius*, rather than all ants that use formic acid, which indicates that these phorid flies must use other shorter-range cues in addition to formic acid to locate their hosts (Weissflog et al. 2008).

Three species of *Pseudacteon* phorid flies (Brown and Philpott 2013) use compounds from the pygidial gland of their host *Azteca instabilis* as long-range host

location cues. The pygidial gland of *A. instabilis* is the source of the alarm pheromone. At least one compound present within the pygidial gland of *A. instabilis*, 1-acetyl-2-methylcyclopentane attracts one or more of these phorid fly species to their host (Mathis et al. 2011, Wheeler et al. 1975), but further research is necessary to determine if all three phorid species are attracted to the same compound or suite of compounds.

The *Solenopsis saevissima* complex has the largest number of congeneric parasitoids recorded, with more than 18 *Pseudacteon* spp. known to parasitize this host group. However, despite significant research on these interactions, the details of the host location cues used in these interactions have remained somewhat elusive. In an early study, several of these phorids were categorized based on whether they were more likely to be found near disturbed ant mounds or trails—with the general hypothesis that phorid flies attacking ants near disturbed mounds must use alarm or defense compounds released by the ants as host location cues, and trail pheromone as a cue if they attack near trails. *Pseudacteon litoralis*, *P. tricuspis*, and *P. wasmanni* were all found attacking predominately near disturbed mounds or, in a few circumstances, trails where aggressive interspecies interactions were taking place between the ants. *Pseudacteon obtusus*, *Pseudacteon borgmeieri*, *Pseudacteon nuicornis* and *Pseudacteon solenopsidis* were more often found attacking ants on trails (Machwitz et al. 2008, Morrison and King 2004). In another set of studies, *P. tricuspis* was attracted to the midden (consisting primarily of dead workers) of *Solenopsis invicta*, lending further evidence to the hypothesis that its host location cue is a volatile chemical from the ants themselves (Gilbert and Patrock 2002, Puckett et al. 2007). Additionally shaken workers both elicit an alarm response in other workers and attract phorid flies (Vander Meer et al. 2002). Electroantennogram (EAG) experiments with *P. tricuspis* show that the flies are attracted to whole body extracts of workers, ant heads (including, to some extent, the mandible alone), and abdomens (Chen and Fadamiro 2007). The same study confirmed that *P. tricuspis* is not attracted to the trail pheromone of *Solenopsis invicta*, (E,E)- α -farnesene (Chen and Fadamiro 2007). The mandibular glands located within the head of *Solenopsis* spp. ants are the source of the ant's alarm pheromone, providing evidence that *P. tricuspis* likely uses a set of (rather than an individual) alarm pheromone compounds as a host location cue (Vander Meer 1996, Vander Meer and Alonso 1998). Recently, 2-ethyl-3,6-dimethylpyrazine has been confirmed as an active alarm pheromone component from within the mandibular glands of *S. invicta* and EAG experiments show this compound elicits a response in *P. tricuspis*, though the compound has yet to be tested in the field (Vander Meer et al. 2010, Sharma et al. 2011).

Yet, not all ant-phorid relationships appear to involve long-range chemical cues. In behavioral observations of *N. elongata* phorid flies and *A. sexdens* ants using a 50 cm³ observation chamber, Gazal et al. (2009) concluded that these phorids do not have a volatile chemical cue involved in host location. However, it is possible these cues are essential when phorids are at a greater distance from potential hosts and behavioral observations of ants and phorids in small and contained areas underestimate phorid specificity [53].

Host Acceptance

Short-range cues used by phorid flies to inspect potential hosts and determine whether they are suitable for oviposition can be visual or chemical or in some cases both

(Table 2). Visual cues are often multifaceted, including several simultaneous or sequential features such as movement, host size, and host shape. The chemical cues used in host acceptance are generally less volatile compounds that can only be detectable at close range.

Movement of target ants is a common visual cue frequently used by the *Pseudacteon* spp. phorid flies that attack both *A. instabilis* and ants in the *Solenopsis saevissima* complex as well as by *N. elongata* phorid flies attacking *A. sexdens* (Wuellner et al. 2002, Mathis et al. 2011, Gazal et al. 2009, Silva et al. 2008). *A. paraponerae* attacking *P. clavata*, however, prefer stationary ant hosts (Morehead and Feener 2000).

Size is also an important factor in phorid host acceptance. Variation in size preferences between phorid species attacking the same host is generally seen as an effective method of niche partitioning (Wuellner et al. 2002, Orr et al. 1997, Morrison et al. 1997, Morrison and Gilbert 1998). Within the guild of phorids that attack the *Solenopsis saevissima* complex, *P. curvatus*, *P. nudicornis* and *P. obtusus* attack small workers, *P. tricuspis* and *P. wasmanni* prefer medium sized workers, *P. borgmeieri* and *P. solenopsidis* tend to attack medium to large workers and *P. litoralis* attacks large workers (Porter 1998, Wuellner et al. 2002, Orr et al. 1997, Gilbert and Morrison 1997, Morrison et al. 1997, Pesquero et al. 1996, Porter et al. 1995). Size of the phorid fly is to a great extent a function of host body size (Morrison and Gilbert 1998, Morrison et al. 1999). In the case of *P. obtusus*, the small and large biotypes that are otherwise morphologically identical proved to be genetically distinct enough to be different species likely due to a variation in host size preference (Kronforst et al. 2007). Moreover, in *P. litoralis* and *P. tricuspis*, sex ratio is determined by the body size of the host, where larger host ants yield female offspring and smaller host ants yield male offspring (Morrison et al. 1999). Phorid flies in other genera also use size cues in host acceptance. *N. elongata* only attack *A. sexdens* foragers with a minimum head width of 1.6 mm and *A. paraponerae* prefer large *P. clavata* workers (Morehead and Feener 2000, Feener and Brown 1993).

The complexity of the visual stimulus related to shape has also been implicated in host acceptance. For example, *N. elongata* will inspect (i.e. hover over) moving visual stimulus of varying degrees of complexity from simple to complex: one model mass sphere, two linked spheres, three linked spheres, a plastic ant model and the host ant. Yet, the phorids only attack the most complex visual stimulus, which in the experiments was the host ant. Indeed, in this set of experiments, movement was unnecessary to trigger inspection if the visual stimulus was identical to the host, indicating that movement may act as a secondary cue to shape or visual complexity cues in order to enhance the speed and accuracy of attacks in this species (Gazal et al. 2009).

Two classes of short-range chemical cues have been identified in phorid-ant interactions, cuticular hydrocarbons and low volatility venom gland secretions. While *A. paraponerae* flies are equally attracted to untreated ants and ants treated with hexane to remove cuticular hydrocarbons, the flies significantly prefer to lay eggs in ants with cuticular hydrocarbons (Morehead and Feener 2000). Recent work on three *Pseudacteon* spp. phorid flies (Brown and Philpott 2012) that attack *A. instabilis* ants also show that these phorid flies may use cuticular hydrocarbons in host acceptance. When cuticular hydrocarbons of other ant species were applied to live *A. instabilis* ants, these *Pseudacteon* spp. phorid flies were much less likely to attack the ants than *A. instabilis* ants that were coated in additional *A. instabilis* cuticular hydrocarbons (Chapter 2). In experiments using electroantennograms and y-tube olfactometer bioassays *P. tricuspis* flies used venom gland

secretions of *S. invicta* in host acceptance. These experiments show several piperidine alkaloids, which are present in the ant's venom glands and used in defense, act as short-range attractants (Chen et al. 2009).

Host Discrimination

The ability for parasitoids to distinguish between unparasitized potential hosts and hosts that have been previously parasitized is evolutionarily favorable as offspring from the same species within a single host are at a competitive disadvantage (Vinson 1976). In fact, many parasitic hymenoptera can distinguish between parasitized and unparasitized hosts. Hymenopteran parasitoids use a variety of inhibitory cues in host discrimination including internal and external host-marking pheromones, or visual cues such as oviposition wounds (Vinson 1976).

In contrast, dipteran parasitoids, including phorid flies, appear to have high rates of superparasitism within populations (Feener and Brown 1997). For example, incidences of superparasitism by *Neodohrniphora curvinervis* on *Atta cephalotes* ants are relatively high at 19% in one field study (Feener and Brown 1993). Superparasitism by *N. elongata* on *A. sexdens* has been reported at 29.4% self-superparasitism and 49.5% conspecific superparasitism in a study conducted under lab conditions (Braganca et al. 2009). However, behavioral observations also show that once a *A. sexdens* host ant is parasitized, it is significantly less likely to be parasitized again by *N. elongata*, indicating that *N. elongata* are able to discriminate between parasitized and non-parasitized host ants but may in some circumstances (lab conditions, for example) choose to superparasitize a host regardless. Thus it appears these *N. elongata* do have some, however imperfect, form of host discrimination though the cues (Braganca et al. 2009). Dipteran parasitoids such as phorid flies do not have the accessory glands commonly used by hymenopteran parasitoids to produce host-marking pheromones (Feener and Brown 1997, Dindo 2011). Thus, while more work is needed to determine the mechanism, it seems most likely that phorid flies use visual cues from the ants' oviposition wounds in host discrimination.

Host Regulation

While relatively little is known about how phorid flies, in general, may manipulate their host's physiology in order to optimize the development of their offspring, strides have been made to understand the role of host regulation of *S. invicta* by *P. tricuspis* phorid flies. Like many other dipteran parasitoids, the developing phorid flies build respiratory structures in order to access fresh air through a hole in the integument of the host ant's head capsule (Consoli et al. 2001). Additionally, developing *P. tricuspis* are suspected to affect the neurophysiology of their ant hosts, as parasitized ants have altered behavior whereby they remain safely within the nest until just before the phorid larvae decapitate their hosts. Shortly before decapitation, ants will leave the nest, presumably to find a suitable location for the phorid fly to continue pupation and emerge (Henne and Johnson 2007). However, much remains unknown about the mechanisms by which these behavioral changes manifest in their host. Furthermore, there is nothing known about how any other phorid species are able to affect the behavior or growth of host ant species.

Conclusions and Future Prospects

In order to successfully parasitize a host, phorid fly parasitoids must undergo a multi-step process to detect and interpret a wide range of cues from their ant hosts. These cocktails of cues, each of which may vary in degrees of host specificity and timing of detection (sequentially or simultaneously), allow the flies to find suitable hosts in a complex environment. Researchers often study the interactions between phorid flies and their ant hosts in order to address the role of phorid flies as potential biological control agents of ants (Folgarait et al. 2003, Morrison and King 2004, Estrada et al. 2006, Folgarait et al. 2005, Porter and Briano 2000, Braganca et al. 2002). However, understanding these interactions could potentially shed light on evolutionary and ecological processes as well as provide a better understanding of multimodal communication.

Cues used by phorid flies are often traits considered to be highly conserved within the host species. These conserved traits are highly reliable and thus adaptive to phorid flies. Yet, little is known about how phorid use of these cues impacts the adaptive nature of these traits within ants. For example, *P. clavata* was originally thought to have no alarm pheromone responses, as these ants are relatively primitive and therefore independent outside of the nests, not requiring the assistance of their sisters during foraging. However, some studies indicate that *P. clavata* do have fairly developed intraspecific interactions during foraging (Breed et al. 1987, Fewell et al. 1992). As previously mentioned, *A. paraponerae* uses the alarm pheromones, 4-methyl-3-heptanol and 4-methyl-3-heptanone, to locate its hosts. Though more intensive investigation is required, it is possible that the use of alarm pheromone by *P. clavata* has been selected against in order to decrease parasitism. On evolutionary timescales, perhaps phorid use of chemical and visual cues has affected ant morphology, behavior and chemical communication.

While phorid flies are ubiquitous and conspicuous users of ant cues, a wide variety of other organisms are attracted to ants (Rettenmeyer et al. 2011, Holldobler and Wilson 1990). Considering the context dependent nature of successful parasitism discussed above, it seems likely that multiple myrmecophiles are utilizing similar cues and may thus affect the parasitism process. Indeed, only considering pair-wise interactions between organisms rather than interactions between a network of multiple parties with distinct cue preferences, perceptions, and responses can be misleading. For example, competitive interactions between male hermit crabs have been shown to affect mating strategy decisions of how male hermit crabs approach females (Wada et al. 2011). Additionally, ant-*Acacia* mutualisms are more accurately understood based on the overall fitness benefits to the *Acacia* plants via a network of ant species rather than one species alone and the nuances of multiple insect-ant interactions give insight into the overall effects of coffee pests in coffee agroecosystems (Perfecto and Vandermeer 2006, Liere and Larson 2010, Palmer et al. 2010, Vandermeer et al. 2010). Thus, future work should be conducted to elucidate how other ant symbionts may affect these phorid-ant interactions. Additionally, as phorid fly behavior is often dependent on a wide array of factors that may be altered in laboratory observations, more studies should be conducted in the field to verify the results of lab experiments.

Finally, phorid flies are often both ecologically relevant species and have remarkably diverse strategies for using diverse arrays of multimodal cues within a complex environment to successfully parasitize host ants. Thus, phorid-ant interactions are ideal systems to bridge the gap between model organisms for used in integrated pest

management and model organisms used in understanding the behavioral ecology of multimodal cue use.

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Table 1: Chemical host location cues used by phorid flies in search of ant hosts.

Phorid Species	Ant Species	Cue	Source	Ant Use	Ref
<i>Apocephalus paraponerae</i>	<i>Paraponera clavata</i>	4-methyl-3-heptanol and 4-methyl-3-heptanone	Mandibular Glands	Alarm Pheromone	2, 4, 37
<i>Pseudacteon brevicauda</i>	<i>Myrmica rubra</i>	3-octanone and 3-nonanone	Mandibular Glands	Alarm Pheromone	38, 39
<i>Pseudacteon formicarum</i>	<i>Lasius niger</i> and <i>Lasius emarginatus</i>	formic acid	Venom Glands	Alarm/Defense Pheromone	40
<i>Pseudacteon</i> spp.	<i>Azteca instabilis</i>	1-acetyl-2-methylcyclopentane	Pygidial Gland	Alarm Pheromone	17, 43
<i>Pseudacteon litoralis</i>	<i>Solenopsis saevissima</i> complex	unknown	unknown	Unknown use at Disturbed Mounds	20
<i>Pseudacteon wasmanni</i>	<i>Solenopsis saevissima</i> complex	unknown	unknown	Unknown use at Disturbed Mounds	20
<i>Pseudacteon obtusus</i>	<i>Solenopsis saevissima</i> complex	unknown	unknown	Unknown use on Trails	20
<i>Pseudacteon borgmeieri</i>	<i>Solenopsis saevissima</i> complex	unknown	unknown	Unknown use on Trails	20
<i>Pseudacteon nuicornis</i>	<i>Solenopsis saevissima</i> complex	unknown	unknown	Unknown use on Trails	20
<i>Pseudacteon solenopsidis</i>	<i>Solenopsis saevissima</i> complex	unknown	unknown	Unknown use on Trails	20
<i>Pseudacteon tricuspis</i>	<i>Solenopsis saevissima</i> complex	2-ethyl-3, 6-dimethylpyrazine	Mandibular Glands	Alarm Pheromone	51, 52

Table 2: Host acceptance cues used by phorid flies to choose ant hosts.

Cue Modality	Phorid Species	Ant Species	Cue	Source	Ant Use	Ref
Visual	<i>Pseudacteon</i> spp.	<i>Solenopsis saevissima</i> complex	Movement	--	--	16, 19
	<i>Pseudacteon</i> spp.	<i>Azteca instabilis</i>	Movement	--	--	17
	<i>Neodohniphora elongata</i>	<i>Atta sexdens</i>	Movement			18
	<i>Apocephalus paraponerae</i>	<i>Paraponera clavata</i>	No Movement	--	--	5
	<i>Pseudacteon nuicornis</i>	<i>Solenopsis saevissima</i> complex	Small Sized Workers	--	--	16
	<i>Pseudacteon obtusus</i>	<i>Solenopsis saevissima</i> complex	Small Sized Workers	--	--	16
	<i>Pseudacteon curvatus</i>	<i>Solenopsis saevissima</i> complex	Small Sized Workers	--	--	16
	<i>Pseudacteon tricuspis</i>	<i>Solenopsis saevissima</i> complex	Medium Sized Workers	--	--	16
	<i>Pseudacteon wasmanni</i>	<i>Solenopsis saevissima</i> complex	Medium Sized Workers	--	--	16
	<i>Pseudacteon borgmeieri</i>	<i>Solenopsis saevissima</i> complex	Medium to Larger Sized Workers	--	--	16
	<i>Pseudacteon solenopsidis</i>	<i>Solenopsis saevissima</i> complex	Medium to Larger Sized Workers	--	--	16
	<i>Pseudacteon litoralis</i>	<i>Solenopsis saevissima</i> complex	Larger Sized Workers	--	--	16
	<i>Neodohniphora elongata</i>	<i>Atta sexdens</i>	Minimum Head Width of 1.6mm	--	--	56
	<i>Apocephalus paraponerae</i>	<i>Paraponera clavata</i>	Large Workers	--	--	5
	Chemical	<i>Apocephalus paraponerae</i>	<i>Paraponera clavata</i>	Cuticular Hydrocarbons	--	Nest mate Recognition
<i>Pseudacteon</i> spp.		<i>Azteca instabilis</i>	Cuticular Hydrocarbons	--	Nest mate Recognition	57
<i>Pseudacteon tricuspis</i>		<i>Solenopsis invicta</i>	Piperidine Alkaloids	Venom Glands	Defense Pheromone	26

CHAPTER 2

Cuticular hydrocarbon cues used in host acceptance by *Pseudacteon* spp. phorid flies that attack *Azteca sericeasur* ants

Abstract

Parasitoids often use complex cues to correctly identify suitable hosts in a their environment. Phorid fly parasitoids that develop on one or a few host species often require use multiple cues, ranging from general to highly specific, to home in on an ideal host. Here, we describe the hierarchy of cues that *Pseudacteon* phorid flies use to successfully identify *Azteca* ant hosts. We show through behavioral observations in the field that phorid flies are attracted to two cryptic *Azteca* species, but will only attack *Azteca sericeasur* (Hymenoptera: Formicidae: Dolichoderinae). To test whether the phorid flies are able to distinguish between the two *Azteca* taxa using their cuticular hydrocarbons, we first documented and compared the cuticular hydrocarbons of two cryptic *Azteca* taxa using gas chromatography-mass spectrometry (GC/MS). Using cuticular hydrocarbon transfer experiments with live ants, we identify the cuticular hydrocarbons of *A. sericeasur* as a short-range host location cue used by *P. lascinosus* (Diptera: Phoridae) to locate the ants.

Keywords: *Pseudacteon* phorid flies; *Azteca* ants; parasitoids; coffee agroecosystem; host location; host acceptance; cuticular hydrocarbons

Introduction

Identifying suitable hosts in a complex environment is a key challenge for parasitoids. Many parasitoids have evolved to use cues from their host or the host's environment to locate and correctly identify hosts (Askew 1971; Godfray 1994; Vinson 1976; van Leteren 1981). Highly specific parasitoids that develop on one or a few host species often require the use of several cues, ranging from general to highly specific, to home in on an ideal host. For example, parasitoids may first use a general cue shared by many insects to locate a search area in which they may successfully find their hosts. Then once the parasitoid is within the appropriate search arena, it may need to use more specific cues to distinguish more finely between similar insects. This fine-scale differentiation among host may require the parasitoid to distinguish among closely related insects, or between viable hosts and unsuitable, previously parasitized hosts. This complex host selection process can be categorized into five general and sometimes overlapping steps: (a) host habitat location, (b) host location, (c) host acceptance, (d) host discrimination and (e) host regulation [Mathis and Philpott 2012].

