The chemistry, recognition behaviors, and population genetics of Neotropical parabiotic ants

by

Virginia Jayne Emery

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Doctor of Philosophy

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Committee in charge:

Prof. Neil Tsutsui, Chair
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Abstract

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In my dissertation, I have explored behavioral, chemical and genetic aspects of a unique nesting symbiosis called parabiosis. In parabiosis, two unrelated ant species share a nest and foraging trails in a potentially mutualistic association. I have focused on the Neotropical parabiosis between *Camponotus femoratus* (Subfamily: Formicinae) and *Crematogaster levior* (Subfamily: Myrmicinae), which occur in ant-gardens throughout Amazonia. These two ants share a common nest but keep their brood in separate chambers. Behavioral tradeoffs suggest that the relationship is a mutualism: both species build the carton nest and forage, but *Cr. levior* is superior in finding food sources, and *Ca. femoratus* carries the epiphyte seeds required to give the nest structural support. Like any mutualism, the relationship is vulnerable to exploiters and cheaters, so reliable recognition systems would help to maintain the relationship.

In Chapter 1, I examine the nestmate recognition behaviors of *Cr. levior* and *Ca. femoratus* living in parabiotic ant garden nests. By using pairwise behavioral assays in neutral arenas, I assayed the proportion of ants exhibiting aggressive behaviors when paired with nestmate and non-nestmate ants. We expect for ants to aggress non-nestmates by biting, stinging, spraying formic acid or otherwise attacking to exclude these intruders. I also sampled the cuticular hydrocarbon chemistry of ants in these nests to determine whether recognition behavior was related to these compounds. Cuticular hydrocarbons (CHC) are often used as recognition cues amongst social insects. I found that there were three different CHC phenotypes in my study population in French Guiana. There were two sympatric chemotypes of *Cr. levior*, with very little overlapping chemistry. Within each nest, there was only a single chemotype of *Cr. levior,* and neither chemotype shared chemical cues with *Ca. femoratus*. Despite sharing a nest, *Ca. femoratus* exhibited a single chemotype throughout the population, and did not chemically match its *Cr. levior* nestmates. Both species maintain intraspecific recognition abilities, and *Cr. levior* shows some evidence of being able to distinguish amongst its *Ca. femoratus* nestmates. However, despite their strong chemical divergence, there was no

evidence *Ca. femoratus* could distinguish between the *Cr. levior* chemotypes. My findings suggest that selection to maintain reliability in conspecific recognition can potentially constrain the evolution of interspecific cooperation.

In Chapter 2, I delve further into the details of the cuticular chemistry of these parabiotic ants. Incidentally, there are three species living within the ant garden nests -- *Cr. levior*, *Ca. femoratus* and a tiny *Solenopsis* thief ant, called *Solenopsis picea*. Do these multispecies nests still form a common colony 'gestalt' odor? Are there differences in the chemical integration techniques of social mutualists and social parasites? I sampled individual ant CHCs to look for patterns of similarity within and between ant species, and within and between colonies. Both parabiotic species show some evidence of forming a single-species common colony odor, which is consistent with the gestalt hypothesis that nestmates share chemical cues. However, this cue sharing does not spread to allospecific nestmates. The two parabiotic species, *Cr. levior* and *Ca. femoratus*, share very few cues in common. In contrast, the social parasite *S. picea* shares cuticular chemistry with both of its host species. The specificity of this chemical cue similarity is limited, and *S. picea* is not chemically different whether it is nesting with *Cr. levior* Type A and *Cr. levior* Type B. These findings are the first to examine the CHC patterns in nests with three ant species, and highlight important differences in the chemical integration of social mutualists and social parasites.

In Chapter 3, I examine the genetic basis of the chemical phenotypes of *Cr. levior* and *Ca. femoratus*. Using the individual profiles of ants from Chapter 2, I compare the chemical phenotypes to genotypic information from both nuclear microsatellite loci and mitochondrial co-1. For both species, there is a correlation between chemical phenotypes and genotypes. In *Ca. femoratus*, there are positive correlations between genetic distances and both chemical and geographic distances of colony pairs. The genetic basis for chemotype includes a correlation between some alleles and the proportion of straight-chain alkanes, which are shorter than other hydrocarbons in the typical *Ca. femoratus* profiles. Likewise, there are correlations between chemical phenotypes and genotypes of *Cr. levior* ants, with a strong genetic distinction between the two *Cr. levior* chemotypes. There is no geographic partitioning of either chemical or genetic differences, which supports the observation of sympatry of the *Cr. levior* Type A and Type B. We find correlations between several alleles and the proportion of different chemical compounds. Specifically, there appear to be opposing genetic trends for the alkane and methyl branched compounds that dominate *Cr. levior* Type A profiles, and the unsaturated alkenes and alkadienes, which typify *Cr. levior* Type B profiles. Together this evidence supports the hypothesis that *Cr. levior* Type A and Type B are genetically distinct and potentially different cryptic species.

In Chapter 4, I attempt to resolve the relationships between geography, chemistry, genetics, and recognition behaviors of the parabiotic ants. In other systems, cuticular chemistry plays an important role in determining the outcome of recognition assays, with increased chemical dissimilarity usually resulting in

increased aggression. Similarly, more genetically distant non-nestmates are expected to be subject to more aggression. I find that for *Ca. femoratus*, conspecific aggression is related to genetic differences, but not to the measured chemical differences. This suggests potential kin-informative cues exist, that we have not yet measured. These genetic cues may also be used by *Cr. levior* to recognize their *Ca. femoratus* nestmates, and highlight the technical limitations of our current chemical machinery. In contrast, *Cr. levior* conspecific recognition is more strongly related to chemical differences, but mainly at the level of chemotypes. These results emphasize the importance of considering all levels of chemical and genetic differentiation when assessing nestmate recognition patterns. Both species of parabiotic ant uses chemical and associated genetic information to assess nest membership. The fully functioning recognition systems in parabiotic nests likely maintain the relationship by excluding exploiters of this unique cooperative relationship.

In sum, my dissertation research characterizes the recognition behaviors, chemical cues, and population genetics of an uncommon but abundant ant-ant mutualism. My findings support the hypothesis that recognition behaviors, although proximately mediated by chemical similarity, are ultimately controlled by genetic factors. I find that both chemical integration techniques and nestmate recognition behaviors differ for mutualistic and parasitic nesting symbioses. Together my dissertation research highlights the unique properties of the parabiotic nesting association, and supports its status as the only ant-ant mutualism.

To my family, for their continued support in my pursuit of outdoor adventures,

To my Papa and Mammaw, for teaching me how to be a lady, and supporting the undergraduate studies that sparked my love for biology,

To my Grandma and Graw, who will probably not understand a single word of my dissertation, but it makes them proud anyways,

And to the ants.

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PREFACE

A year before setting off on my first adventure to French Guiana, I was asking around about parabiotic study systems and received the following email from Stefan Cover, who works at the Harvard Museum.

April 28, 2009

Dear Virginia,

1) Parabiosis involves ant species that nest in close proximity and share foraging trails. As such, I've never seen anything in North America that corresponds at all closely to the well known tropical examples.

2) The most frequently cited example of parabiosis is the *Camponotus femoratus*/*Crematogaster "parabiotica*" system in tropical South America. I can testify from personal experience how thoroughly unpleasant it would be to work with these ants. *Camponotus femoratus* is the most miserable, godforsaken, obnoxious ant I have ever encountered in a long career of ant collecting. As Formicines, they do not sting, but they are hyperactive, insanely aggressive, have a nasty bite, and never seem to occur in units of less than 100,000. They are so bad that I can say with confidence that I'd rather sell insurance for a living than study them!

Best wishes,

Stefan
MCZ Entomology

I am proud to say that despite this fair warning, I have enjoyed my dissertation research much more than I would have enjoyed working in insurance.

INTRODUCTION

Parasitism and mutualism: a continuum of interactions

A central topic in ecological research is the study of interspecific interactions. Interactions exist along a continuum from mutualism, where both parties benefit, to parasitism, where one party benefits at the expense of another (Ewald 1987). Not only do interactions vary along this continuum, but also the nature of the relationship can vary in both space and time (Thompson 1999). Only by measuring the costs and benefits to each participant can we determine the true nature of the relationship. The mechanisms that reinforce this cost-benefit balance are particularly interesting from an evolutionary perspective because these mechanisms may constrain the relationship (Edwards and Yu 2007, Edwards 2009). Such is the case in mutualistic associations where partner recognition can be a key mechanism in maintaining the association (Tebbich et al. 2002, Chaston and Goodrich-Blair 2010).

Cooperation, where participants work together for common mutual benefits, is a relatively rare but important mutualistic association. Unlike most mutualisms, which could be considered reciprocal exploitations providing net benefits, cooperation is defined as a mutualistic behavior that is selected for its beneficial effects on the recipient (West et al. 2007). Usually when cooperation is costly to the actor, benefits are directed to related individuals. In order to be stable, the cost of cooperative actions must be outweighed by the benefits accrued. These benefits can include indirect fitness benefits that result from helping relatives achieve their own reproductive success (Field et al. 2006, Dugatkin 2007, Abbot et al. 2011). This explanation for seemingly non-adaptive behavior is known as Hamilton's rule, and can be incurred to explain the evolution of cooperation (Hamilton 1963). This is also one reason that non-kin cooperation is rare (Dugatkin 2002, Clutton-Brock 2009).

Ants are a key player in many interactions

Amongst the ants, cooperation is absolute- all ant species are eusocial (Hölldobler and Wilson 1990). Eusociality involves three important characteristics: 1) there is reproductive division of labor, with some individuals reproducing more than others, 2) there are overlapping generations which inhabit the same nest, and 3) the nursing duties are shared through cooperative brood care (Crespi 1994, Crespi and Yanega 1995, Reeve et al. 1996). This cooperative lifestyle allows the ants to be numerically dominant, often forming enormous colonies of millions. Ants are also ecologically dominant, playing key roles in nutrient cycling and the population regulation of other species (Pérez-Espona 2010). For example, one key interaction that places ants at the center of plant-herbivore dynamics is the protective nutritional mutualism between ants and honeydew producing insects. The many interactions between ants and other insects, ants and plants, and ants with one another make them a focal group for the study of interactions, and the role of cooperative behavior in these relationships.

Interactions between different ant species are particularly interesting because they involve the intersections of two cooperative societies. Competition is thought to be a dominant force in the structuring of ant communities (Wilson 1961,

Palmer et al. 2003, Sanders et al. 2007, Blüthgen and Stork 2007). Amongst ant species, many traits have evolved to provide an escape from this competition, such as dietary or nesting innovations (Davidson 1998, Parr and Gibb 2012). By harboring beneficial microbes, such as the case for *Camponotus* ants and their *Blochmannia* endosymbionts, many ants can exploit otherwise inaccessible nutritional resources (Schröder et al. 1996, Feldhaar et al. 2007). Similarly, *Oecophylla* ants escape competition for limited arboreal nest-sites by weaving a nest of leaves with silk from their own larvae (Dodd 1902, Hölldobler and Wilson 1990). On the other hand, many traits have evolved to enhance competitive abilities, such as aggressive territoriality and high tempo foraging (Davidson 1997).

In addition to competition, ant-ant parasitism and ant-ant mutualism are important pressures that drive ant evolution.

Social parasite nesting symbioses

Although all ants are eusocial-- with reproductive labor typically divided between queens and workers and several generations coexisting in a nest-- not all ant species share equally in the burdens of nest and brood care. Social parasites adopt a cheating strategy, infiltrating a host nest and benefiting from the social services of the host workers (Hölldobler and Wilson 1990). The hosts generally suffer at the expense of these parasite workers, often losing their queen or the ability to produce new offspring.

Social parasites come in several different flavors. The four main obligate modes are temporary social parasitism, slave-making (dulosis), inquilinism and xenobiosis (Buschinger 2009). Each mode of parasitism is distinguished by alternative natural histories, but in all cases the parasites rely on the hosts for social benefits such as nest maintenance, protection, foraging, and brood care.

Temporary social parasitism is a non-permanent parasitism where the social parasite only relies on the host for colony founding (Foitzik and Heinze 1998). A parasite queen enters the host colony and kills the host queen, often using the dead queen's chemical signature as camouflage (Lenoir et al. 2001). The host workers then rear the parasite's offspring. Since the host queen has been killed, no new host workers are produced, and slowly the balance of workers shifts numerically until only parasite workers remain. Mature colonies consist of only the parasite queen and workers, which can make these ants more difficult to identify as parasites.

In slave-making, also known as dulosis, colonies are initiated in a similar way with queens infiltrating a host nest (Greenberg et al. 2007). However, instead of allowing the host worker population to dwindle to non-existence, the slave-making workers will go on raids to harvest new workers. The mature parasite workers enter neighbouring host colonies, aggressively attack the host workers, and steal larvae and pupae (Foitzik et al. 2001). These kidnapped brood are reared in the parasitized nest and mature to continue the labor required to maintain the colony.

Inquilinism is the most frequent form of social parasitism and involves some of the most specialized adaptations to a parasitic lifestyle (Huang and Dornhaus 2008). The parasite queen infiltrates host nests but often tolerates the host queen, and by keeping her alive can maintain the host population of workers without slave-raiding. The host workers rear the parasite offspring alongside their own. In many

cases, inquiline species are also worker-less and produce only reproductive individuals (Ward 1996). In these cases the parasites rarely emerge from the colony, which make them difficult to collect.

In these three obligate social parasitisms, the host and parasite brood are reared together in the same chamber, which is termed mixed nesting. In contrast, compound nesting involves the host and parasite brood being kept separate in different chambers. Social parasites that live in compound nests are considered to be living in xenobiosis and may also be called 'guest ants' (Buschinger 2009). Unlike the mixed species nests, the guest ants care for their own offspring. However, they are dependent on the host workers for nutrition, and possibly shelter. Generally the xenobiotic ants are distantly related to their hosts, which contrasts with the other types of social parasitism where the two species are usually closely related (Huang and Dornhaus 2008).

These various life history details, coupled with the overall rarity of social parasites, means that we are likely underestimating total social parasite diversity. Social parasitism is a relatively uncommon strategy amongst ants, accounting for the lifestyles of approximately 2% of global ant species (Buschinger 2009). In addition, social parasites are typically collected from less than 10% of colonies in a host population (Davies et al. 1989). However, the fascinating natural histories of these species make them prime systems for investigating ant biology. One area of research with a particular bounty of studies is the workings of recognition systems in these parasitized nests (Lenoir et al. 2001, Buschinger 2009). Social parasite systems can teach us a great deal about how cooperation works, through the study of how cooperation is manipulated.

Parabiosis: the only ant-ant mutualism

On the other side of the interaction spectrum, interspecific ant-ant mutualism is only known from a handful of cases. This mutualistic relationship, often termed parabiosis, is superficially similar to xenobiotic relationships. The involved species are generally unrelated, often from different subfamilies. The ants share a common nest and foraging trails, but the brood is kept in separate chambers in compound nesting (Mann 1912, Wheeler 1921). Unlike xenobiosis, parabiotic relationships are likely mutualistic, with both species benefitting from the association. The mutualistic nature of the relationship has been assumed through observations of behavioral trade-offs, which allow both species to gain from the interaction (Vantaux et al. 2007, Menzel and Blüthgen 2010). To date, there are few studies investigating the parabiotic interaction, with detailed studies from only one system in SE Asia (Menzel 2009).

Ant gardens are hotspots for parabiosis

In both the Paleo- and Neotropics, ant-gardens are a microhabitat that houses many parabiotic interactions (Orivel and Leroy 2011). Ant-gardens are an ant-plant interaction whereby ants collect and plant epiphyte seeds in their carton nests. A wide diversity of plants use this strategy to disperse their seeds, with 53 species reported from 12 plant families (Davidson 1988, Kaufmann and Maschwitz 2006, Orivel and Leroy 2011). These epiphytes are typically highly specialized to

attract the ants with chemical cues, and most are only found within ant nests (Youngsteadt et al. 2008, 2009, 2010). The carton and refuse of the ants nourishes the epiphytes (Blüthgen 2001, Schmit-Neuerburg and Blüthgen 2007, Leroy et al. 2009). The ants also provide constitutive protection from herbivores (Davidson 1988, Leroy et al. 2012). In exchange for the dispersal, nutrition, and protection benefits from the ants, the plants provide structural stability to the nest with their roots (Yu 1994).

Only a handful of ants can produce carton, and even fewer still initiate ant-gardens (Orivel and Leroy 2011). The ant-garden initiators come from four subfamilies of ants (Formicinae, Myrmicinae, Ponerinae and Dolichoderinae). Within ant-gardens, parabiotic associations have been reported between *Crematogaster* (Myrmicinae) ants and the ponerines *Pachychondyla goeldii* and *Odontomachus mayi* (Orivel et al. 1997). More frequently, *Crematogaster* species reside with formicine ants in the genus *Camponotus* (Kaufmann 2002, Menzel and Blüthgen 2010).

MY STUDY SYSTEM

Amazonian ant-garden parabioses

For my dissertation research, I have studied the interactions in parabiotic nests found throughout ant-gardens in the Amazonian region in South America (Figure 1). My main study site has been in French Guiana near Kaw and Petit Saut. I have focused on the interaction between the species *Camponotus femoratus* (Subfamily: Formicinae) and *Crematogaster levior* (Subfamily: Myrmicinae). A third species, *Solenopsis picea* (Subfamily: Myrmicinae) is also occasionally found in these nests (Figure 2).