Dipteran parasitoids in the family Phoridae frequently use social insects as hosts (Brown and Feener 1991, Disney 1994, Feener et al 1996, Morehead and Feener 2000). Phorid fly parasitoids first locate hosts, then hover over a chosen target and dive down to insert an egg beneath the insect's exoskeleton (Disney 1994, Feener and Brown 1997, Porter 1998, Consoli et al. 2001). For phorid flies that parasitize ants, host selection cues often include ant pheromones. Pheromones are effective host location cues for parasitoids because they are both detectable and reliable, as ants live in high densities producing large volumes of volatile pheromones and volatile pheromones are often highly conserved within taxa. Once a phorid parasitoid has located a potential host using long-range cues, oviposition is triggered by the detection of appropriate host acceptance cues. Short-range cues such as movement, host size, and contact chemical cues have all been implicated as triggers in phorid fly oviposition (Porter 1998, Wuellner et al. 2002, Mathis et al. 2011, Gazal et al. 2009, Silva et al. 2008, Orr et al. 1997, Gilbert and Morrison 1997, Morrison et

al. 1997, Pesquero et al 1996, Porter et al. 1995, Morrison and Gilbert 1998, Chen et al. 2009).

Throughout the New World tropics, several species of *Pseudacteon* phorid fly parasitize *Azteca* ants. Three species of phorid fly, *P. lascinosus*, *P. planidorsalis* and *P. pseudocercus* parasitize *Azteca sericeasur* ants within the same region in Chiapas, Mexico (Brown & Philpott 2012). However, *Azteca* workers in the genus *Azteca* are notoriously cryptic and difficult to distinguish from one another (Longino 2007). Indeed, *A. sericeasur* co-occurs with another, nearly identical, species of *Azteca* (currently unidentified but referred to here and on Ant Web as *Azteca* JTL020, J. Longino, personal communication), yet phorid flies that parasitize *A. sericeasur* do not parasitize *A. JTL020* despite being attracted to their nests. This begs the question, if these workers are so similar and the flies are attracted to both species of ants, how are the phorid flies able to distinguish between them to correctly identify *A. sericeasur* to oviposit?

In this study, we identify a three step hierarchy of cues that phorid flies use to successfully identify host ants. We characterize the cuticular hydrocarbons of the two *Azteca* species, and identify the cuticular hydrocarbons as short-range host location cues used by at least one species of phorid fly to locate *A. sericeasur* ants. We tested whether two species of phorid fly, *P. lascinosus* and *P. planidorsalis* use the cuticular hydrocarbons of *A. sericeasur* to discriminate between their host species and *Azteca* JTL020, which shares other long-range visual and chemical cues with *A. sericeasur*.

Methods

(a) Study Site

We conducted field all work on a shaded coffee plantation, Finca Irlanda, in the Soconusco region of Chiapas, Mexico (15° 11' N, 92° 20' W) between July 2012 and March 2013 in both the wet and dry seasons. Finca Irlanda is approximately 280 hectares in size, located between 950-1150 m elevation, and receives approximately 4500 mm of precipitation per year. *Azteca sericeasur* is the most dominant species of the approximately 60 species of arboreal ants that occur on the farm (Philpott 2005). *A. sericeasur* builds carton nests on the tree trunks of shade trees within the coffee plantation, where their colonies tend to be distributed in patches (Perfecto et al. 2014). *Azteca* JTL020 also builds large carton nests on the tree trunks of shade trees within the coffee plantations, but these nests are much less common in occurrence (Mathis, unpublished data).

(b) Behavioral observations

We collected behavioral data on the parasitism of *Azteca* by *Pseudacteon* by placing 10 ant workers (either *A. sericeasur* or *A. JTL020*) in shallow plastic dishes with Fluon-coated sides (Northern Products Inc., Woonsocket, Rhode Island, USA). We then placed these dishes near *A. sericeasur* nests to record phorid parasitism. Phorid attack rates on ants are density dependent and rapidly drop approximately 1 m from *Azteca* nests (Philpott et al. 2009). Twenty trees containing strong *A. sericeasur* colonies, each separated by at least 30 m, were used as trial sites. During each observation we recorded phorid fly arrivals, hover behaviors and attacks of ants within the plastic containers for 20 minutes. We recorded

any time a phorid fly entered the area directly above the plastic container and all phorid fly hover behaviors, which were considered to be any time a fly hovered less than 3 cm over a single ant worker and followed her. We also recorded phorid attacks, which were considered to be any time a phorid dove down to parasitize an ant, causing the ant to recoil from impact.

(c) Extraction, application and analysis of *Azteca* cuticular hydrocarbons

We performed CHC transfer experiments with living ants to test whether species-specific CHCs are used as host recognition cues by *Pseudacteon* phorid flies. We collected *A. sericeasur* and *A. JTL020* cuticular hydrocarbons (CHC) by rinsing 10 frozen ant workers in approximately 1.5 ml of hexane for 10 minutes. We filtered this extract through a silica column constructed from a glass pipette filled with silica gel (70-230 micron mesh, Fisher Scientific), rinsed the column with 1 ml of hexane, and collected the extract in glass vials. We evaporated the extracts under argon or nitrogen while swirling the vial, thus coating the walls of the vial with a layer of CHCs. These coated vials were used immediately in behavioral assays.

We treated worker ants with either CHCs from nestmates, as a negative control, or CHCs from the other *Azteca* species, as an experimental treatment. The negative control addresses the potential role of altering overall CHC concentration and controls for possible effects of handling. We treated individual live ants by first placing them in vials containing 0.1 – 0.15 g of clean silica gel (70-230 mesh, Fisher Scientific) and subsequently tapping the vial for 30 seconds to absorb the ant's cuticular hydrocarbons (Choe et al. 2012). We then removed the ants from the silica vials, placed them in a CHC-coated vial and vortexed them for 90 seconds to transfer the hydrocarbons. These ants were allowed to recover from vortexing (5 minutes) before conducting behavioral assays or stored at -20°C until re-extracted for comparative GC/MS analysis. One CHC-coated vial was used to treat 1 individual. This method did not injure the treated ants and this CHC transfer method is similar to methods used by Torres et al. (2007) in which live Argentine ants, *Linepithema humile*, were treated with hydrocarbons from nestmates and non-nestmates.

For GC/MS analysis, cuticular hydrocarbon extracts using one frozen ant worker were prepared as described in the previous section. After silica filtration, solutions were placed in GC/MS vials with glass inserts, evaporated under nitrogen and subsequently re-eluted with 60 µl of hexane. CHC extracts were then stored at -20°C until use. We then injected two µl of this solution into a Finnigan Trace MS+ gas chromatograph / mass spectrometer equipped with a DB-5 capillary column (30 m x 0.32 mm X 0.25 µm, Agilent Technologies, CA). Extracts were analyzed in splitless mode, with a temperature program that started at 100°C for 1 min, which then increased by 20°C min⁻¹ until it reached 150°C, and then increased by 5°C min⁻¹ until it reached 325°C where it stayed for 5 min. Injector and transfer line temperatures were kept at 325°C and 280°C respectively.

Before performing cuticular hydrocarbon transfer bioassays, we compared the chemical profiles of 10 individual untreated workers to 10 individual workers treated with CHCs for all pair-wise comparisons (2 *Azteca* species, 4 comparisons in all) in order to determine whether these ant taxa differed in their CHC profiles. To examine the effects of cuticular hydrocarbon transfer treatments on the overall hydrocarbon profiles of both *A. sericeasur* and *A. JTL020* workers, we applied extracted hydrocarbons to ants and then re-

extracted the treated ant hydrocarbons and analyzed them using GC/MS. We then compared the hydrocarbon profiles of these treated ants to the profiles of untreated ants (Table 1, Figure 2). To visualize the relationships between profiles of untreated and treated ants, we performed a two-dimensional Non-Metric Multidimensional Scaling (NMDS) (R Development Core Team, 2013).

(d) Cuticular hydrocarbon transfer behavioral assays

To test the response of phorid flies to ant cuticular hydrocarbons, 10 workers treated with either nestmate (negative control) or foreign hydrocarbons placed in plastic containers for behavioral assays. These behavioral assays, included 20 trials for each of the four treatments: a) *A. sericeasur* painted with nestmate CHCs, b) *A. sericeasur* painted with *A. JTL020* CHCs, c) *A. JTL020* painted with nestmate CHCs and d) *A. JTL020* painted with *A. sericeasur* CHCs. During field seasons in 2011 through 2013, we observed phorid fly parasitism of the ants in Fluon-coated plastic dishes for 20 minutes at the same 20 trial sites described above. During each observation we recorded the number of phorid fly attacks on ant workers within the plastic containers. After their first attack, phorid flies were collected and returned to the lab for species identification. Only two of the three species of phorid fly, *P. lascinosus* and *P. planidorsalis*, were present in sufficient numbers to compare between trials.

Results

Behavioral Observations

In initial behavioral observations, phorid flies behaved differently toward *A. sericeasur* workers and *Azteca JTL020* workers. While phorid flies would arrive to observations with both ant taxa, phorid flies arrived much less frequently to observations using *A. JTL020* (Figure 1a; ANOVA; $F(1,143)= 20.01, p < 0.001$). Similarly, we observed phorid flies hovering over both *A. sericeasur* and *A. JTL020*, but phorid flies hovered over *A. JTL020* workers less frequently than *A. sericeasur* workers (Figure 1b; ANOVA; $F(1,143)=20.01, p < 0.03$). Interestingly, although phorid flies would arrive to behavioral observations with *A. JTL020* and hover over the workers, none of the phorid flies chose to attack these ants in our behavioral observations. In contrast, phorid flies frequently attacked *A. sericeasur* workers (Figure 1c; ANOVA; $F(1,143)= 10.15, p < 0.003$). These results indicate that the phorid flies are able to distinguish between these two taxa when at close range, despite their initial attraction to the *A. JTL020* worker ants.

GC/MS profiles of Azteca ants

Analysis of hexane extracts of the cuticular hydrocarbons from both *A. sericeasur* and *A. JTL020* show that workers from these species have distinctly different chemical profiles. For the two species, *A. sericeasur* and *A. JTL020*, we identified 11 and 13 hydrocarbon peaks respectively, each representing at least 1% of the total area of all compounds (Table 1). Compounds consisted of straight chain alkanes, monomethyl alkanes and some dimethyl alkanes. Compounds had chain lengths ranging from 21 to 29 carbons, with *A. sericeasur* containing, on average, compounds with longer carbon chains than *A. JTL020*. Five peaks (*n*-C₂₃; *n*-C₂₅; C₂₇; *n*-C₂₆ and 10 and 12 and 14-MeC₂₆; *n*-C₂₇; 11 and 13-MeC₂₇) were found in both species. Representative chromatograms of cuticular hydrocarbons obtained from each species are depicted in Figure 2. Profiles of treated ants

more closely resembled their treatment chemotype than their original chemotype with very little “bleed through” of their original cuticular hydrocarbons (Figure 2). Our observations are supported by a two dimensional Non-Metric Multidimensional Scaling (NMDS) analysis, which shows that ants treated with *A. sericeasur* or *A. JTL020* cuticular hydrocarbons cluster with untreated *A. sericeasur* and *A. JTL020* ants respectively (Figure 3; stress coefficient = 0.061, indicating a good fit between distance data and the two dimensional rendering).

Behavioral assays with CHC transfers

Phorid flies arrived to behavioral assays in comparable numbers for all treatment types (Figure 4; ANOVA; $F(3,76) = .74$, $p > 0.5$). However, phorid flies attacked *A. sericeasur* ants treated with their own cuticular hydrocarbons more than all other treatment types (Figure 4; ANOVA with Tukey post hoc; $F(3,76) = 6.486$, $p < 0.0003$). Interestingly, when phorid attacks were broken down by species, although both *P. planidorsalis* and *P. lascinosus* attacked *A. sericeasur* workers treated with their own hydrocarbons, *P. planidorsalis* phorid flies also attacked *A. JTL020* workers treated with *A. sericeasur* cuticular hydrocarbons and *A. sericeasur* treated with *A. JTL020* hydrocarbons (Figure 5; ANOVA with Tukey post hoc; $F(3, 76) = 2.086$, $p = 0.109$). Thus, it appears that *P. lascinosus* is more efficiently taking advantage of the cuticular hydrocarbon cue in host choice before attacking the ant (Figure 5; ANOVA with Tukey post hoc; $F(3,76) = 6.275$, $p < 0.0005$).

Discussion

Our results illustrate that a hierarchy of different cues is used by a parasitoid to accurately identify and parasitize its host. We demonstrate that phorid flies are attracted to both *Azteca sericeasur* and *Azteca JTL020* and will hover over both taxa, but will only attack *Azteca sericeasur*. Additionally, although *Azteca sericeasur* and *Azteca JTL020* are nearly morphologically identical and share both of the short-range cues (alarm pheromone and movement) that attract phorid flies, these *Azteca* species taxa differ in their cuticular hydrocarbon composition. For phorid flies, particularly *P. lascinosus*, these cuticular hydrocarbons play a role as a short-range cue in host recognition. Our cuticular hydrocarbon transfer experiments show that *P. lascinosus* phorid flies attacked *A. sericeasur* ants treated with their own hydrocarbons more than ants treated with *A. JTL020* hydrocarbons indicating that presence of the *A. sericeasur* hydrocarbons is a short-range cue used in host choice. However, *P. lascinosus* flies did not attack *A. JTL020* ants treated with *A. sericeasur* cuticular hydrocarbons despite these two ant species being nearly morphologically identical. This result may be due to *P. lascinosus* phorid flies being repelled by remaining trace amounts of *A. JTL020* cuticular hydrocarbons present on the cuticle of some of the ants, which can be seen in the two outliers in Figure 3. Alternatively, these results may indicate that while cuticular hydrocarbons are a necessary cue, these flies may also require an additional synergistic short-range behavioral cue in host selection, such as the body position of the ant, or ant’s speed.

While *P. planidorsalis* phorid flies attacked ants with *A. sericeasur* cuticular hydrocarbons more, and did not attack the *A. JTL020* ants treated with their own hydrocarbons at all, the treatments were not significantly different, likely due to the relatively lower abundance of this species in the field (Reese and Philpott 2012) and the

subsequent overall scarcity in *P. planidorsalis* attacks. Previous work shows that *Pseudacteon* phorid flies also use the ant's alarm pheromone to locate hosts at a distance, and use movement to further home in on individual ants (Mathis et al. 2011). We propose the following hierarchical use of cues in host selection. First, *A. sericeasur* releases alarm pheromone that attracts phorid flies from a distance. Then, once in visual range, the fly will home in on the movement of an ant and hover over an individual worker. Finally, the *P. lascinosus* fly will briefly touch the worker to verify the ant is *A. sericeasur* by inspecting the ant's cuticular hydrocarbon cues before oviposition. The use of the close-range cue may be important for phorid flies because the nature of the initial cues allow for a large number of errors in phorid oviposition. *A. sericeasur* often releases alarm pheromone during aggressive encounters with other ant species. If the phorid flies arrive to an area where *A. sericeasur* is interacting with one or more other ant species, and movement is the only other cue required for oviposition, it follows that these phorid flies would frequently make host choice errors. Therefore it seems likely the flies initially use the movement of the ants as a cue to home in and become close enough to test the cuticular hydrocarbons of target ants to ensure that they are *A. sericeasur*. As phorid flies are also attracted to the alarm pheromone and movement of *A. JTL020* (which are the same as those of *A. sericeasur*), it remains unclear whether *A. JTL020* is an unsuitable host for *P. lascinosus* or if the specificity of their short-range cue merely renders *A. JTL020* invisible to them. Other work has shown that while *Apocephalus paraponerae* phorid flies may not be attracted to ant species closely related to their hosts, they may be able to successfully develop within them (Brown and Feener 1991; Morehead and Feener 2000). Further investigations rearing *P. lascinosus* in both *Azteca* taxa would provide information as to whether flies are compatible with both as hosts.

Here we have identified that *P. lascinosus* phorid flies require the presence of *A. sericeasur* CHCs as a third cue used in successful host selection, however this still may not be the complete picture of successful parasitism for *P. lascinosus* or *P. planidorsalis* as the flies are likely using some kind of synergistic short-range behavioral cue to locate hosts. Despite having the *A. sericeasur* alarm pheromones, phorid flies remained unwilling to parasitize *A. JTL020* ants in transfer experiments, indicating that phorid flies are still able to distinguish *A. JTL020* ants from *A. sericeasur* regardless of CHC. Additionally, behavioral observations using previously parasitized ants have shown that phorid flies prefer to attack unparasitized *A. sericeasur* (K. Mathis, unpublished data), so we may infer that phorid flies that attack *Azteca* ants, as for other phorids, also use some kind of host discrimination cue to determine whether ants have been previously parasitized (Feener and Brown 1993, Bragança et al. 2009).

Through GC/MS analysis, we identified five peaks within the *A. sericeasur* cuticular hydrocarbon profile that are distinct from *A. JTL020* and may be partly responsible for *P. lascinosus* host choice. Additional cuticular hydrocarbon transfer experiments using synthetic versions of these compounds would allow us to determine whether it is the presence or absence of one or many of these compounds that acts as a cue to *P. lascinosus*. While a few other studies have conducted solid-phase cuticular hydrocarbon transfer experiments on live ants (Liang and Silverman 2000; Torres et al. 2007), ours was the first to remove the original cuticular hydrocarbon signature with a silica rubbing technique (Choe et al. 2012) prior to cuticular hydrocarbon transfer. Additionally, our study is the first to use this method to investigate parasitoid host location cues.

In summary, this study shows that phorid flies are able to distinguish between two cryptic taxa of *Azteca* ants, despite these ants sharing two of the cues the phorid flies use in host location. *Pseudacteon lascinosus* use *Azteca sericeasur* cuticular hydrocarbons as a short-range cue directly before oviposition. Further study for synergistic short-range behavioral cues is needed in addition to identifying which of the cuticular hydrocarbon compound candidates are acting as the short-range cue.

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Table 1: Cuticular hydrocarbons of untreated and treated *A. sericeator* and *A. JTL020* ants as described in Figure 2. All hydrocarbons are measured as average percent composition +/- standard deviation.

Peak No.	Compound ID	JTL020	JTL020 on	JTL020 on	SEr	SEr on	SEr on
		SEr	SEr	SEr	SEr	SEr	JTL020
1	n-C ₂₁	1.8±0.9	3.0±2.6	1.4±1.0	0.0±0.0	0.0±0.0	0.0±0.0
2	n-C ₂₂	1.1±1.1	3.5±4.1	1.2±0.9	0.0±0.0	0.0±0.0	0.0±0.0
3	n-C ₂₃	16.4±2.3	20.7±12.2	19.0±1.8	1.6±3.3	1.5±1.7	4.0±2.4
4	13-MeC ₂₃	9.0±0.8	11.9±5.7	8.2±3.6	0.0±0.0	0.0±0.0	0.0±0.0
5	3-MeC ₂₃	2.2±0.7	4.1±2.7	2.5±0.5	0.0±0.0	0.0±0.0	0.0±0.0
6	n-C ₂₄	3.3±0.6	1.4±2.0	2.2±1.0	0.0±2.5	0.0±0.0	0.0±0.0
7	13 and 15-MeC ₂₄	2.6±0.3	5.3±3.5	3.6±0.6	0.0±0.0	0.0±0.0	0.0±0.0
8	3 and 7-MeC ₂₄	3.8±0.5	0.6±1.6	1.4±1.1	0.0±0.0	0.0±0.0	0.0±0.0
9	n-C ₂₅	16.6±2.6	7.0±6.0	18.1±2.9	9.7±1.3	11.8±1.4	16.2±3.0
10	13 and 15-MeC ₂₅	28.3±2.9	28.7±14.5	29.7±4.9	0.0±0.0	0.0±0.0	0.0±0.0
11	13 and 15 and 3-MeC ₂₅	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.0	0.4±1.0	0.3±1.4
12	n-C ₂₆ and 10 and 12 and 14-MeC ₂₆	1.3±0.9	2.8±1.3	0.6±0.7	1.2±1.7	2.1±1.9	3.6±1.8
13	n-C ₂₇	4.2±0.7	3.1±5.4	4.0±0.8	52.7±7.0	55.6±6.1	51.4±8.0
14	11 and 13- MeC ₂₇	9.5±1.1	7.9±5.9	8.0±1.2	7.9±0.0	5.3±0.0	2.9±3.0
15	6, 16 and 8, 15-diMeC ₂₇	0.0±0.0	0.0±0.0	0.0±0.0	5.1±0.7	4.3±0.4	2.1±1.7
16	10 and 12 and 13 and 14-MeC ₂₈	0.0±0.0	0.0±0.0	0.0±0.0	2.0±1.7	1.1±1.5	0.0±1.1
17	n-C ₂₉	0.0±0.0	0.0±0.0	0.0±0.0	13.3±1.7	12.1±1.1	14.9±1.9
18	MeC ₂₉	0.0±0.0	0.0±0.0	0.0±0.0	3.3±2.6	3.9±2.7	3.4±4.4
19	7, 15 and 7, 17-diMeC ₂₉	0.0±0.0	0.0±0.0	0.0±0.0	2.9±2.1	1.9±2.1	1.1±0.0

Figure 1: Plot of average number of phorid behaviors per observation in the field with either *A. sericeasur* ants (SER) or *A. JTL020* ants (JTL). (a) Plot showing number of phorid flies arriving to arena. (b) Plot showing number of phorid fly hover behaviors in the arena. (c) Plot showing number of phorid fly attack behaviors in the arena.