Nest architecture and colony boundaries

Most parabiotic colonies are polydomous, with dozens of nest units making up each colony. Ants move freely between several arboreal nest units, traveling along tree branches and lianas in multi-species trails. Colony boundaries are not immediately obvious due to this polydomy, but generally are differentiated by host tree, with one colony per tree. Colony boundaries of *Ca. femoratus* and *Cr. levior* typically coincide, but occasionally a single colony of *Cr. levior* may span multiple *Ca. femoratus* colonies (Elsa Youngsteadt, unpublished genetic and behavioral data). Each nest can range in size from a few centimeters to several feet in diameter, although the upper limit of nest size is constrained, and very large nests often fall from trees (Davidson 1988).

In my initial pilot studies, I dissected several frozen nests to assay the physical location of ants. The carton nests of these ants are segregated by species. The chambers of *Cr. levior* are more commonly near the exterior surface of the nest, with larger *Ca. femoratus* chambers in the interior. The thief ant *S. picea* occupies a few small chambers that are adjacent to *Cr. levior* brood chambers, or close to the outer surface of the nest. Rarely are workers of two species found together within nest chambers. In all cases the nesting chambers scale to the size of their ant occupants, suggesting that each species builds their own brood chambers. Brood

appears to be sorted according to caste or age, but further nest dissections are necessary to confirm this. If such is the case, it sets up an interesting levels-of-selection scenario where certain nest units represent different fitness investments for the polydomous colony.

Frequency of parabiosis and abundance of ants

Other studies have found 33% - 95% of ant gardens in a population contain the two species *Cr. levior* and *Ca. femoratus* (Davidson 1988, Dejean et al. 2000). Of these nests, 68%-98% represent parabiotic associations (Davidson 1988, Dejean et al. 2000). In my study population near Kaw in French Guiana, I only encountered one single-species ant-garden of *Cr. levior*. This nest was in a fallen tree in the middle of a recent logged swampy area, isolated from other parts of the forest. The epiphytes in this nest were subject to greater herbivory than in other ant-gardens, and the carton was degraded and covered in a green moss or algae.

I have observed several relocation events after a nest was destroyed, and the two species worked together to reinitiate nests. A destroyed nest could be rebuilt, including germination of new epiphyte seeds, within about two weeks. Similarly in experimental studies of ant-garden initiation, both species colonize abandoned epiphytes together (Bader 1999). In arboreal ant community assessments, only *Ca. femoratus* and *Cr. levior* are consistently coexistent (Franken and Gasnier 2010). These findings suggest that the association of *Cr. levior* and *Ca. femoratus* may be an obligate association, and that single-species nests are a temporary state. Further work is needed on the spatio-temporal nature of the relationship (Dejean et al. 2000). It is unknown how new colonies are initiated, when or how reproductives mate and disperse, and whether interspecific cues are involved in nest relocation or colony initiation.

The thief ant *S. picea* is so small (<2 mm) that it easily goes undetected by coexisting ants and researchers alike. It is unclear how frequently it associates with any of the ant-garden ants, but it was collected from 7 of 27 colonies in my studies, and 1 in 5 colonies from Brazilian populations near Manaus (Thiago Izzo, personal communication). These are likely underestimates of the true prevalence of this cleptoparasite.

Within the Amazon region, arboreal ants represent up to 94% of the total canopy arthropod abundance (Davidson et al. 2003). The two ants *Ca. femoratus* and *Cr. levior* represent a disproportionate amount of ants in these samples, occurring in 41% and 69% of Ecuadorian samples respectively (Wilkie et al. 2010). Both species also occur frequently at baiting studies (Davidson 2005, Baccaro et al. 2010), and are dominant at resources (Dejean et al. 2007).

Costs and benefits of the parabiotic relationship

The SE Asian parabioses have been classified as mutualisms based on close observations of several aspects of the relationship (Menzel and Blüthgen 2010). The Neotropical parabiosis of *Ca. femoratus* and *Cr. levior* is less studied, but is likely also a mutualism (Figure 3). However, no direct measure of fitness, such as the number of reproductive individuals produced, has been assessed in any parabiotic system.

A taxonomic note and species synonyms

The ant *Cr. levior* Longino 2003 is thought to be a specialized nesting symbiont with *Ca. femoratus* Fabricius 1804, and its closely related sister species *Cr. carinata* Mayr 1862 is thought to have a more widespread distribution and occur in facultative parabiosis with other species (Longino 2003). The morphological distinction between *Cr. levior* and *Cr. carinata* is a slight difference in carinulae on the pronotal dorsum: *Cr. levior* is completely smooth, but *Cr. carinata* ranges from smooth to strongly carinate. This variation suggests *Cr. carinata* is likely a complex of cryptic species. The two species also show potential behavioral differences, with *Cr. levior* having a reduced sting response and *Cr. carinata* maintaining this aggressive behavior (Davidson, unpublished observations). Some observations of *Crematogaster cf. limata parabiotica* have been synonymized with *Cr. carinata*, while observations of *Cr. limata parabiotica* nesting with *Ca. femoratus* are likely *Cr. levior* (Longino 2003). We have confirmed that all parabiotic *Crematogaster* in our study are the species *Cr. levior* (Longino, personal communication), and not *Cr. carinata*.

The guest or thief ant *Solenopsis picea* Emery 1896 found in ant-gardens of *Cr. levior* and *Ca. femoratus* has previously been called *Solenopsis parabiotica* Weber 1943. However, *S. parabiotica* has recently been synonymized with *S. picea* (Pacheco 2008). These lestobiotic ants are tiny (often <2mm long) and rarely collected, having small colony sizes and restricted habits. They are widespread in Central and South America, and can be found in association with both arboreal and terrestrial hosts. Currently, *S. picea* is considered a generalist social parasite with several host species. However, to my knowledge, no genetic or chemical studies have ever been undertaken in this species, which may include cryptic types and potential specialists.

FIGURES

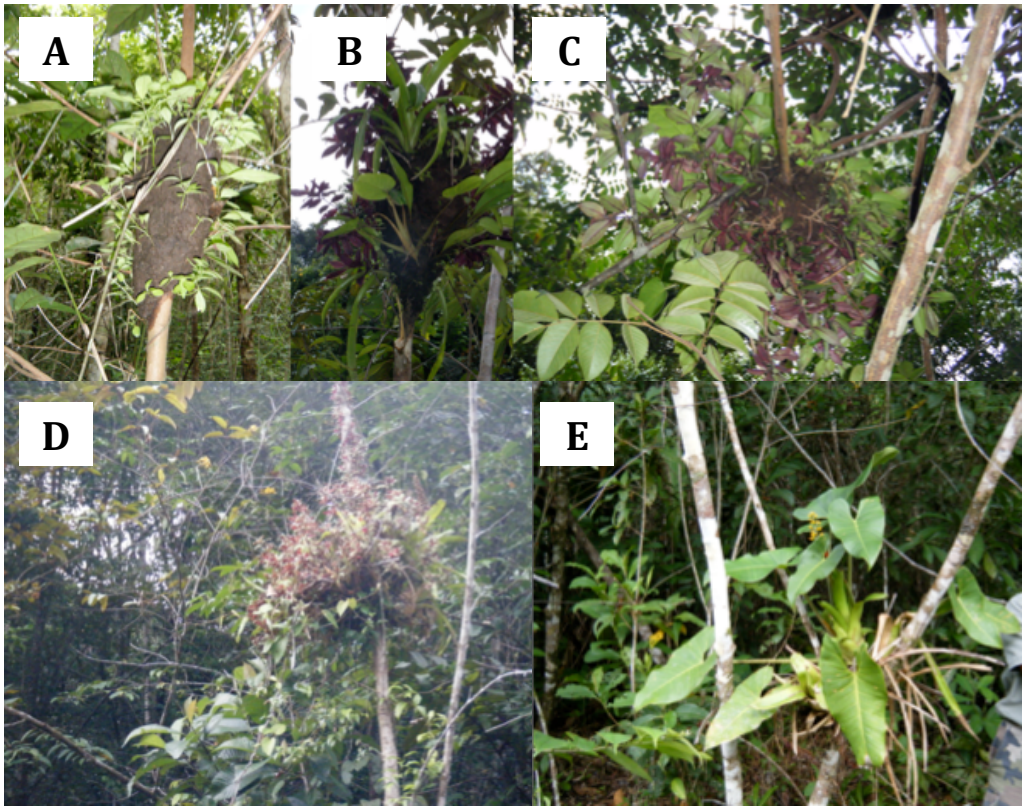


Figure 1. Five pictures of different ant-gardens housing *Ca. femoratus* and *Cr. levior* in French Guiana. Shown here are a) a young ant-garden with epiphyte plantlets, b) a more mature nest housing *Anthurium gracile* (Araceae), c) a nest with *Codonanthe crassifolia and Codonanthe calcarata* (Gesneriaceae), d) ant-garden with *Codonanthe* and e) ant-garden with a large *Philodendron deflexum* (Araceae) and flowering *Achmea mertensii* (Bromiliaceae). The brown carton is visible in pictures a-c but obscured by the mature plants in d and e.