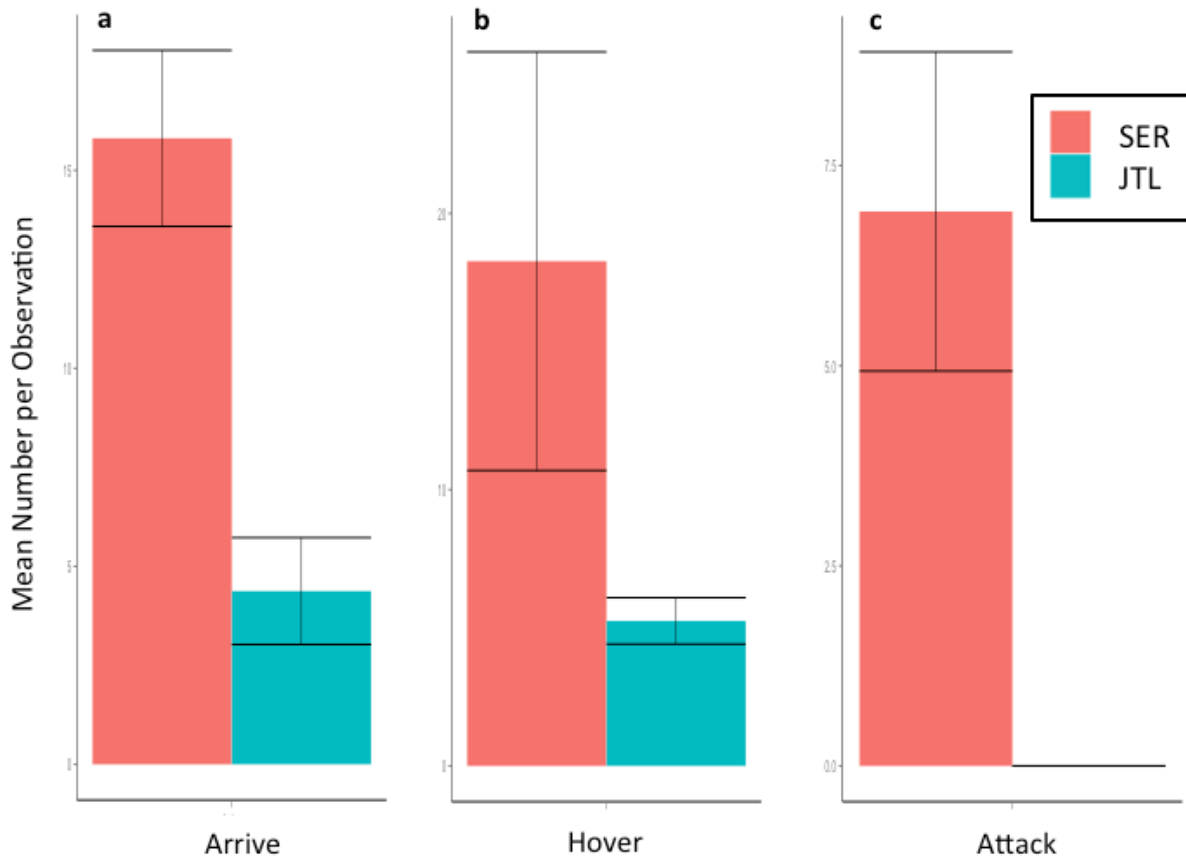


Figure 2: Representative total ion chromatograms of *Azteca* ant cuticular hydrocarbons extracted via hexane including (SER) untreated *Azteca sericeasur*; (JTL) untreated *A. JTL020* ants; (SER/SER) *A. sericeasur* ants treated with *A. sericeasur* CHCs; (JTL/JTL) *A. JTL020* ants treated with *A. JTL020* CHCs; (SER/JTL) *A. JTL020* ants treated with *A. sericeasur* CHCs; and (JTL/SER) *A. sericeasur* ants treated with *A. JTL020* CHCs. In both untreated ant chromatograms, numbers indicate peak numbers seen in Table 1.

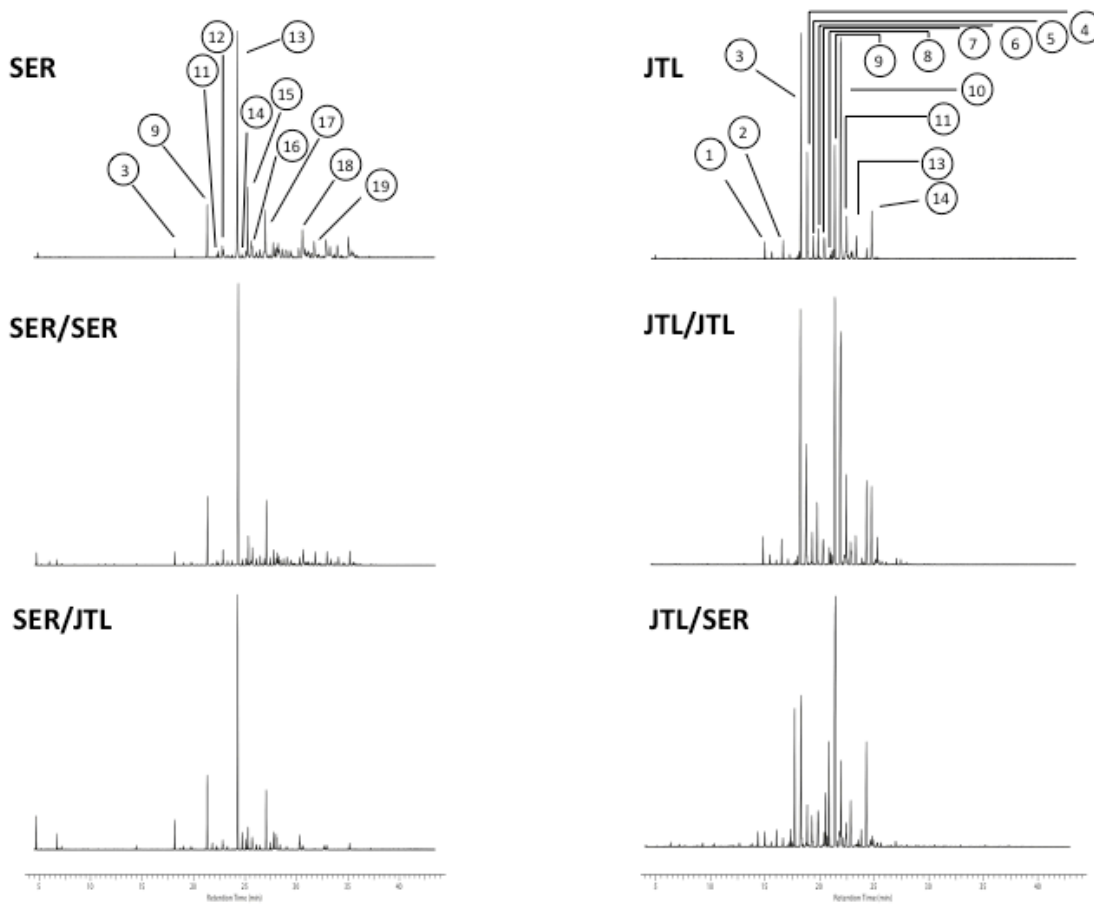


Figure 3: NMDS two factor analysis of hydrocarbon profiles shown in Figure 2. Each symbol represent an individual hydrocarbon profile of one ant worker. Symbol color represents treatment type as described in Figure 2.

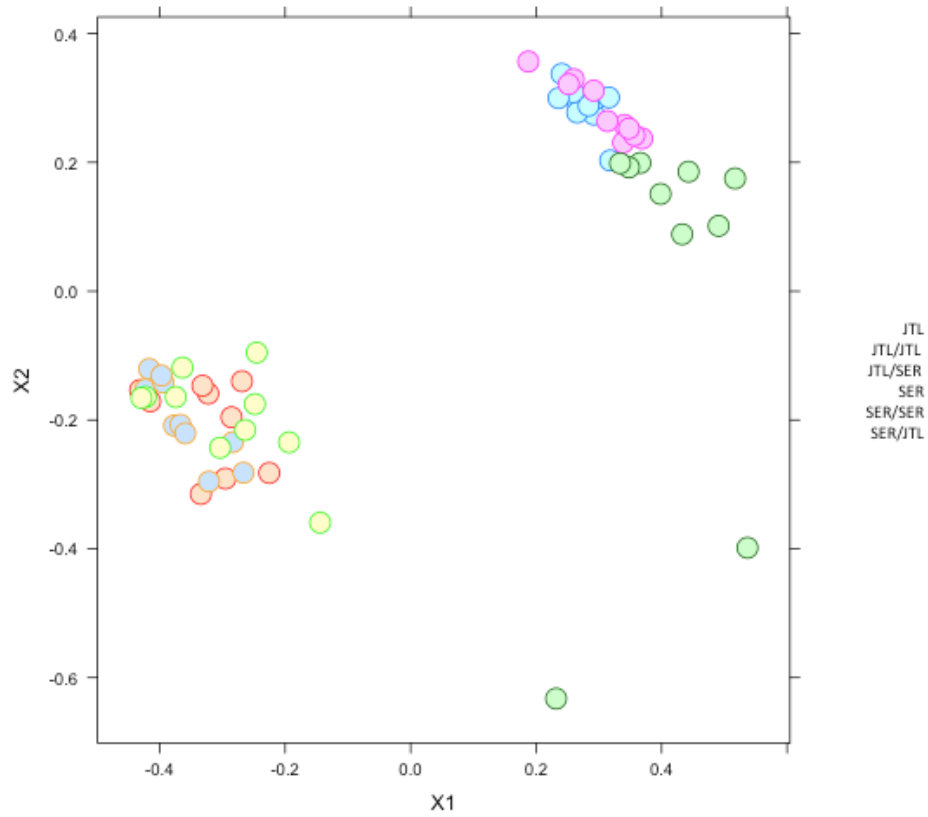


Figure 4: Plot of average number of phorid flies to arrive (red) and attack ants (blue) in cuticular hydrocarbon transfer experiments. Treatment types are the same as described in Figure 2.

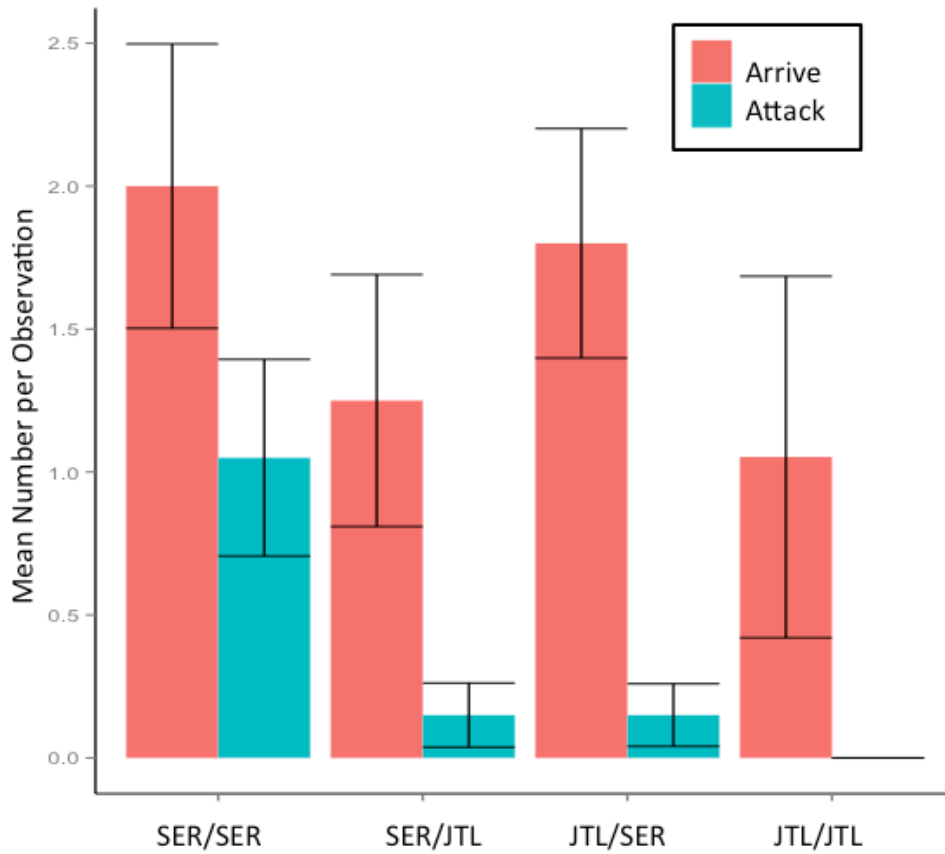
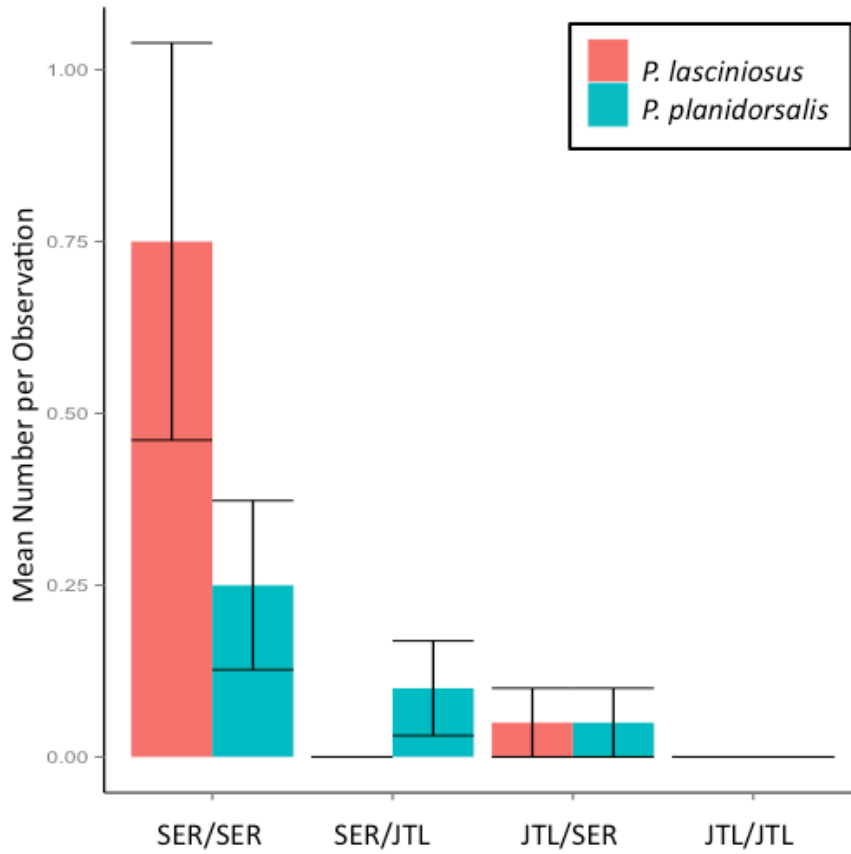


Figure 5: Plot of average number of *P. lasciniosus* (red) and *P. planidorsalis* (blue) phorid flies to attack ants in cuticular hydrocarbon transfer experiments. Treatment types are the same as described in Figure



CHAPTER 3

Descriptions of two new species of *Myrmedonota* Cameron (Staphylinidae: Aleocharinae) from Mexico with comments on the genus taxonomy and behavior.

Abstract

Two new species of *Myrmedonota*, *M. shimmerale* n.sp. and *M. xipe* n.sp., are described, and the genus is recorded from Mexico for the first time. Dorsal habitus photographs, illustrations of the median lobe and spermatheca are presented for diagnostic purposes. We suggest that *Myrmedonota* is in fact diverse in the New World and that its taxonomy is already in a state of confusion. New behavioral observations show that: (1) *Myrmedonota* species will aggregate towards agitated ants, possibly to prey on them; (2) *Myrmedonota* will form mating swarms, either with no apparent landmark or in the vicinity of ants.

Key Words: *Apalonia*, *Azteca*, *Eciton*, myrmecophily, *Papyrius*, *Pheidole*, *Pseudacteon*, symbiosis, *Termitognathus*, termitophily.

Introduction

The genus *Myrmedonota* Cameron, 1920 (Aleocharinae: Athetini) contains 26 species, described primarily throughout the Old World, with 23 species described from Malaysia, Indonesia, Papua-New Guinea and Singapore. Currently only three species have been described from the New World, all from North America (Maruyama et al. 2008; Eldredge 2010), though the actual diversity is believed to be much larger (Eldredge, personal observation). While the life histories of most species are unknown, several have been collected in the presence of either ants or termites (Bourguignon & Roisin 2006; Maruyama et al. 2008). One species, *Myrmedonota papyriomyrmecis* Kistner, 2003 is the first to have a definitive host record, and can be found scavenging in the kitchen middens of *Papyrius nitidus* Mayr, 1862 nests (Kistner 2003).

Recently, we collected two undescribed species of *Myrmedonota* on a shade coffee farm in Chiapas, Mexico from traps for behavioral observations, originally designed to attract *Pseudacteon* Coquillet, 1907 phorid flies to *Azteca sericeasur* Longino, 2007 ants. In this paper we describe the new species, the first ever collected from Mexico, and provide bionomical information.

Material and methods

Specimens were observed using an Olympus SZX7 stereomicroscope, an Olympus BX51 compound microscope, and a Leica MZ12.5 stereomicroscope. Illustrations were made using an Olympus U-DA camera lucida mounted on an Olympus BX51. Scale bars were drawn using an Olympus slide micrometer. Body measurements were made using a Leica stereomicroscope ocular micrometer.

Dissected structures were cleared in KOH, mounted in Euparal (see Eldredge 2012 for details) and pinned underneath the specimen (similar to Maruyama 2004).

Terminology for median lobe orientation follows Gusarov (2002).

Holotypes and paratypes are deposited at the Essig Museum of Entomology, University of California, Berkeley; additional paratypes are deposited at the Snow Entomological Museum Collection, University of Kansas.

Taxonomy

Genus *Myrmedonota* Cameron, 1920

Diagnosis. The following character states in combination can diagnose *Myrmedonota* from other New World genera of Aleocharinae: (1) tarsal formula 4-5-5; (2) mesocoxae widely separated; (3) metaventral process much longer than mesoventral process, which extends little between the mesocoxae; (4) galea length greater than 7 times width at base, maxillae overall appearing extremely slender and elongate; (5) neck absent; (6) occipital suture complete to hypostoma; (7) pronotum with complete marginal line; (8) paramerite vellum much larger than condylite vellum; (9) paramerite vellum obscuring condylite vellum from outer view, condylite vellum small and fan-shaped; (9) apical lobe of paramere free and articulating with paramerite; (10) glossa bifid; (11) labial palpomere II and III subequal in length.

Comments on taxonomy. *Myrmedonota* taxonomy is quite chaotic. Elven et al. (2010) demonstrated that *Myrmedonota* and other New World genera formerly classified under Lomechusini are in fact a lineage within Athetini that has converged on similar morphologies (Elven et al. 2010). Both Lomechusini and these “pseudo-lomechusines” are often symbiotic with ants and termites, one potential explanation for convergence may be adaptively rooted in symbiont ecology.

Since Elven et al. (2010), more formalized morphological characters that diagnose Lomechusini from pseudo-lomechusines are becoming apparent (Eldredge personal observation). It is turning out that most New World taxa fall under the currently outdated diagnostic umbrella of Lomechusini are pseudo-lomechusines, and therefore belong to Athetini. Therefore, there appears to be, at least at a superficial level a New World-Old World biogeographical split between pseudo-lomechusines and Lomechusini respectively. This is interesting in that it implies there have been two independent evolutionary experiments with the ‘lomechusine-morphotype’ both have diversified in the context of social insect symbiosis and demonstrate potential for a comparative study of symbiosis.