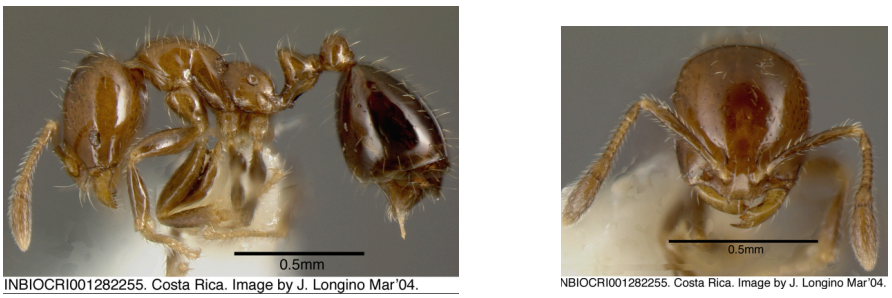
A *Camponotus femoratus*

B *Crematogaster levior*

CASENT0625391 Venezuela. Image by C. Richart. Jan'03

CASENT0625391 Venezuela.
Image taken by C. Richart. Jan'03

C *Solenopsis picea*



INBIOCRI001282255. Costa Rica. Image by J. Longino Mar'04.

NBIOCRI001282255. Costa Rica. Image by J. Longino Mar'04.

Figure 2. Images of the side and front view of the ants a) *Camponotus femoratus*, b) *Crematogaster levior* and c) *Solenopsis picea*. In the first row, thumbnail images of *Cr. levior* and *S. picea* are scaled relative to the *Ca. femoratus* image to give an idea of the relative sizes of each ant. The black scale bars represent a) 2mm, b) 1 mm and 0.5 mm, and c) 0.5 mm. The images of *Ca. femoratus* are courtesy of K. T. Ryder Wilkie 2006 from 'The Ants of Tiputini' website (Ecuador), and of *Cr. levior* (Venezuela) and *S. picea* (Costa Rica) courtesy of AntWeb.org.

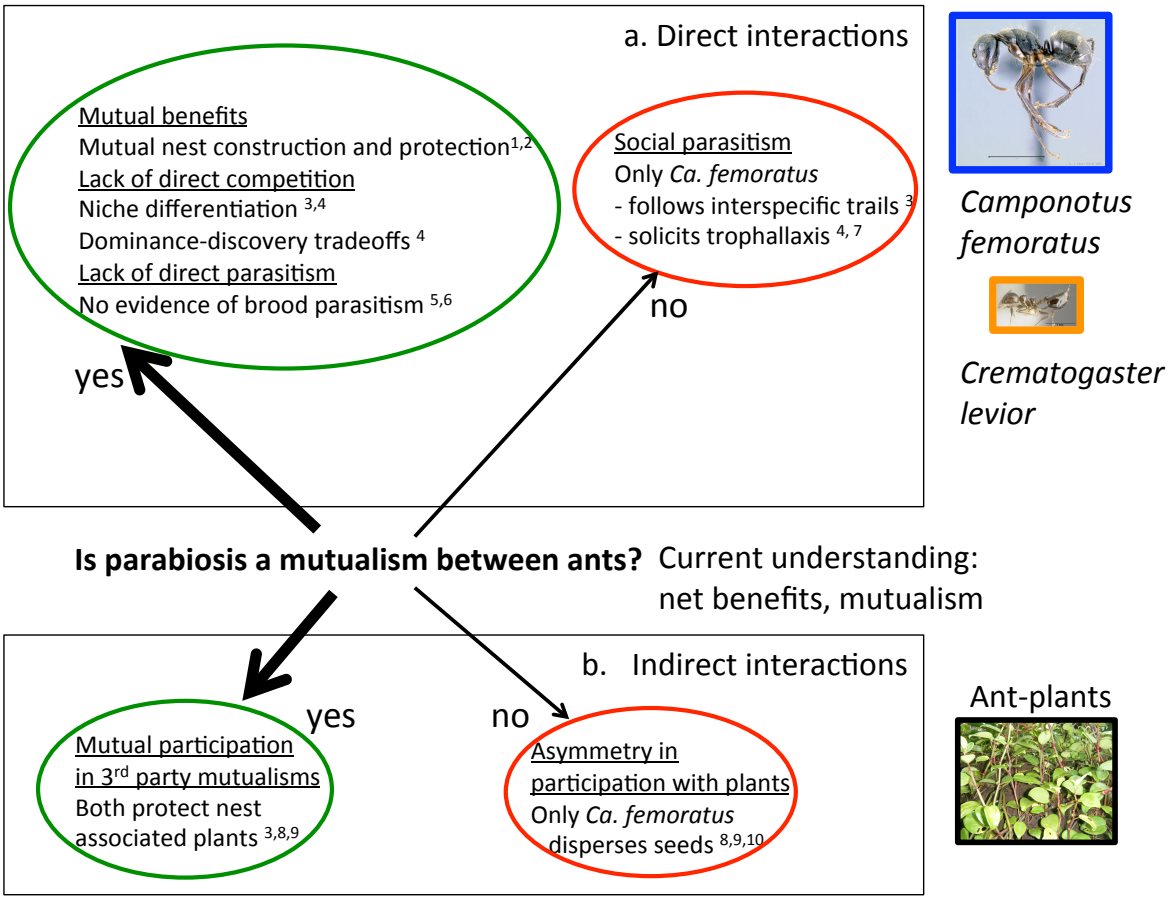


Figure 3. Diagram summarizing the known aspects of the parabiotic relationship between *Ca. femoratus* and *Cr. levior*. Various behavioral trade-offs with each other and with third-party mutualists allow these ants to coexist. The potential costs of the relationship (in red circles) are likely outweighed by the potential benefits (in green circles). Citations: (Wheeler 1921, Weber 1943, Swain 1980, Davidson 1988, Orivel et al. 1997, Dejean et al. 2000, Vantaux et al. 2007, Youngsteadt et al. 2010, 2008, 2009, Menzel and Blüthgen 2010)

CHAPTER 1

Recognition in a social symbiosis: chemical phenotypes and nestmate recognition behaviors of Neotropical parabiotic ants

ABSTRACT

Social organisms rank among the most abundant and ecologically dominant species on Earth, in part due to exclusive recognition systems that allow cooperators to be distinguished from exploiters. Exploiters, such as social parasites, manipulate their hosts' recognition systems, whereas cooperators are expected to minimize interference with their partner's recognition abilities. Despite our wealth of knowledge about recognition in single-species social nests, less is known of the recognition systems in multi-species nests, particularly involving cooperators. One uncommon type of nesting symbiosis, called parabiosis, involves two species of ants sharing a nest and foraging trails in ostensible cooperation. Here, we investigated recognition cues (cuticular hydrocarbons) and recognition behaviors in the parabiotic mixed-species ant nests of *Camponotus femoratus* and *Crematogaster levior* in North-Eastern Amazonia. We found two sympatric, cryptic *Cr. levior* chemotypes in the population, with one type in each parabiotic colony. Although they share a nest, very few hydrocarbons were shared between *Ca. femoratus* and either *Cr. levior* chemotype. The *Ca. femoratus* hydrocarbons were also unusually long–chained branched alkenes and dienes, compounds not commonly found amongst ants. Despite minimal overlap in hydrocarbon profile, there was evidence of potential interspecific nestmate recognition –*Cr. levior* ants were more aggressive toward *Ca. femoratus* non-nestmates than *Ca. femoratus* nestmates. In contrast to the prediction that sharing a nest could weaken conspecific recognition, each parabiotic species also maintains its own aggressive recognition behaviors to exclude conspecific non-nestmates. This suggests that, despite cohabitation, parabiotic ants maintain their own species- specific colony odors and recognition mechanisms. It is possible that such social symbioses are enabled by the two species each using their own separate recognition cues, and that interspecific nestmate recognition may enable this multi-species cooperative nesting.