Even with this enlightened perspective of a pseudo-lomechusine lineage, *Myrmedonota* as currently understood is plesiomorphic in overall body form, and many genera may in fact be lineages that have derived from *Myrmedonota*, rendering it paraphyletic. For example, the genus *Termitognathus* Borgmier, 1959 may be one such lineage, only diagnosable by morphologies derived from the pressures of living with termites.

To attest to the difficulties with distinguishing *Myrmedonota* from some other pseudo-lomechusine genera, Pace (1997, 2008a, 2008b, 2009) in a series of publications may have in fact described species of *Myrmedonota* under *Apalonia* Casey, 1906 (Eldredge 2012). Habitus and genitalic figures that accompany Pace’s descriptions support this hypothesis. In order to address issues concerning *Myrmedonota* taxonomy, the new species described below have been compared with Pace’s (1997, 2008a, 2008b, 2009) descriptions.

We bring up these issues on *Myrmedonota* taxonomy, even though much of the above is still speculative, because we think that *Myrmedonota* may be phylogenetically

important. More apomorphic symbiont lineages may nest within *Myrmedonota* as we currently diagnose it, making the genus important in understanding the evolution of social insect symbiosis among pseudo-lomechusines. Additionally, this has implications if evolution of social insect symbiosis is to be studied comparatively between Lomechusini and pseudo-lomechusines. We suggest that new genera of pseudo-lomechusines be erected with caution, and urge the phylogeny reconstruction of this diverse clade.

Comments on behavior. Observations of *Myrmedonota* behavior have been accumulating for some time, although most are unpublished. From these, two interesting tendencies have come to light: (1) *Myrmedonota* swarm to ant colonies, especially during periods of colony agitation; (2) *Myrmedonota* mate by swarming, frequently in the presence of ants.

Myrmedonota are predators, and as ant symbionts either predators of ants (Eldredge personal observation, Mathis in preparation) or scavengers (Kistner 2003). In Costa Rica, Eldredge has observed a *Myrmedonota* species fly into the midst of an agitated *Pheidole* Westwood, 1839 colony during nest excavation. Mathis (in preparation) has made detailed observations of aggregation and predation behavior of the two new species described below but these will be presented subsequently.

Mating swarms of *M. lewisi* Maruyama et al. 2008 have been observed to occur on open ground with no apparent landmark (Eldredge 2013). Adults would fly above ground and periodically land, at which time they begin a searching behavior until the opposite sex is found. Upon locating a mate, a circular, almost ritualistic chase begins, followed by brief copulation. A white sheet placed on the ground did not elicit concentrated swarming around the sheet, but did allow easier observation of matings. Eldredge also observed mating swarms of a *Myrmedonota* species in Costa Rica, concentrated around the foraging column of an *Eciton* Latreille, 1804 species. The two new species described below have been observed mating in the presence of agitated *Azteca sericeasur* often while *Pseudacteon* phorid fly attacks took place (Mathis, 2013). Adults appear to be attracted to a chemical compound present within the alarm pheromone of *Azteca sericeasur* (Mathis, in preparation). When the adults arrive to the location and find a mate, a chase begins, often resulting in a lengthy copulation.

***Myrmedonota shimmerale* Mathis & Eldredge, sp. n.**

Type series. Holotype, m#: MEXICO: Chiapas: Finca Irlanda Biological Station (15° 11' N, 92° 20' W) 25.ix.2012, on sticky trap, coll. K. Mathis. Paratypes: 10, same data as holotype.

Etymology. The name refers to the dull green iridescence of the integument that makes the beetle appear to shimmer in light.

Diagnosis. This species can be distinguished from all other species of the New World *Myrmedonota* by its dull irredescent green integument, small size, presence of a shallow basomedial impression on the pronotum, form of the aedeagus and spermatheca.

Description. Body (Fig. 1) compact-slender and subparallel. Length with a mean of 2.09 mm (n = 5), color yellowish to black, integument overall shining with a dull green iridescence. Head, pronotum and abdominal tergites III–VII black; elytra black to light

brown; abdominal tergites I–II yellowish light brown to black; mouthparts, legs yellowish to black; antennae black, segments I–III may be yellowish light brown to yellowish brown.

Legs short; hind tibia 0.785 times as long as the elytral width. Macrosetae less prominent; antennal macrosetae 1.8 times the width of antenomeres.

Male. Median lobe (Figs. 2–3) somewhat limuloid in parameral view; apical lobe gently curved paramerally in lateral view, pointed at apex in lateral and parameral views; copulatory piece wide, apically subulate and base with lateral paired rounded arms, annulus large and laterally wide; comb of teeth present, directed paramerally in lateral view and apical of copulatory piece. Tergite VIII apicomediaally concave and margin variably serrate.

Female. Spermatheca (Fig. 4) curved twice. Mildly S-shaped. Internal cone with circumventral sculptural grooves.

***Myrmedonata xipe* Mathis & Eldredge, sp. n.**

Type series. Holotype, m#: MEXICO: Chiapas: Finca Irlanda Biological Station (15° 11' N, 92° 20' W) 25.ix.2012, on sticky trap, coll. K. Mathis. Paratypes: 10, same data as holotype.

Etymology. The name refers to Xipe Totec, the Aztec life-death-rebirth deity that was worshipped in part by flaying human sacrifices in an act symbolizing the casting off of the old and new growth. The name is a metaphor for the species role in Azteca ant societies. The beetles prey on phorid parasitized workers whose deaths reduce phorid fly populations and benefit the colony as a whole (Mathis, in preparation).

Diagnosis. This species can be distinguished from all other species of New World *Myrmedonota* by color pattern, bifid apex of the median lobe, form of the median lobe and copulatory piece.

Description. Body (Fig. 5) slender and subparallel. Length with a mean of 3.2 mm ($n = 5$), color yellowish to black, integument overall dull. Head and abdominal tergites VI–VII dark brown to black; pronotum, elytra, and abdominal tergite V yellow to dark brown; abdominal tergites I–IV yellowish to light brown; mouthparts and legs yellowish; antennae dark brown, segments I–III and apex of segment XI may be yellowish.

Legs long; hind tibia 0.78 times as long as the elytral width. Macrosetae prominent; antennal macrosetae 2.47 times the width of the antenomere.

Male. Median lobe (Figs. 6–7) somewhat limuloid in parameral view, apex slightly bifid; apical lobe gently curved paramerally in lateral view, generally pointed at apex in lateral and parameral views; longitudinal bands large and conspicuous; copulatory piece overall narrow, apex slender and attenuated, base with lateral paired blunt arms slightly constricted at midpoint, annulus small and longitudinally elongate; comb of teeth present, directed paramerally in lateral view and apical of copulatory piece. Tergite VIII apicomediaally concave and margin variably serrate.

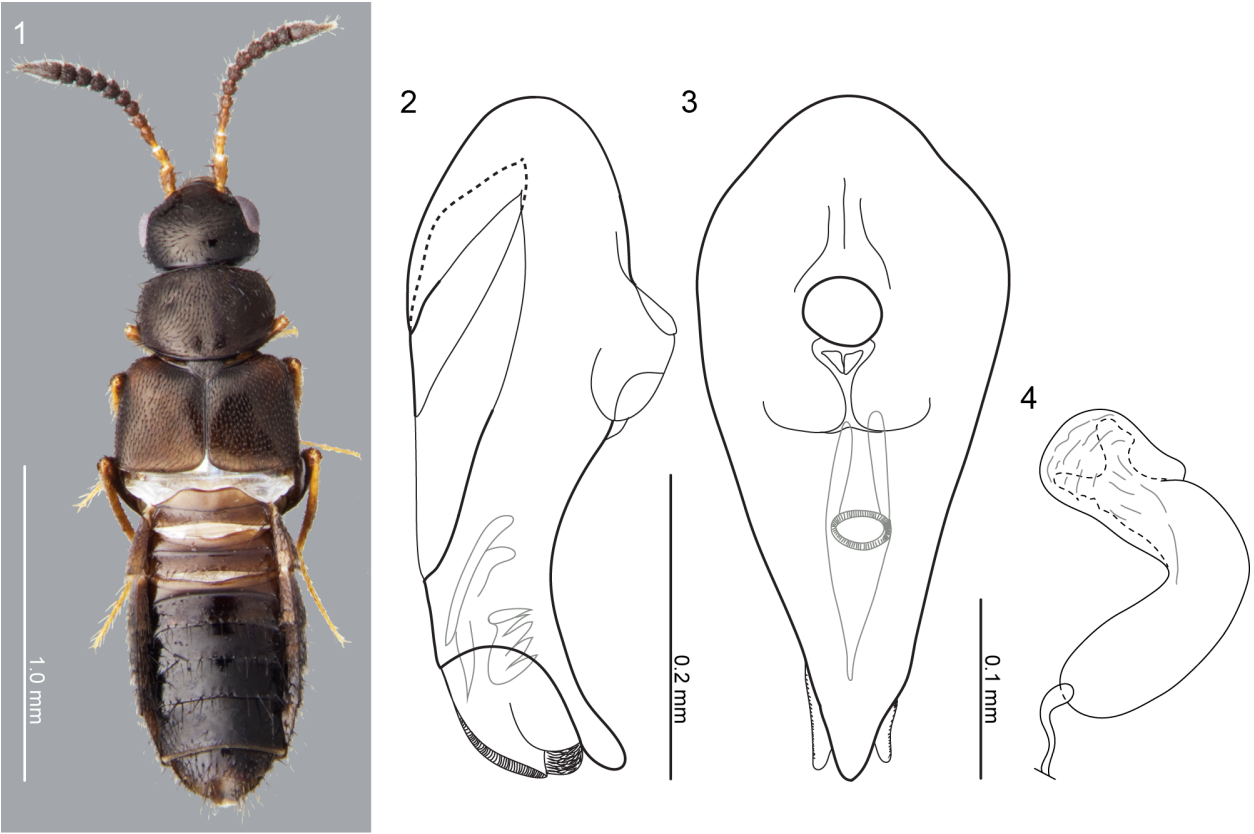
Female. Spermatheca (Fig. 8) bent submedially at near-right angle. Internal cone with circumventral sculptural grooves.

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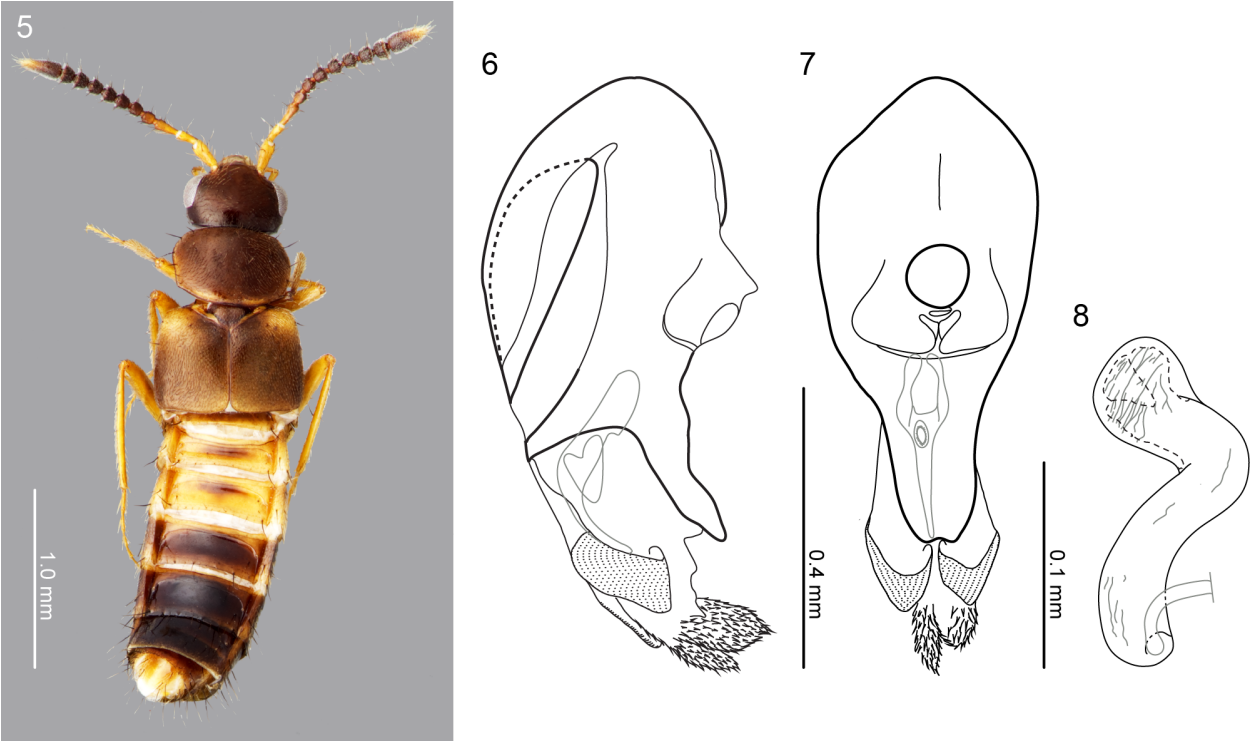
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Figures 1–4: *Myrmedonota shimmerale* Mathis & Eldredge, n.sp.: (1) dorsal habitus, (2) median lobe, lateral view, (3) median lobe, parameral view, (4) spermatheca, gland opening oriented right.



Figures 5–8: *Myrmedonota xipe* Mathis & Eldredge, n.sp.: (1) dorsal habitus, (2) median lobe, lateral view, (3) median lobe, parameral view, (4) spermatheca, gland opening oriented right.



CHAPTER 4

Dead Ant Walking: Trait plasticity in parasitized ants facilitates predation

Abstract

The role of beetles that associate with ant societies vary but are, in general, poorly understood. Here we document the role of two species of ant-associated beetles that have been found near *Azteca sericeasur* ants and the dynamics of their interactions. *A. sericeasur* is a common arboreal ant in the New World tropics and a keystone species in pest management in coffee agroecosystems in Chiapas, Mexico. However, a suite of *Pseudacteon* phorid fly parasitoids is the natural enemy of *A. sericeasur* and potentially reduce the ant's ability to act beneficially in coffee agroecosystems. We show that a species of beetle, *Myrmedonota xipe*, is attracted to *A. sericeasur* alarm pheromone. In the field, these beetles are able to locate ants that have been parasitized by at least one species of phorid fly. We show that approximately 14% of parasitized ants are consumed by the beetles within two days of being placed in the field. We also show that, in choice tests, beetles will preferentially attack parasitized ants over unparasitized ants, and that these beetles may distinguish between parasitized ants and unparasitized ants by the reduced levels of aggression unparasitized ants displayed toward the beetles. We show that the aggression in parasitized ants is so reduced that beetles are essentially able to eat these ants alive without interruption. These results indicate that, although beetles are predators of the ants, by preying primarily on ants harboring phorid fly eggs, the beetles may also provide indirect positive effects for the ant colonies as a whole.

Keywords: Ant-associated beetles, complex interactions, phorid fly parasitism, predation, adaptive suicide

Introduction

While often studied in isolation, predator-prey and host-parasitoid interactions have a wide array of effects within food webs and are thus increasingly approached from a community perspective (Orlofske et al 2014). Natural enemies can directly influence hosts or prey by reducing population size or inducing changes in phenotype (i.e behavior, morphology, etc.). These interactions, when considered within the greater ecosystem, often have cascading effects on other species within the community (Bruno and Cardinale 2008, Liere and Larson 2010, Perfecto et al. 2014). Cascading or indirect effects can be mediated by both changes in host/prey density (density mediated indirect effect, DMIE) and changes in the traits of host/prey species (trait mediated indirect effects, TMIE)(Werner and Peacor 2003).

Trait-mediated interactions may be particularly relevant to host-parasitoid systems because host behavior and/or physiology is frequently modified as a result of parasitism, with subsequent consequences for structuring biological communities (Hatcher et al. 2013). Trait modifications can occur before or after parasitism, and are either adaptive or non-adaptive to the parasitoid and/or host (Shorter and Rueppell 2011, Poulin et al. 1992, 1994). For example, prior to parasitism, in the presence of parasitoids, hosts will often suspend normal activity in order to implement chemical or behavioral defensive strategies. After parasitism, immature parasitoids within the host may alter the host's physiology to encourage behaviors that optimize the conditions for parasitoid development (Vinson 1975, Fritz 1982). Alternatively, host immune response or even self-sacrifice behavior may increase to prevent parasitoids from developing (Horton and Moore 1993, Smith Trail 1980).

Ant societies attract a suite of symbiotic organisms that take advantage of a colony's abundant resources. Beetles in the family Staphylinidae are common ant-associates, yet relatively little is known about the role of these beetles in their host colonies. Due to the formidable chemical and behavioral defenses of ants, beetles often possess complex strategies to safely interact with their ant symbionts (Kistner 1979, Stoeffer et al. 2011). Many beetles act as scavengers that hide in refuse piles, or as ant-mimicking social parasites within a host colony draining them of their resources. Others are predators of the ants themselves, locating their prey by eavesdropping on the ants' communication system. (Holldobler and Wilson 1990). While commensal, parasitic and predatory beetles are common within ant societies, little is known about how beetles might benefit their ant associates (Hughes et al. 2008). Furthermore, although most biological communities function as complex networks of context dependent interactions, ant-beetle associations are rarely observed outside of their pair-wise context.

Here, we document and experimentally demonstrate a case of context dependent intra-guild predation between an ant's two natural enemies: (1) phorid fly parasitoids, where parasitism reduces ant aggression, and (2) predatory beetles, where beetles selectively prey upon parasitized ants, thus reducing phorid fly populations without impacting ant populations. We also demonstrate that this interaction is mediated by the alarm pheromone of the ant, which both natural enemies use in host/prey location.

The aggressive arboreal ant, *Azteca sericeasur* (formerly referred to as *A. instabilis* in prior publications, but recently identified as *A. sericeasur*; J. Longino, personal communication) is a highly aggressive and territorial arboreal ant species that lives in large polydomous carton nests (Perfecto et al. 2014). This species frequently nests in the shade trees on coffee plantations and forages in the coffee below, preying on and removing coffee herbivores (Jiménez-Soto et al. 2013). Ants in the genus *Azteca* are known for their pungent alarm pheromone, that they will disperse liberally from large pygidial gland sacs when disturbed. Three *Pseudaceton* phorid fly species use this alarm pheromone to locate and parasitize *A. sericeasur* (Mathis et al. 2011). *P. lascinosus* is the largest of the three species and the most abundant at our field sites, *P. planidorsalis* is a smaller species and the second most abundant, while *P. pseudocercus* is the rarest of the phorid species (Reese and Philpott 2012). Presence of phorid fly parasitoids not only reduces the ants' ability to forage by as much as 50% (Philpott et al. 2004, Philpott 2005), but also indirectly affects interactions between the ants and a wide range of competitors and mutualistic partners (Perfecto et al. 2014). A newly described species of rove beetle, *Myrmedonota xipe* (Staphylinidae), has been observed in association with *A. sericeasur* ants. *M. xipe* beetles are found near disturbed *A. sericeasur*, often mating or preying on *A. sericeasur* ants after the arrival of phorid flies (Mathis and Eldredge 2014). These interactions suggest several questions. First, because *A. sericeasur* workers are notoriously aggressive, how are the beetles able to prey upon the ants? Second, are the beetles utilizing the same alarm pheromone as the phorid flies to locate the ants?

We test the hypothesis that if ants are parasitized by phorid flies, then ants become less aggressive, allowing beetles to gain access to ants, an otherwise non-accessible prey item. Furthermore, we hypothesize that this context dependent interaction is induced by the ant's alarm pheromone, which is released during phorid attack.

Significance

We document the behavioral interactions between a species of ant-associated beetles, ants, and two species of phorid fly parasitoids. We show that ant-associated beetles use ant pheromones to locate ants that have been parasitized by certain species of phorid flies. Furthermore, these ant-associated beetles will selectively consume parasitized ants due to the reduced aggression displayed by parasitized ants. Predation by these beetles on parasitized ants is likely advantageous for the ant colonies as a whole, because these beetles are also consuming phorid fly larvae that are developing within the ants' bodies, thus reducing overall phorid fly populations.

Methods

(a) Study Site

We conducted field all work on a shaded coffee plantation, Finca Irlanda, in the Soconusco region of Chiapas, Mexico (15° 11' N, 92° 20' W) between July 2012 and March 2013 in both the wet and dry seasons. Finca Irlanda is approximately 280 hectares in size, located between 950-1150 m elevation, and receives approximately 4500 mm of precipitation per year. *Azteca sericeasur* is the most abundant ant of the approximately 60 species of arboreal ants on the farm (Philpott 2005). *A. sericeasur* builds carton nests on the trunks of shade trees within the coffee plantation, where their colonies tend to be distributed in patches (Vandermeer et al. 2008).

(b) Extraction and analysis of *Azteca* alarm pheromone

We collected *A. sericeasur* volatile alarm pheromone compounds using a Solid Phase Microextraction (SPME) fiber by placing disturbed individual *A. sericeasur* ants (n=10) in 50 ml glass beakers covered with aluminum foil. We then inserted a SPME fiber into the beaker through the aluminum foil for 10 minutes to adsorb the headspace volatiles. SPME fibers were immediately inserted into a Finnigan Trace MS+ gas chromatograph / mass spectrometer equipped with a DB-5 capillary column (30 m x 0.32 mm X 0.25 μ m, Agilent Technologies, CA). Extracts were analyzed in splitless mode, with a temperature program that started at 100°C for 1 min, which then increased by 20°C min⁻¹ until it reached 150°C, and then increased by 5°C min⁻¹ until it reached 325°C where it stayed for 5 min. Injector and transfer line temperatures were kept at 325°C and 280°C respectively.

(c) Alarm pheromone bioassays

To determine whether the two species of beetle are attracted by the alarm pheromone of *A. sericeasur*, we prepared four treatment solutions, (1) 1 mL of pesticide-grade hexanes, (2) 20 crushed *A. sericeasur* pygidial glands in 1 mL of hexanes, (3) 1 μ L 2-heptanone in 1 mL of hexanes, and (4) 1 μ L of 2-pentanone in 1 mL of hexanes. We then placed treatment solutions in 2-dram glass vials, open, with a filter paper wick at 20 field sites. All field sites were at least 25 m apart, at the base of trees within the coffee farm that contain an *A. sericeasur* nest. At each site, we placed the treatment solution vial on the ground with leaf litter removed from the surrounding area. Once we opened the vial with treatment solution, we observed a 10 cm² area surrounding the vial for 15 minutes, and collected and identified beetles attracted to the area using an aspirator. We calculated the

total number of beetles from each species collected at each site with each treatment type. We examined the differences in treatment types using a two-way analysis of variance (ANOVA) and made pairwise comparisons between treatment types using Tukey's post-hoc tests.

(e) Arena Experiment

To determine whether *Myrmedonota spp.* beetles will selectively attack parasitized ants when in the presence of both parasitized and unparasitized ants, and whether parasitized and unparasitized ants respond to beetle attacks differently, we placed parasitized and unparasitized ants in an arena with the beetles, filmed their interactions and analyzed the resulting footage (Figure 1a). We placed *A. sericeasur* ants (n=5) in small plastic container with an individual phorid fly until ants were parasitized (approximately 1 hr). We then froze the containers in the -20° C freezer for 2 minutes until the fly and ants were anesthetized. While the ants were anesthetized, we added a single dot of paint (green, white, or blue chosen at random for each observation) on the head of each ant and identified the anesthetized phorid flies to species under a microscope (Figure 1b). We also anesthetized and painted five unparasitized ants using the same method. We allowed the ants a one hour recovery period and then placed the five unparasitized ants and the five ants parasitized by either *P. lascinosus* or *P. planidorsalis* with *M. xipe* beetles in a plastic container (arena) coated with fluon (brand) with a transparent glass lid. We then filmed the arena for 15 minutes. We later analyzed the video footage using the Observer XT software (version 11, Noldus Information Technology, Wageningen, The Netherlands), by recording the duration, frequency and order of each behavior. The behaviors we chose to include were: (1) attack (where an animal bites another animal); (2) antennate (antennating another animal for longer than a second); (3) mandible flare (where an ant opens and closes her mandibles repeatedly in the direction of another animal). All researchers recording behaviors with the Observer Software were blind to the treatment type. We calculated the average percentage of each behavior by organism type (parasitized ant, unparasitized ant, or *M. xipe*) and the corresponding target organism type for each observation. We examined the differences in interactions using a two-way analysis of variance (ANOVA) and made pairwise comparisons between corresponding interaction types using Tukey's post-hoc tests.

(f) Beetle Trap Experiment

Parasitized ants often leave or are restricted entry from their nests (Heinze and Walter 2010). In order to determine whether *M. xipe* beetles are able to selectively locate individual parasitized ants in the field, we constructed beetle traps from small plastic cups with a 5 mm layer of plaster of paris on the bottom to retain moisture (Figure 1c). Each cup contained a single individual *A. sericeasur* that we assigned to one of the four treatment types. Four treatments included, (1) *A. sericeasur* parasitized by *P. lascinosus*, (2) *A. sericeasur* parasitized by *P. planidorsalis*, (3) *A. sericeasur* manually injured by puncturing their mesothorax with a Minuten pin to simulate a phorid attack wound (.20 mm diameter, Bioquip Products, Rancho Dominguez, CA), and (4) *A. sericeasur* untreated as a control. We placed lids on the cups with holes large enough for the beetles to enter the cups, but small enough to prevent the ants from escaping. We placed one cup of each treatment type 0 m and 5 m from each sites' focal tree, both on the ground and suspended 1 m in the nearest

coffee plant. We chose sites in both high shade (n=20) and low shade (n=20) habitats where focal trees contained *A. sericeasur* nests or did not contain *A. sericeasur* nests (n=10 within each habitat type). We placed the cups for each site in the field on the same day, and retrieved them two days later (Figure 1d). We counted and identified the beetles found in each cup, and recorded whether the ant was living, dead but remaining, or completely consumed. To examine the ability of each species of beetle to locate and consume ants from each treatment type, we used the total number of *M. xipe* found in each minicup trap and examined the differences by treatment type using ANOVA and determined the statistical differences between treatment types using Tukey's post-hoc tests. Treatments significantly different from the control were further analyzed by examining the differences in *M. xipe* presence by habitat variables (sites with high and low shade density, focal tree with or without *A. sericeasur* nests, traps 0 m and 5 m from the focal tree, and traps on the ground and suspended 1 m into the coffee bushes) using ANOVA and Tukey's post-hoc tests.

Results

(a) Do beetles prefer to prey on parasitized ants?

In the arena experiments, beetles attacked ants parasitized by *P. lascinosus* more often than unparasitized ants (Figure 2a; ANOVA; $F(1,48) = 24.59, p < 0.001$). While ants parasitized by *P. planidorsalis* were not attacked more than unparasitized ants in the arena experiments (Figure 2b; '% of observation spent attacking': $11.21 \pm 5.67\%$ spent attacking parasitized ants, $8.97 \pm 6.23\%$ spent attacking unparasitized ants; ANOVA; $F(1,31) = .81, p = 0.37$). In field observations beetles were only found consuming ants parasitized by either *P. lascinosus* (14.1% of ants) or *P. planidorsalis* (2.5%), and no control or injured ants. Consumption of *P. lascinosus* ants was significantly higher than consumption of control or injured ants (Figure 3, ANOVA with Tukey post hoc; $F(3,620) = 11.3, p < 0.001$).

(b) Do parasitized and unparasitized ants display different levels of aggression toward predatory beetles?

In arena experiments, parasitized ants were less aggressive than healthy ants. During the observations, parasitized ants attacked beetles significantly less than unparasitized ants, regardless of which phorid species parasitized them (Figures 4a and 4b; *P. lascinosus* parasitized ants \times unparasitized ants: $F(1,34) = 4.124, p < .05$; *P. planidorsalis* parasitized ants \times unparasitized ants: $F(1,26) = 4.495, p < 0.05$). However, ants parasitized by the two species of phorid fly differed in their mandible flare performance when compared to unparasitized ants. Ants parasitized by *P. lascinosus* mandible flared less than unparasitized ants in the arena experiments (Figure 5a; $F(1,40) = 11.38, p < 0.002$), but ants parasitized by *P. planidorsalis* did not (Figure 5b; $F(1,28) = 0, p = 0.99$).

(c) Are beetles able to successfully locate parasitized ants in different habitat types?

In beetle trap experiments, in all traps where *M. xipe* were collected, the ant had been completely consumed after two days. Beetles successfully located and consumed ants parasitized by *P. lascinosus* in both habitat types. Attacks in high shade were five times more frequent than attacks in low shade (Fig. 6a, $F(1,154) = 9.115, p < 0.003$), and higher

on the ground than above ground (Figure 6b; $F(1,154) = 7.599, p < 0.006$). However, there were no differences based on distance from focal tree (Figure 6d; 5.1%) ($F(1,154) = 0.501, p = 0.5$), or with and without *A. sericeasur* presence (Figure 6c; $F(1,154) = 1.581, p = 0.21$).

(d) How do beetles locate ants?

Analysis of SPME fibers containing volatiles from disturbed *A. sericeasur* workers shows that workers have two primary components present within their alarm pheromone, 2-pentanone and 2-heptanone in a roughly 2:8 ratio (Figure 7; $.383 \pm 0.062$ 2-pentanone:2-heptanone ratio; $n=5$). In alarm pheromone bioassays, *M. xipe* was attracted to both pygidial gland extracts and synthetic 2-heptanone, the most abundant compound in the pheromone blend. *M. xipe* beetles were not attracted to 2-pentanone, the secondary component of the blend, or the hexane control (Figure 8).

Discussion

This study demonstrates that the rove beetle, *M. xipe*, is able to detect the alarm pheromone released by *A. sericeasur*. The compound that attracts *M. xipe*, 2-heptanone, is a relatively common compound in the alarm pheromone of dolichoderine ants. Although phorid flies also use the alarm pheromone to locate *A. sericeasur* hosts, they are not attracted to 2-heptanone but instead, are attracted to 1-acetyl-2-methylcyclopentane, a less abundant compound within the alarm pheromone blend that is only found in *Azteca* species ants (Mathis et al. 2011, Mathis and Philpott 2012, Wheeler et al. 1975). While phorid flies likely use the more rare compound because they are highly host specific to *A. sericeasur* ants, the use of 2-heptanone by *M. xipe* suggests that these beetles are less selective and may prey on other dolichoderine ant species in addition to *A. sericeasur*. However, of the approximately 15 species at our study sites, *A. sericeasur* is overwhelmingly the most abundant and are likely the ant species these beetles encounter the most frequently.

Our results also show that, *M. xipe* selectively preys on ants parasitized by *P. lascinosus* phorid flies. These parasitized ants display reduced aggression, and particularly a reduced frequency of mandible flaring, which may allow beetles to gain access to these parasitized ants as a prey item. Because ant workers, in general, will mandible flare prior to attack as a warning, thus it appears that this preliminary act of aggression by both unparasitized ants and ants parasitized by *P. planidorsalis* is the primary behavioral cue used by *M. xipe* to determine whether to engage an ant. Furthermore, in the field, beetles are able to consume up to 14% of all ants parasitized by *P. lascinosus* in the field, which suggests that these beetles may have an important role in reducing *P. lascinosus* populations. This estimate is likely conservative given that *M. xipe* are generally already present just after phorid parasitism and have been observed preying on ants near disturbed colonies shortly after parasitism takes place (Mathis, personal observation).

Given that these beetles are only preying on parasitized ants and phorid parasitism is already fatal, the beetles likely have no direct impact on *A. sericeasur* population density. Thus, despite preying upon individual parasitized ant workers, our results indicate that *M. xipe* is a beneficial ant-associated beetle to the *A. sericeasur* population as a whole. Indeed, this study provides evidence that parasitized ants may be self-sacrificing by allowing themselves to be preyed upon by *M. xipe* beetles in order to reduce phorid fly populations.

Although adaptive suicide in social insects is relatively common as a preemptive strategy (e.g. honeybees using their stingers to defend the colony), it is difficult to determine whether this strategy is adaptive to hosts or their parasitoids post-parasitism (Tofilski et al. 2008; Chapuisat 2010; Tomlinson and Latta 1987, Stamp 1981, Heinze and Walter 2010, McAllister and Roitberg 1987). The host suicide hypothesis postulates that mature parasitoids emerging from host are more likely to infect host's kin than non-kin. Therefore, when maturation of the parasitoid is prevented, the inclusive fitness of the host should be increased (Smith Trail 1980). Even a very small increase in inclusive fitness will be enough to drive the system and favorable situations for adaptive suicide include systems where the host is a colonial or social insect, when there is high host inbreeding, and when parasitoids have small search ranges. *Azteca sericeasur* meet the criteria as they are not only social insects, but also monogynous and highly polydomous ants where colonies can span several hectares (Remfert, master's thesis?). Furthermore, *P. lascinosus* phorid flies have extremely limited search range, approximately two meters surrounding any given *A. sericeasur* nest (Philpott et al. 2009). Therefore, it is highly unlikely that phorid flies are dispersing beyond the boundaries of an *A. sericeasur* colony prior to oviposition. In principle, reduced aggression by parasitized ants might merely be a consequence of the physical damage caused by oviposition. However, our results show that both manually injured ants and ants parasitized by another species of phorid fly are not preyed on by beetles and do not display reduced levels of aggression. Thus we conclude that the reduced aggression displayed toward *M. xipe* beetles by *P. lascinosus*-parasitized ants is likely a direct consequence of parasitism by this particular species. However, further investigation is necessary to confirm whether this is an adaptive strategy used by ants parasitized by *P. lascinosus* rather than a fortuitous exploitation by *M. xipe* of a non-adaptive behavior in *A. sericeasur*. In particular, it will be worthwhile to determine the physiological mechanism by which this behavioral switch occurs and to quantify the fitness consequences for both the host (*A. sericeasur*) and that parasitoid (*P. lascinosus*).

Nonetheless, our study shows that the effects of the *M. xipe* association with *A. sericeasur* is dependent upon *P. lascinosus* presence and that these beetles are indirectly beneficial to *A. sericeasur*, by reducing the number of developing *P. lascinosus* parasitoids in the population by approximately 14%. To our knowledge, ours is one of the few studies that document the role of ant-associated beetles outside of its pairwise context (Mynhard 2013, Rettenmeyer et al. 2011, O'Keefe 2000, Stoeffler et al. 2011, Parmentier et al. 2014), and the first study documenting an ant-associated beetle predator that is beneficial to its ant associate.

Increasingly, it is becoming apparent that investigating the ecological complexity within a system provides instructive examples of how organisms can change their behavior or morphology in response to challenges from other organisms and subsequently how these changes can have cascading effects throughout a network of interacting species (Perfecto et al. 2014, Hsieh et al. 2012, Werner and Peacor 2003). Current literature on the role of beetles within ant societies tells us that these beetles are exceedingly common and behaviorally diverse, however few ant-beetle associations have been examined in depth. Further investigation into both the roles of these beetles in their ant societies, as well as within the network of organisms surrounding ant societies is paramount to understanding how social insect colonies function within the ecosystem as a whole.

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Figure 1: Photos of study organisms and experimental set up. (a) Set up of arena experiments with plastic container of *A. sericeasur* ants and *M. xipe* beetles with camera overhead to document each observation. (b) *A. sericeasur* painted for arena experiment. (c) Beetle trap experiment set up with four transect types: (A) Low shade, *Azteca* absent; (B) Low shade, *Azteca* present; (C) High Shade, *Azteca* absent; (D) High Shade, *Azteca* present. (d) *M. xipe* beetles inside beetle trap baited with *P. lascinosus* ant.

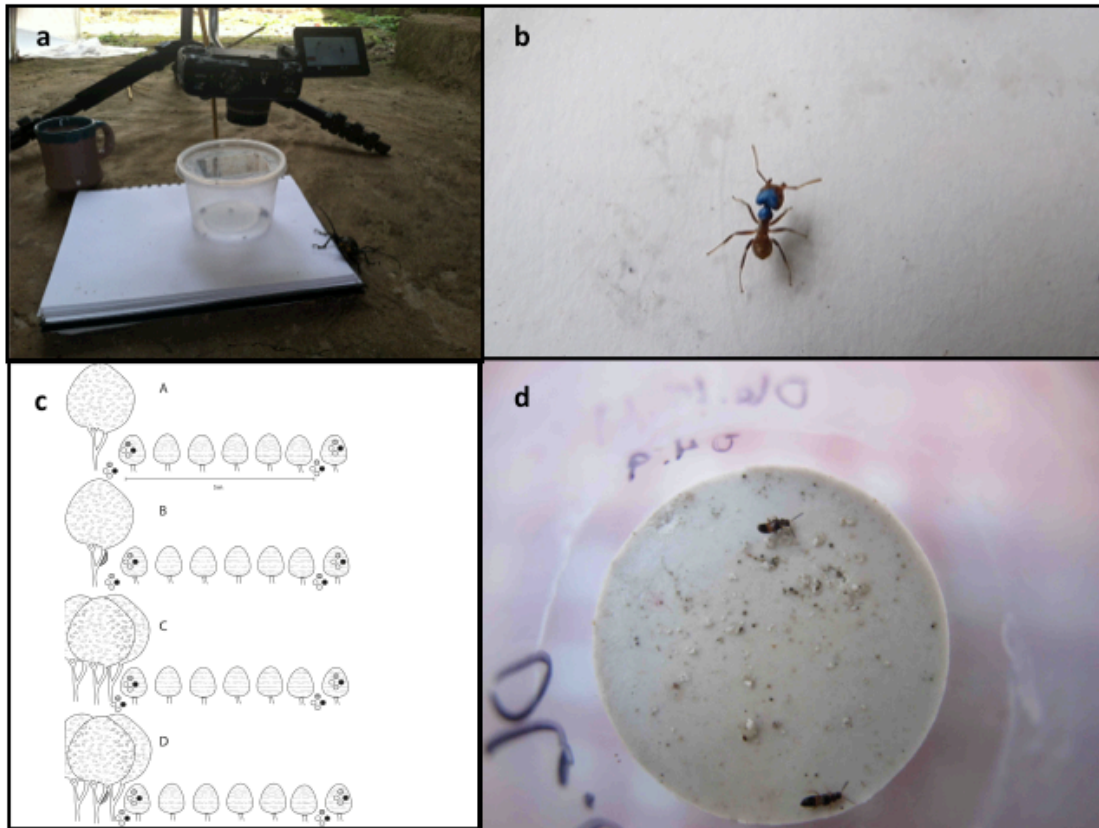


Figure 2: Plot of average percent of observations beetles spend attacking ants in arena experiments. (a) Observations with beetles attacking ants parasitized by *P. lascinosus* (left column) or healthy ants (right column). (b) Observations with beetles attacking ants parasitized by *P. planidorsalis* (left column) or healthy ants (right column).