INTRODUCTION

Social organisms, ranging from microbes and insects to humans, dominate our planet. The success of any society is contingent on the ability to recognize members and non-members, and to maintain an efficient recognition system in the face of exploiters who might manipulate it (Breed 1983, Lenoir et al. 2001, Bos and D'Ettorre 2012, Sturgis and Gordon 2012). Optimal social recognition systems minimize both rejection errors (that falsely reject members) and acceptance errors (that falsely accept non-members) by increasing the reliability of signals used in the recognition system. This can be done on the sender side, with more consistent relationships between cues and identity (Tsutsui 2004, Weddle et al. 2012), or on the receiver side by honing sensory perception and decision rules used by receivers to evaluate cues and assign identity (Blumstein et al. 2004, Liebert and Starks 2004, Mateo 2004, Magrath et al. 2009, Johnson et al. 2011).

For example, in a typical ant nestmate recognition system, the recognition cues are chemicals called cuticular hydrocarbons (CHCs), which can be both

genetically and environmentally determined (Torres et al. 2007, Brandt et al. 2009a). A common nest odor, (the 'gestalt odor'), is maintained through frequent social interactions, such as allogrooming, during which odors are exchanged among the interacting individuals. These interactions minimize recognition errors by homogenizing chemical cues across individuals (Crozier and Dix 1979, Buckle and Greenberg 1981, Carlin and Hölldobler 1983, Breed et al. 1985). Perceptually, both sensory habituation (Ozaki et al. 2005) and learning (Errard et al. 2008) allow ants to familiarize themselves with the gestalt odor and form a neural template of expected nestmate phenotypes. Ant nestmate recognition systems are reliable because of the frequent mixing of recognition cues, and the constant updating of individual's neural templates as colony odors shift (Bos and D'Ettorre 2012).

Social parasites gain entry to a host nest by manipulating or circumventing the recognition process, thus gaining access to the host's social benefits, such as protection or brood care, to the detriment of the host species. In the ants, social parasites have evolved many times, with 230 described socially parasitic species, potentially representing up to 2% of total ant diversity (Buschinger 2009). Chemical mimicry or camouflage are the most commonly used methods of social integration. For example, the slave-making ants *Protomagnathus americanus* have locally adapted to increase their chemical similarity to their sympatric *Temnothorax* hosts (Achenbach et al. 2010). Most ant social parasites gain entrance to their hosts' nests by targeting closely related species and placing their brood in the same chamber as the host brood, producing a 'mixed nest' (Huang and Dornhaus 2008) which facilitates the chemical integration of the parasite into the host society (Lenoir et al. 2001). However, some social parasites form 'compound nests' with their hosts, in which brood are kept in separate locations (Buschinger 2009). In these cases, called xenobioses, the two nest-sharing species are often distantly related, but still obtain a similar, shared colony odor (Lenoir et al. 1997).

In theory, however, cue mimicry is not absolutely necessary, and social integration can be achieved by other mechanisms (Beeren et al. 2012). For example, the perceptual component of recognition is not completely self-referent, as it can be expanded to include other species' cues (Errard and Hefetz 1997, Orivel et al. 1997, Errard et al. 2005, 2008). This template broadening may reduce the host's own conspecific recognition abilities, which can be a major cost of being parasitized (Bos and D'Ettorre 2012). Parasites can also escape detection by becoming imperceptible to their hosts, by either decreasing the amount of CHCs produced, or changing the type of compound expressed (Lambardi et al. 2006). However, this 'chemical insignificance' could also reduce the ability of the parasite to discriminate conspecifics (a cost to the parasite) (Bos and D'Ettorre 2012). The altered recognition systems in socially parasitized nests can therefore be costly to both the host and parasite species.

In some cases, however, different species of ants can coexist in a single nest without any apparent parasitism (Buschinger 2009). This rare relationship is called parabiosis, and is known from fewer than 20 species, many in the genera *Camponotus* and *Crematogaster*. In parabiosis, two distantly related species, often of different subfamilies, share a nest and foraging trails, but keep brood separate in a compound nest (Mann 1912, Wheeler 1921, Weber 1943). Superficially, these nests

resemble xenobiotic parasitism, but the parabiotic partners are thought to coexist in a mutualism, with both species benefitting from the nesting association. This has been measured by quantifying the contribution of each species to foraging, nest defense, and third party mutualisms, such as with plants or honeydew producers (Swain 1980, Vantaux et al. 2007, Menzel and Blüthgen 2010, Menzel et al. 2010). However, one unmeasured cost of the parabiotic relationship could arise from a compromised recognition system.

Within the 'compound nests' there have been very few investigations of recognition (summarized in Table 1). Due to the limited number of studies, it is unclear which features of the recognition systems differ in parabiotic (mutalistic) and xenobiotic (parasitic) nests, but there are a few trends. The parabiotic ants seem to share fewer chemical cues with each other than xenobiotic ants (Espelie and Hermann 1988, Lenoir et al. 1997, Martin et al. 2007). Parabiotic associations may allow for the development of heterospecific nestmate level recognition (Orivel et al. 1997, Errard et al. 2005), or chemotype level recognition (Menzel et al. 2008a), whereas xenobiotic associations have not shown this specificity. It is also unclear whether the parabiotic association has impacted conspecific recognition, which is reduced in the host species of xenobiotic nests (Errard et al. 1992, Martin et al. 2007). There are also differences between different parabiotic systems. For example, in the genus *Camponotus*, species that live in parabiosis or who are tolerated by other species have unusually long-chained hydrocarbons that are mostly branched alkenes and dienes (Menzel and Schmitt 2012). The facultatively parabiotic ant *Odontomachus mayi*, does not have these specialized hydrocarbons (Orivel et al. 1997).

Here, we examined the recognition system of the parabiotic association between *Camponotus femoratus* (subfamily: Formicinae) and *Crematogaster levior* (*Cr. limata* spp. group, subfamily: Myrmicinae). These ants co-occur in parabiotic ant-gardens in the Amazon region of South America (Davidson 1988, Youngsteadt et al. 2008, Orivel and Leroy 2011). We assessed nestmate recognition systems in these parabiotic nests by examining the cuticular hydrocarbon cues of ants and the aggressive rejection of non-nestmates in pair-wise behavioral assays. By combining an investigation of con- and heterospecific recognition, we tested the hypothesis that parabiosis can lead to heterospecific nestmate recognition (Orivel et al. 1997), and the hypothesis that the ants in these mixed species nests may have compromised conspecific recognition systems through template broadening (Bos and D'Ettorre 2012). Specifically, we ask: 1) Do parabiotic ants share cuticular hydrocarbon cues? 2) Is there evidence of heterospecific recognition? 3) Is there evidence of altered conspecific recognition, such as reduced aggression to conspecific non-nestmates?

Our investigation is only the second study to look at recognition in a common and obligate social symbiosis (the first being in SE Asia (Menzel et al. 2008a, 2008b)), and contributes to identifying features that distinguish non-parasitized from parasitized recognition systems. We find that in this parabiosis, both species maintain their own species-specific odors and conspecific recognition behaviours. We also find some evidence that ants may be able to distinguish between their heterospecific nestmates and non-nestmates. These recognition patterns are

consistent with the hypothesis that these social symbioses are different than social parasitisms, and may be true inter-society mutualisms.

METHODS

Study site

Parabiotic nests of *Ca. femoratus* and *Cr. levior* ants were observed in the lowland Amazonian rainforest of French Guiana, near the village of Kaw (3° 30' 43. 1994" N, 30° 15' 54"W) in March 2010 and July 2010. All research conformed to the policies for field-work and collection in that country, and no specific permits were required for the described field studies. None of the species collected for this study are listed as endangered or protected, and the study location is not privately-owned or protected in any way. Twenty colonies were selected, and a single accessible nest from each polydomous colony (colonies span over several individual nest units) was used as a source of ants for the behavioral observations and chemical extractions. All chosen nests were separated by 100m or more of nest-free space, and assumed to belong to different colonies because these polydomous colonies have clustered nests, and no ants were observed walking between the chosen nest pairs. The 20 selected colonies were haphazardly assigned to 10 independent colony pair comparisons. The location of each nest was recorded using GPS.

Cuticular hydrocarbon extraction

For each nest (n=20) we collected a pooled single-species sample of 3-5 ants for *Ca. femoratus* and 20-30 ants for *Cr. levior*, because *Cr. levior* workers are individually much smaller (2-3 mm) than *Ca. femoratus* workers (>1 cm). Each group of ants was freeze-killed and submerged in 10-200 µL of hexane for 10 minutes. The ants were removed and stored in 95% EtOH, and the hexane was evaporated for transport back to UC Berkeley. Each CHC sample was re-eluted in 200 µL of hexane, and filtered through a 1 cm hexane-rinsed silica column to separate polar and non-polar compounds. To maximize sample recovery, each column was further rinsed with 300 µL of hexane. The 500 µL sample containing the non-polar hydrocarbons was blown down under nitrogen gas to a 60 µL volume, of which 2 µL were injected and analyzed.

Cuticular hydrocarbon extract processing

Extracts were analyzed using electron impact-mass spectrometry (70 eV) on an Agilent 5975 C mass selective detector interfaced to an Agilent 7890A gas chromatograph fitted with a DB-5 column (30-m×0.32-mm i.d., Agilent Technologies). Two µL of each sample were injected at 325˚C in splitless mode using helium as a carrier gas, with a flow rate of 54.8 mL/min, and the following temperature program: 100 ˚C hold for 1 min, ramp of 15 ˚C/min to 200 ˚C, and then a 2nd ramp of 2 ˚C/min to 325 ˚C with a hold at 325˚C for 10 min, for a total run time of 80.167 minutes. Each resulting chromatogram was first automatically integrated using Chemstation vE.02.00 (Agilent Technologies), and then manually integrated using ACDC Labs (Advanced Chemistry Development) to ensure consistent

integration of smaller peaks. The identity of each compound was verified using both library comparisons and also by manual comparison of the mass spectra diagnostic ions and calculation of Kovats indices (Katritzky et al. 2000).

Behavioral observations

Approximately 50 ants of each species were collected directly from their nests using an aspirator and kept separate from the other species in vials (*Cr. levior*) or Fluon-coated boxes (*Ca. femoratus*). Only actively moving and undamaged ants were used in assays. All behavioral assays were 1 to 1 individual interactions in neutral arenas; we used small (5 cm x 5 cm) covered petri dishes for the *Cr. levior* x *Cr. levior* and the *Cr. levior* x *Ca. femoratus* assays, and 15 cm x 15 cm Fluon coated glass bowls for the *Ca. femoratus* x *Ca. femoratus* assays. Each assay dish was cleaned with soapy water and hexane, and air dried between trials to remove any chemical cues from previous ants.

All observations were for 3 minutes, and were only used in analysis if both ants made antennal contact with the other ant. Assays were performed blind to the source colony of the interacting ants. All interactions and their approximate duration were noted by transcribing observations of the following behaviors: presence/absence of trophallaxis, mandible flares, biting, spatulate sting extrusion (*Crematogaster*), defensive spraying (*Camponotus*), prolonged fighting, antennal boxing, and active running away. An overall behavioral score was assigned at the time of observation (0= amicable, 1= neutral, 2=mandible flare, 3=biting, 4=sting extrusion or spraying, 5=prolonged fighting). A second observer verified the transcribed interactions by watching a subset of the same interactions, and by reading all of the transcribed interactions and assigning an independent aggression score. Any inconsistent observations (ie: when the two observers were not in agreement) were excluded from the analysis (n=33).

Colony combinations and behavioral pairings

We did both nestmate (two ants from the same nest) and non-nesmate (each ant from a different nest) comparisons, and both conspecific (*Cr. levior* x *Cr. levior* n= 211, and *Ca. femoratus* x *Ca. femoratus* n=214), and heterospecific comparisons (*Cr. levior* x *Ca. femoratus*, n=188) for both the nestmate and non-nestmate pairings. We aimed for a minimum of 60 assays for each colony pairing with 10 nestmate and 10 non-nestmate assays for each species combination. For the non-nestmate *Cr. levior* x *Ca. femoratus* comparisons, we did 5 comparisons of each type (ie: five comparisons with *Cr. levior* nest 1 x *Ca. femoratus* nest 2, and five with *Cr. levior* nest 2 x *Ca. femoratus* nest 1). The final dataset consisted of a total of 613 observations.

Statistical analysis for chemical data

All chromatogram peaks eluting after a retention time of 15 minutes (>C20 backbone length) were included in the analysis. We included only compounds with >1% total abundance for at least one colony, but noted 'trace' compounds found in amounts <1% of the total profile for all colonies. Cross-chromatogram peak identity was confirmed by comparing retention times and the mass spectra. Both the presence/absence of peaks and the relative proportion of each peak within a

chromatogram were used for analysis. First, using the presence/absence data for all peaks, we compared the profiles using principle component analysis (PCA). Next, we compared the relative proportion data for all peaks of the same pooled profiles using nonmetric multidimensional scaling (NMDS). Since results for both analyses were similar, only the NMDS results are shown in the figures.

Statistical analysis for behavioral data

For our analysis, we used the presence/absence of aggression as our categorical response variable, using both a definition of aggression as any score 2-5, and a more conservative measure of aggression (presence of aggression only for scores 3-5). We used both measures because a behavioral score of 2 corresponds only to 'mandible flare', which is more ambiguous than biting (score of 3) or stinging (score of 4). The results were always comparable, so we are only presenting results from a definition of aggression as 2-5, but other analyses (with aggression scores 3-5) can be found in Appendix 1. For all assays if one ant showed aggression, we considered there to be 'presence of aggression' in that interaction. However, for the *Cr. levior* x *Ca. femoratus* interactions, we were able to determine whether one or both ants showed aggression, so we also analyzed the behavior of each species separately for the heterospecific assays.

We used generalized linear mixed models (GLMMs) with a binomial error distribution and a logit link function with observation category (nestmates vs non-nestmate) as a fixed effect and chemotype combination (within vs between chemotype), and colony pair combination (#1-10) as random effects. We used likelihood ratio tests with reduced models to assess effect significances. Since there was an effect of colony pair number in some subsets of the data, indicating that certain colony pairs showed different aggression levels than other colony pairs, we did a matched pairs t-test on the proportion of aggressive interactions towards nestmates and non-nestmates to confirm the direction of behavioral trends. Each analysis was repeated separately for each of the three species combinations (conspecific for *Cr. levior*, conspecific for *Ca. femoratus* and heterospecific), and for the two categories of behavioral scoring (2-5, or 3-5=aggression). We used R v 2.14.0 for all statistical analysis (R Development Core Team 2011).

RESULTS

Cuticular hydrocarbons

Surprisingly, we consistently recovered two distinct *Cr. levior* chemotypes, henceforth designated *Cr. levior* Type A and *Cr. levior* Type B (Fig 1 a,b). Within each nest, however, there was only one *Cr. levior* chemotype (confirmed by analysis of individual chromatograms, data not shown). None of the nest cuticular hydrocarbon profiles appeared to be intermediate between *Cr. levior* Type A and *Cr. levior* Type B. Of the 20 colonies, 7 were of Type A, and 13 were of Type B. Ants from these two chemotypes were behaviorally and morphologically indistinguishable in the field. Examination by a taxonomic expert on *Crematogaster* who was blind to chemotype confirmed the lack of morphological differentiation between *Cr. levior* chemotypes

(J. Longino, personal communication). The *Cr. levior* chemotypes overlapped in geographic distribution (Fig 2), with one very distant nest (200 km away from main population, not shown in Fig 2) sharing an almost identical CHC profile to the main population *Cr. levior* Type B. No obvious topographical or landscape feature isolated the two chemotypes, and they appeared to occur sympatrically and sometimes very close together (<10 m between colonies of Type A and Type B, as verified by a sampling of other colonies not used in this study).

Across the three types of hydrocarbon profiles found in the parabiotic nests (two *Cr. levior* types and one *Ca. femoratus* type), there was a total of 78 different identifiable compounds, with some co-eluting for a total of 45 resolvable peaks. In general, *Ca. femoratus* compounds were of longer chain length than either *Cr. levior* type (Fig 1c), and within the range observed previously for *Ca. femoratus* (Menzel and Schmitt 2012). The profiles of *Ca. femoratus* and *Cr. levior* contained very few shared compounds (Table 2). Of the 45 peaks, only 2 compounds were shared amongst *Ca. femoratus* and *Cr. levior* Type B and no compounds were shared between *Ca. femoratus* and *Cr. levior* Type A. were found in more than trace amounts (>1% of total profile, and for >1 colony), and were shared only amongst *Ca. femoratus* and *Cr. levior* Type B. The two *Cr. levior* chemotypes shared only 4 compounds in more than trace amounts.

When analyzed both qualitatively and quantitatively, the *Cr. levior* and *Ca. femoratus* profiles clustered separately from one another (Fig 3). The *Cr. levior* Type A and *Cr. levior* Type B profiles were consistently different. In contrast, all *Ca. femoratus* possessed the same qualitative chemotype, regardless of whether they shared a nest with Cr. Type A or Cr. Type B (Fig 1 c). This result was consistent when the analyses were repeated using only the *Ca. femoratus* profiles, and when including trace compounds (results not shown).

Conspecific recognition behavior

In total, there were three between-type (*Cr. levior* Type A by *Cr. levior* Type B) colony pairs, two within-*Cr. levior* Type-A colony pairs, and five within-*Cr. levior* Type-B colony pairs. All colony pair comparisons were independent (ie: no colony was used twice). We were unaware of any chemotype differences at the time of the behavioral sampling, and only had colony pairings of all three combinations (axa, axb, bxb) by chance.