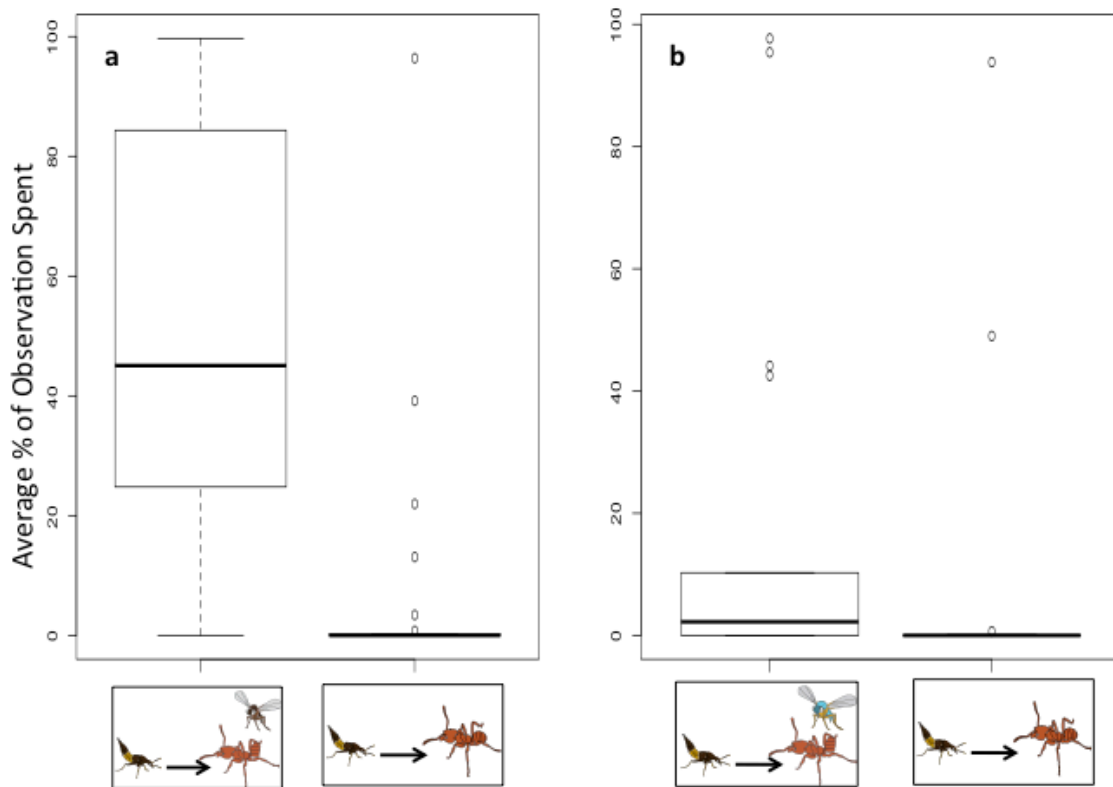


Figure 3: Plot of the proportion of ants consumed by *M. xipe* beetles in beetle trap experiments. Four bars represent the four treatment types where control treatments are traps baited with healthy ants, injure treatments are traps baited with manually injured ants, planidorsalis treatments are traps baited with ants parasitized by *P. planidorsalis* and lascinosus treatments are traps baited with ants parasitized by *P. lascinosus*.

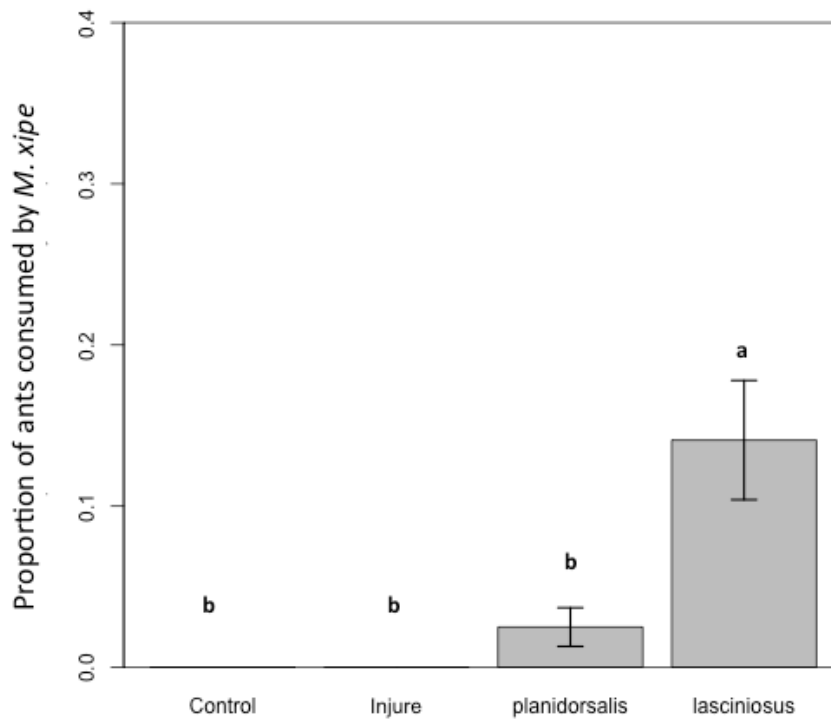


Figure 4: Plot of average percent of observations ants spend attacking beetles in arena experiments. (a) Observations with ants parasitized by *P. lascinosus* (left column) or healthy ants (right column) attacking beetles. (b) Observations with ants parasitized by *P. planidorsalis* (left column) or healthy ants (right column) attacking beetles.

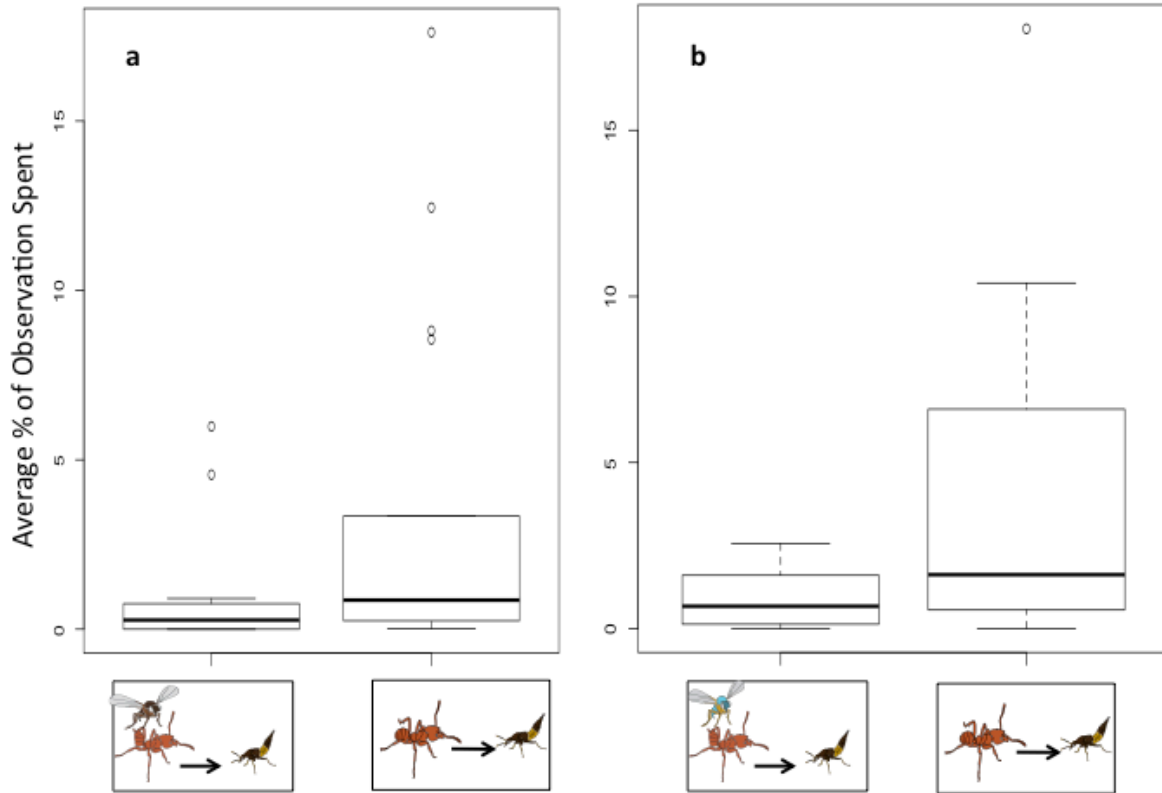


Figure 5: Plot of average percent of observations ants spend mandible flaring beetles in arena experiments. (a) Observations with ants parasitized by *P. lascinosus* (left column) or healthy ants (right column) mandible flaring beetles. (b) Observations with ants parasitized by *P. planidorsalis* (left column) or healthy ants (right column) mandible flaring beetles.

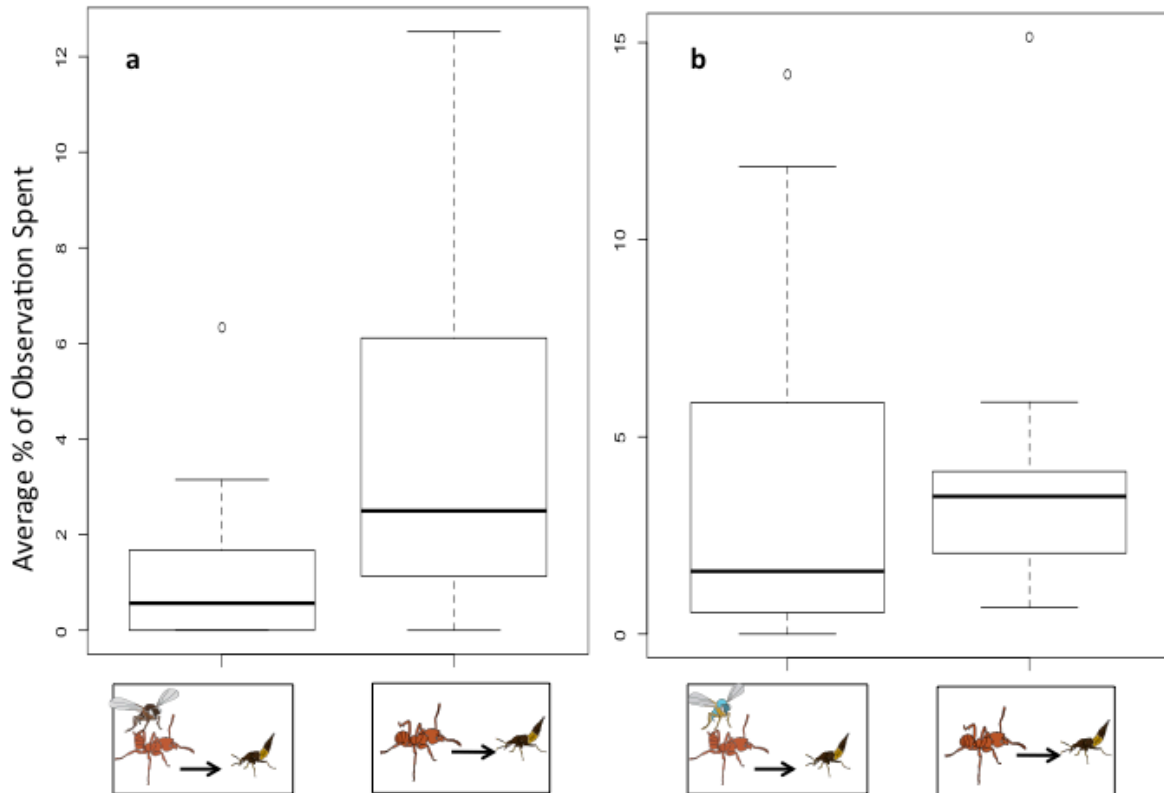


Figure 6: Plots of the proportion of ants parasitized by *P. lascinosus* consumed by *M. xipe* beetles in beetle trap experiments broken down by habitat type. (a) Plot of average number of beetles found in traps with ants parasitized by *P. lascinosus* in low shade (left bar) and high shade (right bar) habitats. (b) Plot of average number of beetles found in traps with ants parasitized by *P. lascinosus* on the ground (left bar) and 1m above the ground (right bar). (c) Plot of average number of beetles found in traps with ants parasitized by *P. lascinosus* at sites with *A. sericeasur* nests (left bar) and without *A. sericeasur* nests (right bar). (d) Plot of average number of beetles found in traps with ants parasitized by *P. lascinosus* at 0m (left bar) and 5m (right bar) from the focal tree.

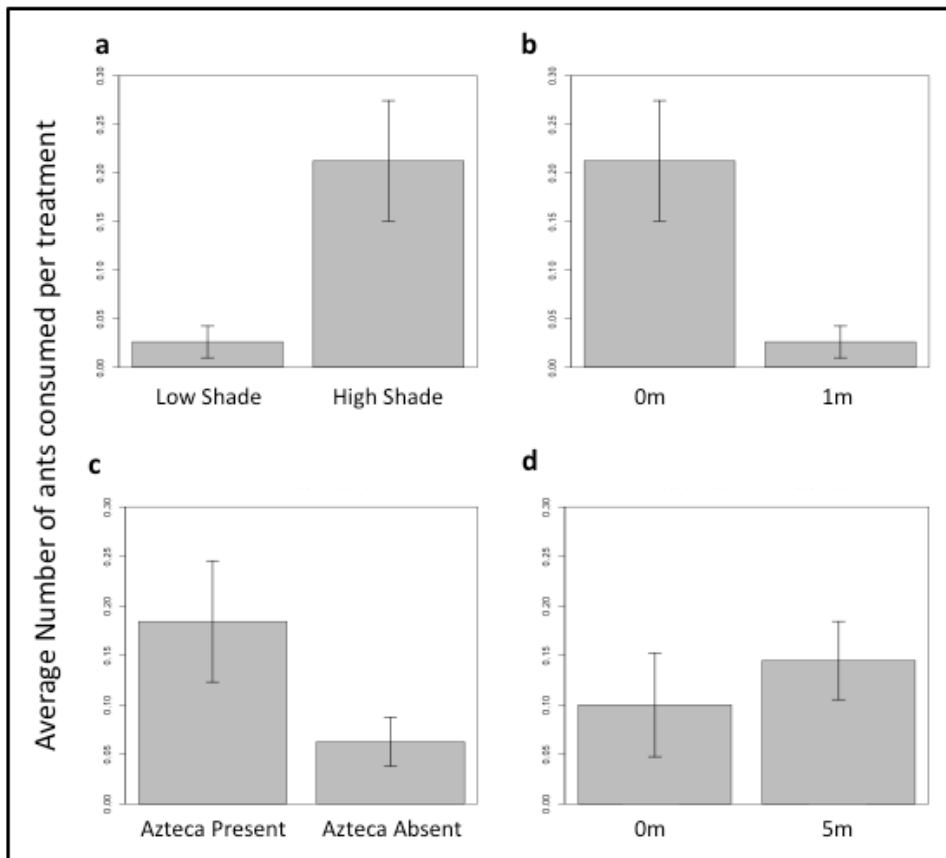


Figure 7: Representative chromatogram of *A. sericeasur* alarm pheromone volatiles collected using a SPME fiber where (1) is 2-pentanone and (2) is 2-heptanone.

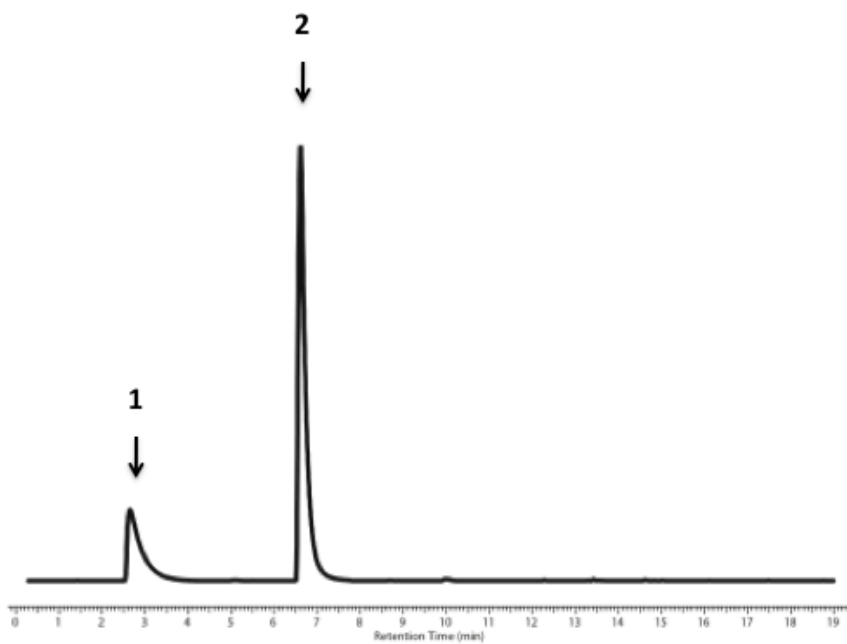
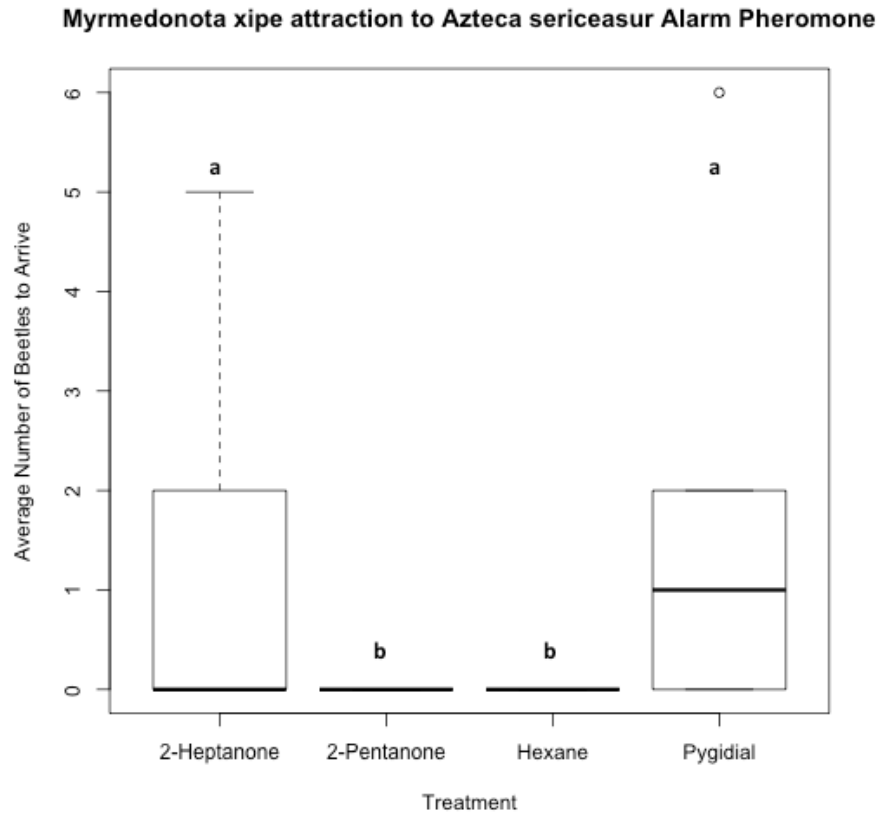


Figure 8: Plot of the average number of *M. xipe* beetles to arrive at alarm pheromone bioassays. Each bar represents a treatment type, where 2-heptanone is synthetic 2-heptanone, 2-pentanone is synthetic 2-pentanone, hexane is a solvent control and pygidial is an extract from crushed pygidial glands of *A. sericeasur*.



CHAPTER 5

Colony boundaries and spatial variation in cuticular hydrocarbons of twig-nesting ants in coffee agroecosystems

Abstract

Social insects utilize complex signals to distinguish between colony members and potential nest intruders. Most social insects rely on colony-specific chemical cues (*e.g.* cuticular hydrocarbons) to recognize and discriminate between nestmates from non-nestmates. Previous studies have shown that lower variation in cuticular hydrocarbon blends within a population can result in reduced nestmate discrimination, leading to lower aggression and higher nest densities within the population. Here we investigate the variation of cuticular hydrocarbon profiles in three species of polydomous arboreal twig-nesting ants; *Pseudomyrmex simplex*, *Pseudomyrmex ejectus* and *Pseudomyrmex sp.* (hereafter PSW-53). Our goal was to determine whether variation in the ecology and social structure of these species is reflected in the variation in their cuticular hydrocarbon profiles. We tested the hypothesis that the more abundant species with higher nest densities exhibit lower cuticular hydrocarbon variation. We found that worker ants of abundant *P. ejectus* and *P. simplex* from our study site exhibit significantly lower variation in cuticular hydrocarbon profiles than workers of the rare ant species *P. PSW-53*. Our study reinforces the idea that examining cuticular hydrocarbon profiles can provide insight into the colony structure of social insects.

Keywords

Polydomous ants; social organization; *Pseudomyrmex*; cuticular hydrocarbons

Introduction

Social insects rank among the most ubiquitous and ecologically dominant terrestrial animals on Earth. Complex communication and cooperation are two defining features of social insect societies (Holldobler & Wilson 1990). To maintain cooperation between nestmates while excluding intruders, social insects have well-developed nestmate recognition systems, which primarily rely on chemical signals (Howard & Blomquist 2005).

Cuticular hydrocarbons often play an important role as signals (or “labels”, Tsutsui 2004) in nestmate recognition (Gamboa et al. 1986b, Krasnec & Breed 2013, Howard and Blomquist 2005). These cuticular hydrocarbons (CHCs) form a thin hydrophobic layer on the cuticle surface of most insects, and act as a barrier to prevent desiccation (Howard and Blomquist, 2005; Blomquist and Bagnères, 2010). CHCs are generally a blend of individual compounds ranging from 20-40 carbons in length and thus have relatively low volatility. These blends can also have mixtures of straight chain alkanes, alkenes or methyl branched molecules (Provost et al. 1993; Dahbi et al. 1996; Breed 1998a). Cuticular hydrocarbon mixtures vary between species (Espelie et al. 1994; Gamboa, 2004), and their relative concentration ratios also vary between colonies, creating both species- and colony-specific profiles (Carlin and Holldobler 1983; Gamboa et al. 1986; Crosland 1989; Butts et al. 1993; Soroker et al. 1994; VanderMeer and Morel, 1998; Kasnec and Breed 2013).

The source and formation dynamics of cuticular hydrocarbons and the discrimination capabilities of colony members can vary greatly even between closely related species (Foitzik et al. 2007). Studies on invasive ants suggest the loss of genetic and cuticular hydrocarbon variation result in a reduction of functionality in the nestmate recognition system of ants (Tsutsui et al. 2003, Torres et al 2007). This loss is correlated with dramatic changes in the ecology and social structure of these ants, which may ultimately impact the ecological community as a whole. These invasive ant populations

are often unicolonial with workers moving freely between neighboring nests. The absence of aggression between nests in these populations results in a lack of costly energy expenditure on territorial fights, which often results in the ecological dominance (eg. high density and abundance) of these unicolonial invasive ants (Holway 1998, Holway et al. 2002, Sanders et al. 2003). Although colony level variation in cuticular hydrocarbons plays an important role in nestmate recognition in most species of ants, and differences in cuticular hydrocarbon variation in non-invasive ant species may result in similar types of social organization, little is known about the colony-level variation in non-invasive polydomous ants.

Here we investigated the variation of CHC profiles in three species of polydomous arboreal twig-nesting ants; *Pseudomyrmex simplex*, *Pseudomyrmex ejectus* and *Pseudomyrmex sp.* (hereafter PSW-53) that co-occur in Central American coffee plantations. These species were chosen because they vary from one another according to nest density and abundance in addition to success in competitive interactions. Our aim in this study was to determine whether differences in the complexity of recognition cues (cuticular hydrocarbons) is associated with the variation in ecology and social structure of these ants. First, we tested the hypothesis that the three species of ant's cuticular hydrocarbon colony "labels", or "chemotypes" are more variable between colonies than within colonies. We also tested the hypothesis that the observed within species variation in CHC profile labels for each species correlates with their social structure and competitive dominance, with less competitive, more abundant, and densely populated ants exhibited reduced CHC variation and rare, sparsely populated, and more competitive ants exhibited increased CHC variation.

Methods

Sites and Study System

Finca Irlanda (email: finca_irlanda@hotmail.com Office: ciruelos No. 18 fraccionamiento Los Laureles, Tapachula, Chiapas, Mexico 30700), a private owner of the land, granted us permission to perform all field work. We had permission from SEMARNAT (Secretaria de Medio Ambiente y Recursos Naturales) for arthropod collection.

We sampled cuticular hydrocarbons from ants collected during May and June 2012 in two areas (one 45 ha area and one 7 ha area) of Finca Irlanda, a 300 ha, shaded coffee plantation in the Soconusco region of Chiapas, Mexico (15°11'N, 92°20'W). The plantation is located between 950-1150 m elevation and receives approximately 4,500 mm of rain each year. The farm contains approximately 100 species of shade trees, the most common being trees in the genus *Inga* (Vandermeer et al. 2008). Canopy cover at the time of ant sampling ranged from 35-70% in the 45 ha area and was around ~90% in the 7 ha area.

In the study site, there are at least 60 arboreal ant species including 40 that nest in dry, hollow coffee twigs (Philpott & Foster 2005, Livingston & Philpott 2010). Heavy rains, natural processes of decomposition, and farm workers moving through the coffee plantations frequently break these twigs from the coffee plants, making the nesting sites ephemeral. There are nine species of *Pseudomyrmex* that nest in coffee twigs, and the three most common at the study site are *P. simplex* (occupies ~35% of all occupied coffee twigs), *P. ejectus* (~20% of twigs), and *P. PSW-53* (~6% of twigs). *P. simplex* range from Florida to Paraguay and are commonly collected from forest habitats (AntWeb 2014). *P. simplex* nests are commonly found without queens, suggesting a high level of polydomy (Ward 1995). *P.*

ejectus are also commonly found in forests and coffee plantations, and range from Florida to Costa Rica (Ward 1995, AntWeb 2014). *P. PSW-53* have been collected from Baja California to Costa Rica, and commonly inhabit forests and woodlands (AntWeb 2014). The majority of nests collected for this study were queenless, accounting for 76% of *P. simplex* nests, 66.1% of *P. ejectus* nests, and 65.0% of the *P. PSW-53* nests. The three ant species differ in their abilities to gain access to nesting sites. In nesting colonization trial experiments *P. simplex* won 37% of paired trials with other twig-nesting species, *P. ejectus* won 24% of trials, and *P. PSW-53* won 70% of nest competition trials (Yitbarek & Philpott, in review). We chose to work with these three species because of the differences they exhibit in their relative abundance in dry, hollow coffee twigs, and also because of their different apparent success in winning nest sites in competitive encounters.

Sample Collection

During 2012, we surveyed twig-nesting ants in 20x20 m plots within established permanent plots at the study site. In 2004, a 45-ha plot was established, and in 2009 a 7-ha plot. We surveyed 8 hectares of the 45-ha plot and in 1 hectare of the 7-ha plot (Fig. 1). We sampled twig-nesting ants on every coffee plant (~100-250 coffee plants) within each 20x20 m plot and removed all dry twigs, counted hollow and occupied twigs, identified all occupant ants, and counted the number of queens, males, brood, and workers within occupied twigs. The twig nests are most easily sampled destructively by breaking into the twigs to determine their occupants (Philpott and Foster 2005). We mapped the coordinates of each coffee plant in the plots, and also noted whether collected twigs were from the top, middle, or bottom third of each coffee plant to further refine the spatial distribution of collected twigs. We took all twigs occupied by *P. simplex*, *P. ejectus*, and *P. PSW-53* back to the lab where we freeze-killed the ants and placed them at -18°C until extraction. For *P. simplex* and *P. ejectus*, we ultimately chose to extract ants from the three plots with the most colonized twigs for each species (Fig. 1). For *P. PSW-53*, the least common of the three species, we extracted ants from all five plots in which this ant was collected (See Fig. 1).

Cuticular Hydrocarbon Extraction and Processing

For each twig that contained at least three ant workers (n=197), we collected three individuals for CHC extraction (270 ants for *P. simplex*, 207 ants for *P. ejectus* and 114 ants for *P. PSW-53*). Each ant was freeze-killed and submerged in 100-200 µL of hexane for 10 minutes. The ants were then removed, the hexane was evaporated, and the samples were stored at -18°C until they were transported to the lab. Once in the lab, samples were placed at -20°C until processing. Directly before processing, we resuspended each sample in 200µL of hexane. We filtered samples through a 1.5 cm hexane-rinsed silica column to remove non-hydrocarbon material and flushed the column with 300 µL of hexane to maximize sample recovery. We then used nitrogen gas to reduce our samples to a 60 µL volume. To analyze the samples, we injected 2 µL of the 60 µL into the GC/MS.

Cuticular Hydrocarbon Analysis

For each sample, we acquired electron impact mass spectra (70 eV) with an Agilent 5975 C mass selective detector interfaced to an Agilent 7890A gas chromatograph fitted with a DB-5 column (30 m x 0.32 mm i.d., Agilent Technologies). Extracts were analyzed in

splitless mode, with a temperature program that started at 100°C for 1 min, then increased by 20°C min⁻¹ until it reached 150°C, and then increased by 5°C min⁻¹ until it reached 325°C where it was held for 5 min. Injector and transfer line temperatures were kept at 325°C and 280°C respectively.

Data Analysis

All chromatogram peaks that eluted between the retention times of 13 and 35 minutes were included in the analysis. Quantitative comparisons of spectra were based on peak integration of total ion chromatograms. For each sample, we only included compounds with >1% total abundance. When comparing species as a whole, only compounds with an average of >1% total abundance were included after standard deviation was added. All subsequent analyses were performed after peak areas were standardized to relative proportions. Individual hydrocarbon peaks were identified through a combination of (a) comparing retention times and mass spectra with those of synthetic standards to determine carbon chain length and calculate Kovat Indices, (b) matching our spectra with those previously published and (c) studying fragmentation patterns. To assess within-species variation, we calculated the mean and standard deviation of each compound for each species. The mean and standard deviation were plotted against each another and we used a general linear model to establish a line of best fit for each species (R Development Core Team, 2013, glm). We conducted an ANCOVA with Tukey's post-hoc tests to determine whether the mean-variance relationships differed between species (R Development Core Team, 2013). To determine whether species clustered by chemotype, we used Ward's hierarchical cluster analysis, and assessed the uncertainty of clusters using multiscale bootstrap resampling (R Development Core Team, 2013, pvclust). For each species, we used the entire data matrix containing all the compounds present in their workers for each hectare. All compounds were included in the analysis, as previous experiments show that excluding compounds may increase error (Krasnec & Breed 2013). Chemotypes were divided based on statistically significant clusters, and individuals were assigned to each chemotype if the majority (two out of three) of the twig-mates were assigned to a particular cluster.

Results

Compound Identification

For the three species, *P. simplex*, *P. ejectus*, and *P. PSW-53*, we identified 18, 7, and 12 hydrocarbons (or combinations of co-eluting hydrocarbons) respectively, each representing at least 1% of the total area of all compounds (Table 1). Compounds consisted of straight chain alkanes, monomethyl alkanes and some multimethyl alkanes. Compounds had chain lengths ranging from 20 to 36 carbons, with *P. simplex* containing, on average, compounds with longer carbon chains than *P. ejectus* and *P. PSW-53*. Three compounds (C25, C27, and 3me C27) were found in all three species. Representative chromatograms of cuticular hydrocarbons obtained from each of the three species are depicted in Figure 2.

Comparisons of within species variation

To assess the amount of within-species variation in each species, and to determine whether these species could be broken into discrete chemotypes based on CHC variation, we plotted the average proportion of each compound against its standard deviation (Fig. 3). While the mean-variance relationships for *P. simplex* and *P. PSW-53* did not differ significantly from one another ($p=0.16$), *P. ejectus* had a significantly less positive mean-variance correlation than both *P. simplex* and *P. PSW-53* ($p<0.001$ and $p<0.05$ respectively), indicating that *P. ejectus* workers have less overall variation in their cuticular hydrocarbon profiles.

Cluster Analysis

We performed Ward's hierarchical cluster analysis to determine whether groups of individual ants could be grouped into discrete units based on chemical similarities. The analysis showed that *P. simplex* and *P. ejectus* did not reliably cluster into chemotypes, either within or between hectares. However, *P. PSW-53* individuals clustered into distinct chemotypes both within and between hectares (Fig. 4). Individual ants of *P. PSW-53* divided initially into two primary clusters ($p<0.05$), with plots 2 and 4 grouping together into cluster 1 and plots 1, 5, and 7 grouping together into cluster 2 (with the exception of three individuals from one twig found in plot 4). Chemotypes are further divided into five distinct secondary clusters (cluster O, N, M, P and Q) ($p<0.05$). The secondary clusters O and N were found exclusively in plots 2 and 4, and cluster N was found only within plot 4. The clusters P, Q and M were found predominately in plot 1, 5, and 7, with the exception of one twig of ants in plot 4, that contained the sole members of cluster M. Cluster P was found only within plot 1 (Fig 1C). Furthermore, although some coffee plants did contain multiple *P. PSW-53* nests, individual coffee plants did not support more than one *P. PSW-53* chemotype. In other words, all ants from twigs on the same plant shared the same chemotype. Yet, some ants found in twigs on plants within 1m of one another had different chemotypes. (Fig. 1C). At the primary cluster level, all *P. PSW-53* individuals separate accurately into their clusters, however individuals separate into their secondary clusters with only 91% accuracy; although, we should note, this is a similar level of accuracy as found in monodomous, monogynous ant species using CHC profiles with ants known to belong to different colonies (see Krasnec & Breed 2013).

Discussion

Our chemical analysis of cuticular hydrocarbons of three species of twig-nesting ants revealed species-specific profiles that unambiguously separated all three species. We found that within-species variation of CHCs differed between the three species examined. *P. PSW-53* individuals had the most within species variation and we were able to accurately separate individuals from nests into chemical clusters. *P. PSW-53* divided into two clearly defined clusters, each comprised of two and three secondary clusters. *P. PSW-53* CHC profiles did not have the largest number of compounds, but they did have the most within-compound variation between individuals, indicating that the number of compounds is less important than variation of compound ratios to chemotypic variation. *P. PSW-53* within the same chemical clusters also exhibited spatial clustering within the sampled area, indicating that this species may have multiple colonies within the sampled area and supporting our hypothesis that low nest density indicates higher within species cuticular

hydrocarbon variation. *P. ejectus*, had significantly less within-species variation than the other two species. However, both *P. ejectus* and *P. simplex* workers exhibit similar within species chemical profiles and clustered into one chemotype within the study area.

Our hypothesis that the high abundance and densely populated species would have low CHC variation was supported, as the CHC profiles of individuals from both *P. simplex* and *P. ejectus* could not be broken into chemotypes due to high within-species similarity. In ants generally, CHC variation exists due to (1) genetic distance of individuals, (2) age differences between individuals, and (3) environmental factors (e.g. food type, nesting substrate). Ant species with reduced CHC variation among individuals often possess reduced genetic variation and reduced levels of aggression between conspecifics. These species include ants that form supercolonies and some species of monogynous ants that occur in extraordinarily high nest density and that often inhabit virtually all nesting sites (Foitzik et al. 2007). The single chemotype found in *P. simplex* and *P. ejectus* supports the idea that these species may operate as single colonies in our research area. Similar to other species with low CHC variation, both *P. simplex* and *P. ejectus* occur commonly, each representing about a third of the occupied twigs in the field, while also exhibiting some of the lowest success in competitive interactions of any common twig nesting species (Table 2). However, further investigation using a secondary method such as aggression assays or molecular analysis along with wider sampling would indicate whether these species are a single colony in our research area.

Our study illustrates how the analysis of variation in CHC profiles, of non-invasive polydomous ants, sheds new light on how signal variation is associated with social organization. Our analyses demonstrate that the more abundant ants with higher nest densities, *P. ejectus* and *P. simplex*, did not exhibit as much individual chemical variation as the less abundant and more competitive *P. PSW-53*. Individual *P. PSW-53* clustered into two primary clusters and further into five secondary clusters, which also closely correlate to their physical location in the study areas. Further investigation of intraspecific CHC variation along spatial scales, in combination with other behavioral studies could be particularly useful to infer and determine colony boundaries in polydomous ants.

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Table 1: Summary of compounds present in three species of *Pseudomyrmex* ants with average abundances. Percentages indicate the average relative proportion of each compound, as determined by the area under each peak in the chromatogram divided by the sum of all peaks, +/- standard deviation

#	Retention time (min)	Compound Class	Compound ID	<i>P. simplex</i>	<i>P. ejectus</i>	<i>P. PSW-53</i>
1	13	straight	C20	0.84 +/- 0.49	0.93 +/- 1.06	
2	16.25	single methyl	mix of 8me and 10me C22		1.16 +/- 1.16	
3	18.5	single methyl	mix of 11me and 13me C23			1.22 +/- 1.87
4	19	single methyl	3me C23			0.90 +/- 1.56
5	21	straight	C25	1.87 +/- 6.90	47.13 +/- 14.65	8.19 +/- 9.46
6	21.5	single methyl	mix of 9me, 11me, 13me and 15me C25			15.07 +/- 11.03
7	22	single methyl	3me C25			5.48 +/- 4.79
8	22.75	single methyl	mix of 9me and 11me C25		2.69 +/- 1.41	
9	23	straight	C26			1.00 +/- 0.44
10	24	straight	C27	10.73 +/- 6.62	30.46 +/- 9.41	8.51 +/- 4.43
11	24.5	single methyl	mix of 9me, 11me, 15me and 17me C27	6.83 +/- 3.09		
12	24.5	single methyl	Mix of 13me and 15me C27			29.94 +/- 16.45
13	25	single methyl	3me C27	3.30 +/- 2.65	2.67 +/- 3.97	5.84 +/- 4.39
14	25.5	straight	C28	2.06 +/- 1.27		
15	25.75	multimethyl	(3,7) dime C27			1.03 +/- 0.78
16	26	single methyl	mix of 10me and 12me C28	2.59 +/- 1.23		
17	26.75	straight	C29	5.91 +/- 5.33	2.05 +/- 1.94	
18	27.25	single methyl	mix of 11me, 13me and 15me C29	21.29 +/- 11.98		13.76 +/- 10.33
19	27.75	single methyl and multimethyl	mix of (11, 15) dime C29, (7,11) dime C29 and 3me C29	5.63 +/- 3.58		
20	28.25	multimethyl	(11,13,15) trime C29	1.09 +/- 0.86		
21	28.5	straight	C30	1.96 +/- 1.23		
22	29.75	single methyl	mix of 11me, 13me, 15me and 17me C31	8.57 +/- 3.99		
23	30	multimethyl	(11, 13) dime C30			1.16 +/- 2.30
24	30.25	single methyl and multimethyl	mix of (13, 15) dime C30, 3me C31 and 5me C31	4.75 +/- 2.59		
25	32.25	single methyl	mix of 11me, 13me, 15me and 17me C34	3.58 +/- 2.50		
26	32.5	multimethyl	(11, 13) dime C34	4.74 +/- 4.17		
27	34.5	single methyl	mix of 11me, 13me, 15me and 17me C36	1.02 +/- 1.10		
28	34.75	multimethyl	(15,17) dime C36	1.99 +/- 2.36		

Table 2: Summary of Abundance, Density and Competitive ability of each of the three species of *Pseudomyrmex*. Abundance is measured by total number of twigs occupied by this species in our study and in parentheses the percentage of occupied twigs occupied by the species. Density was measured as the inverse of the average distances between each twig and its closest conspecific neighbor. The competition score is percent success in competition trials described in Yitbarak & Philpott, *in review*.

<i>Species</i>	<i>Abundance</i>	<i>Density</i>	<i>Competition</i>
P. simplex	215 (36%)	1.528	37%
P. ejectus	105 (17%)	0.814	24%
P. PSW-53	43 (7%)	0.164	70%

Figure 1: Spatial distribution of *Pseudomyrmex* PSW-53. A) Regional map of the study area. Black dot indicates the location of the study sites. B) Satellite image of the study areas. Red boxes are around the larger 45 ha area (left) and smaller 7 ha area (right). Smaller boxes represent the plots where ants from each of the three species were sampled. Boxes are numbered according to site number and color-coded according to species sampled within. *P. PSW-53*, *P. simplex*, and *P. ejectus* are represented by blue, yellow, and green boxes respectively. The black oval encompasses plots where the cluster one chemotype was found, and the areas surrounded by the black dashed line include plots where the cluster two chemotype was present, with the exception of one twig with ants from cluster two found in plot 4. C) Plots 1, 4 and 7 are expanded to show detailed spatial distribution of individuals within the plots. Circles are color coded according to chemotype assignment found in Figure 4.