Crematogaster levior

There was a significant effect of observation category (whether nestmate or non-nestmate, $\chi_{3,4}=60.2$, $dF_{2,3}= 1$, $p<0.001$,), with colony pair and chemotype combination explaining 11.9% and 37.9% of the variance respectively. In all 10 of the nest combinations, *Cr. levior* ants displayed more aggression toward non-nestmates than toward nestmates (Fig 4 a) (one tail paired t-test, t-ratio= -11.48, dF=9, p=<0.01). This aggression was often typified by biting and fighting which often resulted in the death of one or both ants. This pattern of aggression was consistent whether the non-nestmate was of the same or of a different chemotype, but more aggression was displayed in pairings of non-nestmate ants of different

chemotypes. Trophallaxis was rarely observed between non-nestmates (only 3/30 observed trophallaxes), and never between ants of the different chemotypes.

Camponotus femoratus

There was a significant effect of observation category (whether nestmate or non-nestmate, $\chi_{3,4}=4.2$, $dF_{2,3}= 1$, $p=0.04$), with no effect of chemotype combination (0% of variance), but with a significant effect of colony pair as a random effect ($\chi_{3,4}=10.6$, $dF_{2,3}= 1$, $p=0.001$, 43.9% of the variance). Of the 10 nest combinations, only 7 displayed significantly more aggression toward non-nestmates than toward nestmates (Fig 4 b5), but there was an overall trend of more aggression toward non-nestmates (one tail paired t-test, t-ratio=-2.23, df=9, p=0.02). The conspecific *Ca. femoratus* aggression was less often fatal than conspecific *Cr. levior* comparisons, with ants often engaging in antennal boxing instead of direct biting and fighting conflicts. The boxing behavior was almost exclusively seen in the non-nestmate comparisons, and only in 3 of the 10 colony pairs, none of which were within-*Cr. levior* Type A comparisons. Aside from this occurrence of antennal boxing, there was no pattern related to the chemotype of the *Cr. levior* nesting partner (ie: *Ca. femoratus* is not more aggressive to non-nestmates that cohabitate with a different *Cr. levior* chemotype).

Heterospecific recognition behavior

Cr. levior and *Ca. femoratus*

In general, there was less aggression observed in the heterospecific assays than in the conspecific assays. When aggression was analyzed without separating the behavior of the ants by species, there was no significant effect of observation category (nestmate or non-nestmate) ($\chi_{3,4}=1.4$, $dF_{2,3}= 1$, $p=0.24$). We found that there was higher aggression displayed towards non-nestmates , but this effect was not significant at the 0.05 level for *Cr. levior* ($\chi_{3,4}=3.1$, $dF_{2,3}= 1$, $p=0.08$ with 38.7% variance due to colony pair) or *Ca. femoratus* ($\chi_{3,4}=1.1$ $dF_{2,3}= 1$, $p=0.28$, with 18.4% variance due to colony pair). Chemotype was not explanatory for either dataset (0% of variance). However, when considered significant at the 0.10 level, there was a difference in aggression of *Cr. levior*, especially when accounting for variation in colony pairs (Fig 6, one tail paired t-test, t-ratio 1.77, df=9, p=0.06). For *Ca. femoratus*, this result was not significant (one tail paired t-test, t-ratio 0.26, df=9, p= 0.40), but the trend was for increased aggression to non-nestmates (Fig 7). This pattern was consistent regardless of whether the interaction was between or within chemotypes. In a few cases, extreme heterospecific aggression (resulting in the death of the *Cr. levior* ant) was observed, sometimes amongst nestmates. Heterospecific trophallaxis was only observed twice, with one occurrence between non-nestmates.

DISCUSSION

Ants typically have species-specific cuticular hydrocarbon profiles, with mostly quantitative differences between nests within a species. The surprising

result of finding two very distinct *Cr. levior* chemotypes within parabiotic nests is unexpected because the two chemotypes were morphologically, behaviorally, and ecologically indistinguishable. It is highly probable that more cryptic types exist within the parabiotic *Crematogaster limata* complex (Longino 2003), and we recommend using cuticular hydrocarbons as an informative phenotype to investigate possible cryptic differences within this group. Genetic analyses of these different chemotypes may provide insights in to the extent of gene flow and genetic differentiation between chemotypes, but, at present, thus far we continue to regard both chemotypes as the species *Cr. levior*.

We found that *Cr. levior* and *Ca. femoratus* shared very few chemical cues, despite their nest-sharing lifestyle. This was also unexpected because other ants are known to actively acquire CHCs through social interactions with other ants (Bagnères et al. 1991, Sledge et al. 2001), as well as passively from the nesting material (Bos et al. 2011), physical contacts (Meer and Wojcik 1982), and food sources (Liang and Silverman 2000). This lack of chemical cue homogenization contrasts with the shared chemical cues in other multi-species social systems, such as socially parasitized mixed nests (Lenoir et al. 2001) and artificially mixed nests (Errard and Hefetz 1997). However, our results are consistent with findings from other socially symbiotic compound nests (see Table 1) (Espelie and Hermann 1988, Errard et al. 1992, 2003, Lenoir et al. 1997, 2001, Orivel et al. 1997, Martin et al. 2007, Menzel et al. 2008a, 2009) in which the brood of the two species are kept physically separated, supporting the idea that mixed brood rearing facilitates chemical cue transfer. In artificially mixed nests, the degree of heterospecific chemical similarity scales with social interaction (Errard et al. 2005). In these cases, ants only acquire heterospecific compounds through social interaction, and cannot synthesize hydrocarbons de-novo to match their heterospecific nestmates (Vienne et al. 1995). Given that the parabiotic ants in our study share nest space, immediate environmental conditions, and food sources, our findings suggest that non-environmental effects, such as social interaction, are required for chemical integration of social individuals.

Despite a lack of chemical cue homogenization, we found evidence that ants may recognize their heterospecific nestmates. Both species were more aggressive toward heterospecific non-nestmates and than nestmates, with a more evident effect amongst *Cr. levior* ants. In NE Amazonia, recognition behavior has been studied in only one other parabiotic system: *Odontomachus mayi* and *Crematogaster limata parabiotica* (synonym for *Cr. carinata*) (Orivel et al. 1997). These studies showed that ants attacked non-nestmates of the other parabiotic species, but tolerated heterospecific nestmates (Orivel et al. 1997). Our findings are consistent with this evidence, but we recommend caution before concluding that heterospecific nestmate recognition occurs amongst all socially symbiotic ants. In SE Asia, parabiotic ants could only distinguish amongst heterospecifics of common and foreign chemotypes (Menzel et al. 2008a, 2008b, 2009), not specifically amongst nestmates. In all cases, some degree of heterospecific recognition seems to be a consistent difference between parabiotic and xenobiotic associations.

In the chemotype recognition of parabiotic ants of SE Asia, the dual chemotype species was the larger of the two ants (*Camponotus*)(Menzel et al.

2008a), in contrast to our system, in which the smaller *Crematogaster* has two chemotypes. Although we ensured in all observations that both species made antennal contact with the other, our assays highlight size-specific perceptual constraints because, despite being in close proximity to one another, *Ca. femoratus* (>1cm in length) would frequently walk over its *Cr. levior* testing partner (2-3 mm) without hesitation. Indeed, size difference is a proposed mechanism for successful commensal compound nesting between *Pyramica* and *Platythyrea* (Yéo et al. 2006). Size differences have also been suggested as a mechanism to reduce foraging competition between the parabiotic species (Swain 1980, Vantaux et al. 2007). The workers of the inquiline parasite *Acromyrmex insinuator* are also smaller than that of their sister-species host, which may help them escape heterospecific aggression (Lambardi et al. 2006). Thus, there may be size-specific constraints on chemical cue perception, with size differences allowing the smaller *Cr. levior* to go undetected by the larger *Ca. femoratus*. This may explain why we found no significant evidence of heterospecific nestmate recognition by *Ca. femoratus*.

In our parabiotic system, and in previously studied parabiotic systems, the two species share few chemical cues but maintain some ability to recognize their heterospecific nestmates (Orivel et al. 1997, Menzel et al. 2008a, 2008b, 2009). Heterospecific recognition is consistent with the hypothesis that the recognition template used to assess nest- membership is learned and not self-referent, since it can expand to include another species phenotype (Bos and D'Ettorre 2012). Is there a cost to having an expanded recognition template? There is no evidence that either parabiotic species has lost the ability for conspecific recognition, which might happen if the recognition template was more generalized (Bos and D'Ettorre 2012). Both ant species involved in parabiotic social symbiosis maintain effective conspecific nestmate recognition behaviors, aggressively rejecting non-nestmates.