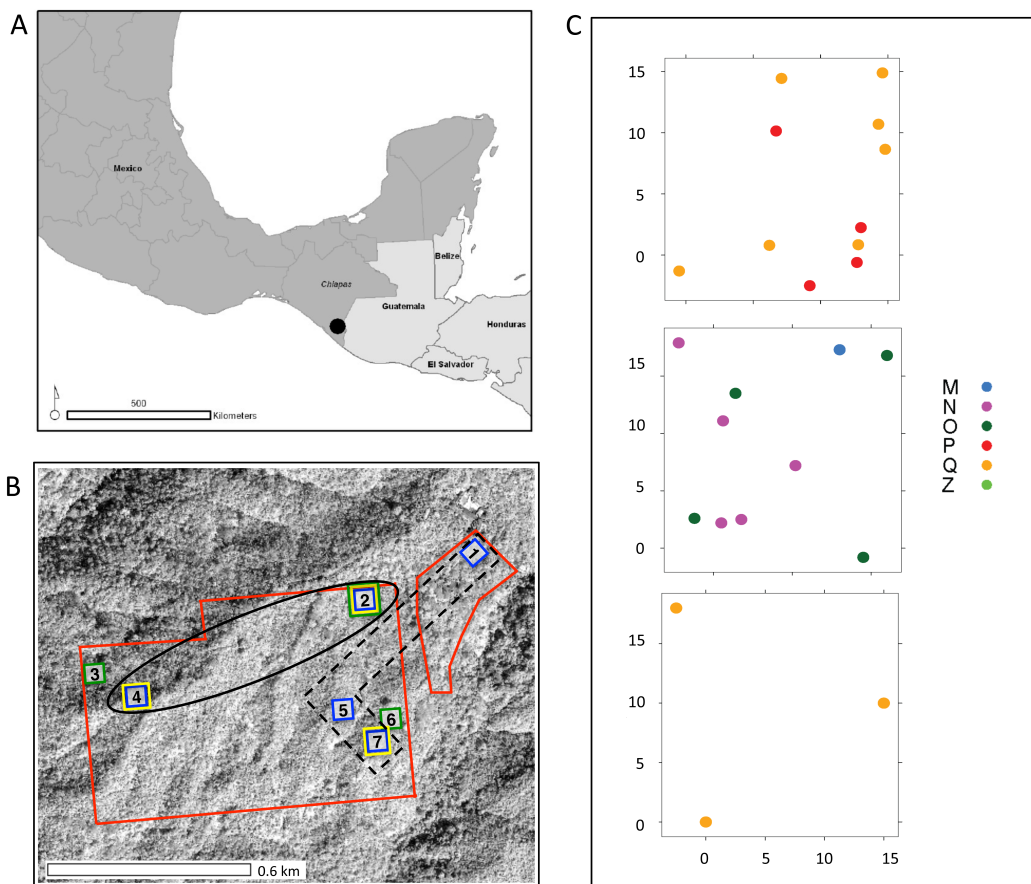


Figure 2: Representative chromatograms of the three species of *Pseudomyrmex*. A) *Pseudomyrmex simplex*, B) *Pseudomyrmex ejectus*, C) *Pseudomyrmex* PSW-53. Peak numbers refer to compound numbers found in Table 1.

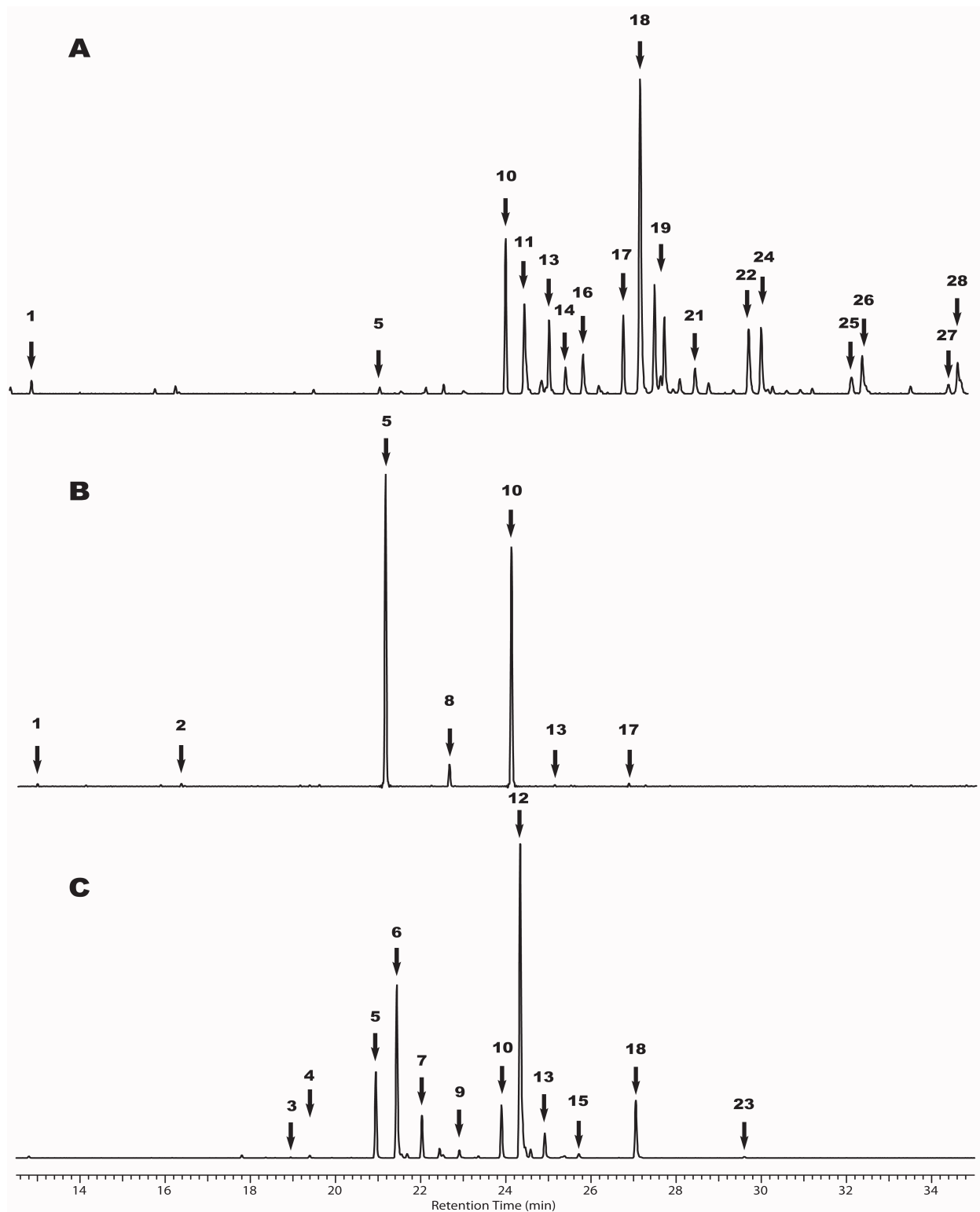


Figure 3: Scatter plot of the average proportion of each compound (present at proportions greater than 1%) by the standard deviation of the proportion of each compound within the species and corresponding lines of best fit. Compounds in *Pseudomyrmex simplex*, *Pseudomyrmex ejectus* and *Pseudomyrmex* PSW-53 are represented by black, green, and blue lines respectively.

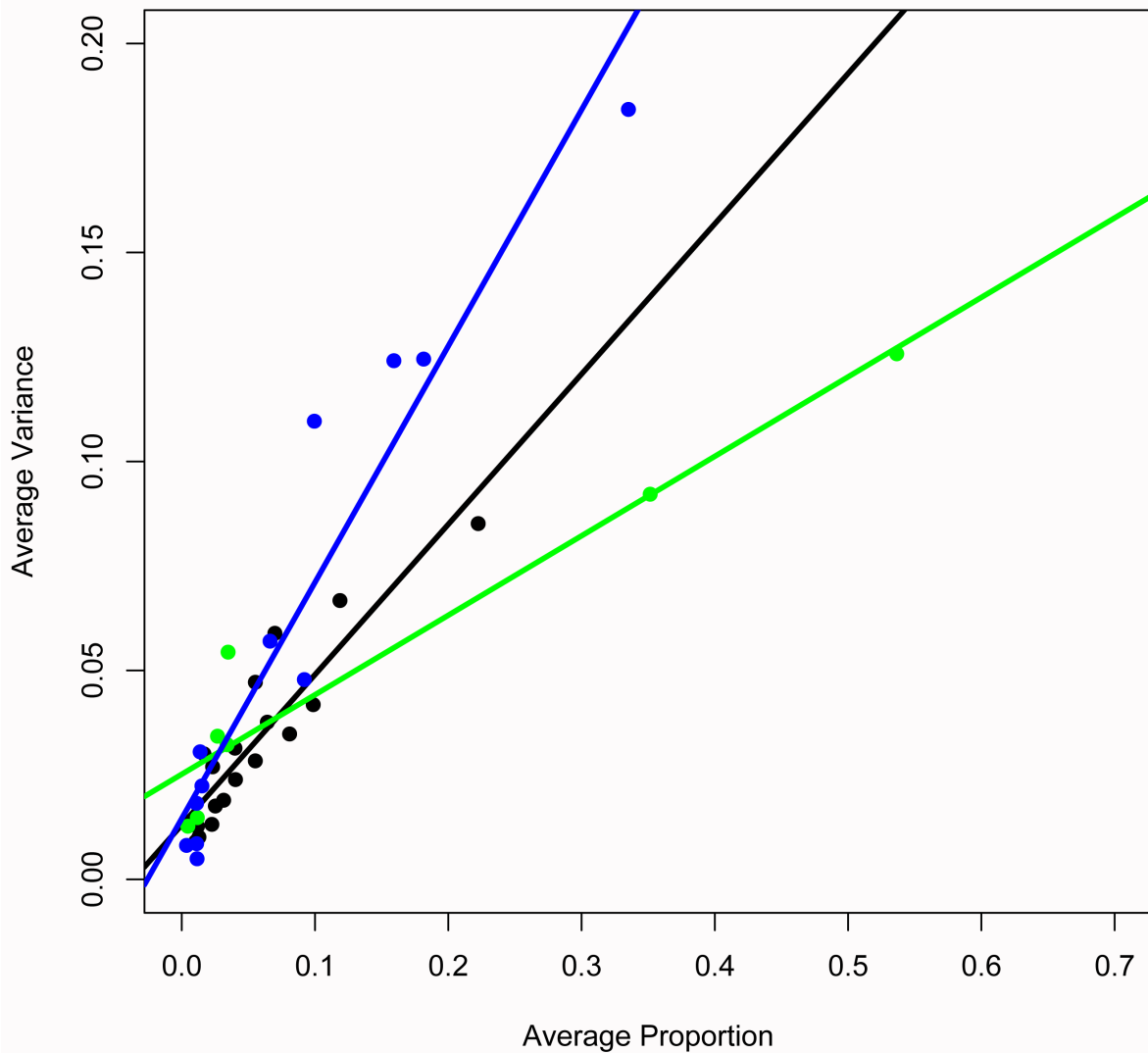
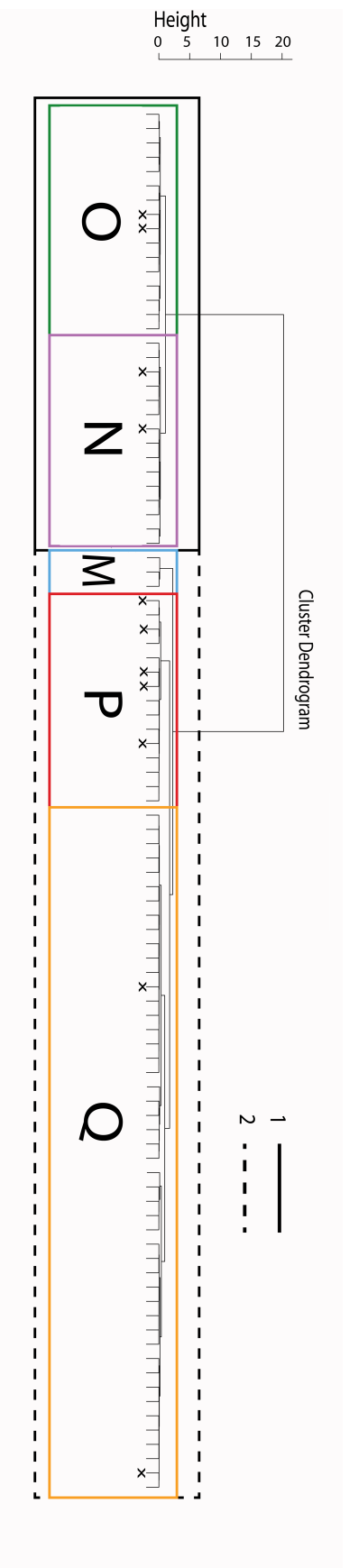


Figure 4: Ward's hierarchical cluster analysis to assign *Pseudomyrmex* PSW-53 individuals to chemotype using the entire CHC profile. Out of 98 individuals, no individual clustered outside of the first division (clusters 1 or 2). Within all five secondary clusters (O, M, N, P, Q) only 11 individuals clustered outside of the chemotype with their twigmates and are marked by Xs. Individual specimens are assigned by secondary cluster (O, M, N, P, Q).



CONCLUSION

Ants are at the center of a complex network of interactions that ultimately provides pest control within coffee agroecosystems (Perfecto et al. 2014). However, relatively little is known about the chemical ecology of these ants or how their chemical communication systems mediate their interactions within these agroecosystems. Combining studies of behavioral and chemical ecology of two ecologically important ant taxa and their natural enemies within coffee plantations, this dissertation begins to address these gaps in the literature.

In the first chapter of my dissertation, I focus on how one natural enemy of ants, phorid fly parasitoids, use chemical and visual cues to successfully parasitize their host ants. For successful parasitism, phorid fly parasitoids must undergo a multi-step process to detect and interpret a wide range of cues from their ant hosts. These cocktails of cues, each of which may vary in degrees of host specificity and timing of detection (sequentially or simultaneously), allow the flies to find suitable hosts in a complex environment. This chapter identifies five general and occasionally overlapping steps that are crucial in successful parasitism: (a) host habitat location, (b) host location, (c) host acceptance, (d) host discrimination and (e) host regulation. This review highlights how understanding the use of these cues by phorid flies can shed light on evolutionary and ecological processes within host-parasitoid interactions while providing insight into multimodal communication. Additionally, phorid flies are often both ecologically relevant species and have remarkably diverse strategies for using diverse arrays of multimodal cues within a complex environment to successfully parasitize host ants (Morehead and Feener 2000, Mathis et al. 2011, Gazal et al. 2009, Chen and Fadamiro 2009, Folgarait et al. 2003, Sharma et al. 2011). Thus, phorid-ant interactions are ideal systems to bridge the gap between model organisms for use in integrated pest management and model organisms used in understanding the behavioral ecology of multimodal cue use.

The second chapter of my dissertation examines phorid fly cue use when parasitizing their host, *Azteca sericeasur*. These ants are important for coffee pest control and ecosystem functions (Vandermeer et al. 2010). However three species of phorid fly reduce the ability of *A. sericeasur* to forage by approximately 50% (Philpott 2005). The results of this study show that phorid flies are able to distinguish between two cryptic taxa of *Azteca* ants, *Azteca sericeasur* and *Azteca* JTL020, despite the ants sharing two of the cues the phorid flies use in host location. The phorid fly, *Pseudacteon lascinosus* use *Azteca sericeasur* cuticular hydrocarbons as a short-range cue directly before oviposition. Together my previous work on this system (Mathis et al. 2011), this chapter identifies that *P. lascinosus* require a hierarchy of both chemical and visual cues, that increase in specificity and proximity to their target, in order to successfully oviposit on a host ant.

In the third chapter of my dissertation, I describe two new species of rove beetle that play a role in *Azteca sericeasur* – phorid fly interactions. The results from this chapter are the first descriptions of the genus, *Myrmedonota* in Mexico. This genus was previously only described in the Old World, with the exception of three recent descriptions of species within the United States, therefore the results suggest that these beetles may be much more prevalent within the New World than previously thought (Maruyama et al. 2008, Eldredge 2010). By documenting that these beetles are found mating near and interacting with

Azteca sericeasur colonies, this chapter is also one of the first to provide these beetles with a definitive host record (but see Kistner 2003).

The fourth chapter examines the role of one of the two previously described species, *Mymedonota xipe*, in *Azteca sericeasur*-phorid fly interactions. Here I demonstrate *M. xipe*, is able to detect 2-heptanone, a component of the alarm pheromone released by *A. sericeasur*. Our results also show that, *M. xipe* selectively preys on ants parasitized by *P. lascinosus* phorid flies. These parasitized ants display reduced aggression, and particularly a reduced frequency of mandible flaring, which may allow beetles to gain access to these parasitized ants as a prey item. Ant workers frequently mandible flare prior to attack as a warning. Thus, it appears that this preliminary act of aggression by both healthy ants and ants parasitized by *P. planidorsalis* is the primary behavioral cue used by *M. xipe* to determine whether to engage an ant. Our study also shows that, in the field, beetles are able to consume up to 14% of all ants parasitized by *P. lascinosus* in the field, which suggests that these beetles may have an important role in reducing *P. lascinosus* populations. Given that *M. xipe* are only preying on parasitized ants and phorid parasitism is already fatal, the beetles likely do not directly impact *A. sericeasur* population density. Thus, despite preying upon individual parasitized ant workers, our results indicate that *M. xipe* is a beneficial ant-associated beetle to the *A. sericeasur* population as a whole. Indeed, this study provides evidence that parasitized ants may be self-sacrificing by allowing themselves to be preyed upon by *M. xipe* beetles in order to reduce phorid fly populations. The host suicide hypothesis postulates that mature parasitoids emerging from host are more likely to infect host's kin than non-kin. Therefore, when maturation of the parasitoid is prevented, the inclusive fitness of the host should be increased (Smith Trail 1980). *Azteca sericeasur* meet the host suicide hypothesis criteria as they are not only social insects, but also monogynous and highly polydomous. Furthermore, *P. lascinosus* phorid flies have extremely limited search range, approximately two meters surrounding any given *A. sericeasur* nest (Philpott et al. 2009). Therefore, it is highly unlikely that phorid flies are dispersing beyond the boundaries of the *A. sericeasur* colony prior to oviposition. Ultimately, this study shows that the effects of the *M. xipe* association with *A. sericeasur* is dependent upon *P. lascinosus* presence and that these beetles are indirectly beneficial to *A. sericeasur*. To our knowledge, ours is one of the few studies that document the role of ant-associated beetles outside of its pairwise context (Mynhard 2013, Rettenmeyer et al. 2011, O'Keefe 2000, Stoeffler et al. 2011, Parmentier et al. 2014), and the first study documenting an ant-associated beetle predator that is beneficial to its ant associate.

In the final chapter of my dissertation, I examine the chemical communication system of another ecologically important ant taxa within coffee agroecosystems, twig nesting *Pseudomyrmex* spp. I use chemical analysis of cuticular hydrocarbons of three species of twig-nesting ants, *Pseudomyrmex simplex*, *Pseudomyrmex ejectus* and *Pseudomyrmex* PSW-53 to reveal species-specific profiles that unambiguously separated all three species from one another. This study also shows that within-species variation of CHCs differed between the three species examined. *P. PSW-53* individuals had the most within species variation and we were able to accurately separate individuals from nests into chemical clusters. The results of support the hypothesis that the high abundance and densely populated species would have low CHC variation, as the CHC profiles of individuals from both *P. simplex* and *P. ejectus* could not be broken into chemotypes due to high within-species similarity. In ants generally, CHC variation exists due to (1) genetic distance of

individuals, (2) age differences between individuals, and (3) environmental factors (e.g. food type, nesting substrate). Ant species with reduced CHC variation among individuals often possess reduced genetic variation and reduced levels of aggression between conspecifics. These species include ants that form supercolonies and some species of monogynous ants that occur in extraordinarily high nest density and that often inhabit virtually all nesting sites (Foitzik et al. 2007). The single chemotype found in *P. simplex* and *P. ejectus* suggests that these species may operate as single colonies in our research area. Similar to other species with low CHC variation, both *P. simplex* and *P. ejectus* occur commonly, each representing about a third of the occupied twigs in the field, while also exhibiting some of the lowest success in competitive interactions of any common twig nesting species. This chapter illustrates how the analysis of variation in CHC profiles, of non-invasive polydomous ants, sheds new light on how signal variation is associated with social organization. Our analyses demonstrate that the more abundant ants with higher nest densities, *P. ejectus* and *P. simplex*, did not exhibit as much individual chemical variation as the less abundant and more competitive *P. PSW-53*.

Examining the behavioral and chemical interactions between ants and their natural enemies in coffee agroecosystems offers a unique opportunity to provide insight into the mechanisms that mediate species interactions within complex ecosystems. Overall, these results address several gaps in the knowledge we have of the behavioral and chemical ecology of two key ant taxa within the complex foodweb that provides ecosystems services to coffee. My dissertation also describes two natural enemies of the keystone ant species, *A. sericeasur* and illustrates the importance of studying these species outside of their pairwise interactions with coffee pests.

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