Ants distinguish amongst nestmates and non-nestmates by detecting both quantitative and qualitative differences in chemical phenotype (Brandt et al. 2009a, Guerrieri et al. 2009, Bos and D'Ettorre 2012, Van Wilgenburg et al. 2012), but species are genetically constrained to produce only a limited range of compound classes and sizes (Blomquist et al. 1987). The informational constraints on the chemical phenotype can be overcome by producing not only differing quantities of compounds, but also a broader range of compounds. We hypothesize that the long-chain unsaturated hydrocarbons of *Ca. femoratus*, found amongst several species of heterospecifically tolerated *Camponotus* ants (Menzel and Schmitt 2012), may be evolutionary novelties that facilitate heterospecific relationships, perhaps by opening new chemical information channels to communicate identity. Because both *Cr. levior* and *Ca. femoratus* were able to distinguish nestmates and non- nestmates of *Ca. femoratus* using only these unusual compounds, it is unlikely they are chemically insignificant or imperceptible (Menzel and Schmitt 2012). The repeated evolution of these unusually long-chain alkenes and dienes suggest that they are a key trait that facilitates heterospecific tolerance (Menzel and Schmitt 2012).

In sum, we have found evidence that in parabiotic nests, 1) the recognition cues are not mimicked as in socially parasitized nests, but instead both species maintain a species-specific odor, 2) there is evidence of potential heterospecific nestmate recognition, and 3) conspecific recognition is maintained despite mutual

heterospecific tolerance. The intact recognition systems in parabiotic social symbioses are distinct in many ways from the manipulated recognition systems in socially parasitized nests.

How is the cooperation of these social symbionts maintained in the face of potential exploiters? Cooperation is maintained through a combination of factors, such as compound nesting and novel chemicals, which minimize the heterospecific interference in nestmate recognition processes. In particular, the social symbiosis has likely been facilitated by each species using unique informational channels, by producing a different range of chemical cues and maintaining species-specific colony odors. This may be one reason that these social symbioses are so rare amongst social insects, and yet so common amongst *Camponotus* ants (Menzel et al. 2010) who have repeatedly evolved both heterospecific tolerance and unusual long-chain hydrocarbons (Menzel and Schmitt 2012). Interference in the recognition system, a potential cost of living together, is minimized by such chemical innovations. There is certainly more work to be done investigating the frequency and distribution of such communication innovations, and their potential links to cooperative behavior. The maintenance of reliable recognition systems in these socially symbiotic nests supports the theory that parabioses are different from social parasitisms (Menzel and Blüthgen 2010). Our findings suggest that selection to maintain reliability in conspecific recognition can potentially constrain the evolution of interspecific cooperation.

TABLES

Table 1. Summary of published work on chemical phenotypes, and heterospecific and conspecific nestmate recognition behaviors in naturally occurring parabiotic and xenobiotic compound nests.

	Species	Cues shared between species? How many/total?	Range of HC chain lengths	Aggression to heterospecific non-nestmates?	Aggression to conspecific non-nestmates?	References
Parabiosis (compound nests in possible mutualism)						
1	*Camponotus femoratus*	Few (2/8)	37-45	No	Yes	(Emery and Tsutsui 2013)
	Crematogaster levior Type A	None (0/16)	25-33	Yes	Yes	
	Crematogaster levior Type B	Few (2/15)	29-41			
2	*Camponotus rufifemur* black	Few (3/46)	21-49	No	Yes	Menzel et al. 2008, 2009
	Camponotus rufifemur red	Few (2/17)	24-41			
	Crematogaster modigliani	Few (5/28)	35-40	Yes, but only to foreign chemotype	Yes	
3	*Odontomachus mayi*	Few		Yes	Yes	Orivel et al. 1997
	Crematoagster carinata	Few		Yes	Yes	
Xenobiosis (compound nests in likely parasitism)						
4	*Solenopsis gayi*	Yes (14/21)	23-28	No	No	Errard et al. 2003
	Camponotus morosus	Yes (15/36)	23-31	No	Yes	
5	*Formicoxenus provancheri*	Yes (60/60)	21-37	No	Low	Lenoir et al. 1997
	Myrmica incompleta	Yes (60/65)	21-37	No	Yes	Errard et al. 1992
6	*Formicoxenus quebecensis*	Yes (38/40)	23-31			Lenoir et al. 1997
	Myrmica alaskensis	Yes (38/62)	21-37			
7	*Formicoxenus nitidulus*	Yes (17/24, 19/28)	25-35	No	No	Martin et al. 2007
	Formica rufa	Yes (14/22)	23-35	No	Yes	
	Formica lugubris	Yes (19/35)	23-33			
8	*Pseudomyrmex ferrugineus*	Yes (8/8, 81.8%)	~25-31	No		Espelie et al. 1988
	Parachartegus aztecus	Yes (8/8, 94.3%)	~25-31	No		

Table 2. Summary of average abundance of the 34 most abundant peaks from the pooled profiles of parabiotic ants.

Compound number	Retention time (min)	Class of compound	Compound ID	*Ca. femoratus* *(n= 20)*	*Cr. levior* Type A (n= 7)	*Cr. levior* Type B (n=13)
3	19.72	straight	C25	*trace*	9.66 +/- 6.37	
7	25.14	straight	C27		4.45 +/- 3.01	*trace*
8	26.09	single methyl	mix of 11me and 13me C27	*trace*	4.25 +/- 2.06	
12	28.94	single methyl	mix of 10me, 11me, 12me, 13me, 14me and 15me C28		1.15 +/- 0.5	
13	30.16	unsaturated	C29 alkene		10.8 +/- 4.42	
14	**30.98**	**straight**	**C29**		**6.35 +/- 2.01**	**3.95 +/- 1.4**
15	31.92	single methyl	mix of 7me, 9me, 11me, 13me, and 15me C29	*trace*	23.11 +/- 6.73	*trace*
16	32.45	multimethyl	11,13 dime C29		1.17 +/- 2.71	
17	32.69	unsaturated	C30 alkene		1.38 +/- 2.12	
18	32.69	single methyl	5meC29		1.55 +/- 2.65	*trace*
19	32.75	multimethyl	11, 13 dime C30		1.17 +/- 2.01	
21	**36.06**	**unsaturated**	**C31 alkene**		**16.18 +/- 9.8**	**2.13 +/- 0.89**
22	36.82	straight	C31		*trace*	1.6 +/- 0.63
23	37.99	single methyl	mix of 7me, 9me, 11me, 13me, 15me, and 17me C31		5.31 +/- 4.71	*trace*
24	38.37	multimethyl	unidentified		3.74 +/- 2.09	
25	41.39	unsaturated	C33 diene			16.5 +/- 12.93
26	**41.98**	**unsaturated**	**C33 alkene**		**3.99 +/- 3.22**	**18.81 +/- 4.33**
27	42.56	straight	C33			1.54 +/- 1.25
28	43.37	single methyl	mix of 11me, 13me, 15me, and 17me C33	*trace*	*trace*	4.76 +/- 1.4
29	**44.03**	**multimethyl**	**unidentified**		**1.55 +/- 3.31**	**1.7 +/- 0.5**
30	47.14	unsaturated	C35 alkene and diene	*trace*	*trace*	21.15 +/- 5.53
31	48.88	single methyl	mix of 11me, 13me, 15me, and 17me C35	*trace*	*trace*	3.96 +/- 1.15
32	49.44	multimethyl	13,15,20,22 tetrame C34			1.33 +/- 1.17
33	52.1	unsaturated	C37 diene	*trace*		8.08 +/- 6.22
34	52.82	unsaturated	C37 alkene	*trace*		2.95 +/- 2.65
35	53.97	single methyl	mix of 10me, 13me, 15me, 17me, and 19me C37	1.89 +/- 0.77		*trace*
38	54.78	multimethyl	13, 15 dime C38	18.13 +/- 4.72	*trace*	*trace*
39	**57.74**	**unsaturated**	**C39 alkene and diene**	**15.43 +/- 3.26**		**6.18 +/- 7.56**
40	58.91	single methyl	mix of 11me, 13me, 15me, 17me, and 19me C39	1.27 +/- 0.66		*trace*
41	59.6	multimethyl	unidentified	5.85 +/- 1.24		*trace*
42	61.72	straight	C40	1.68 +/- 4.8		
43	**62.51**	**unsaturated**	**C41 diene**	**41.76 +/- 6.76**	*trace*	**1.36 +/- 2.7**
44	66.2	unsaturated	C43 diene	1.84 +/- 2.69		*trace*
45	71.19	unsaturated	C45 diene	*trace*		

**The percentages indicate the average relative proportion of each compound, as determined by the area under the peak in the chromatogram, +/- SD. The bolded compounds are highlighted in Figure 1. The word 'trace' indicates compounds only found in trace amounts (<1% of all profiles).*

FIGURES

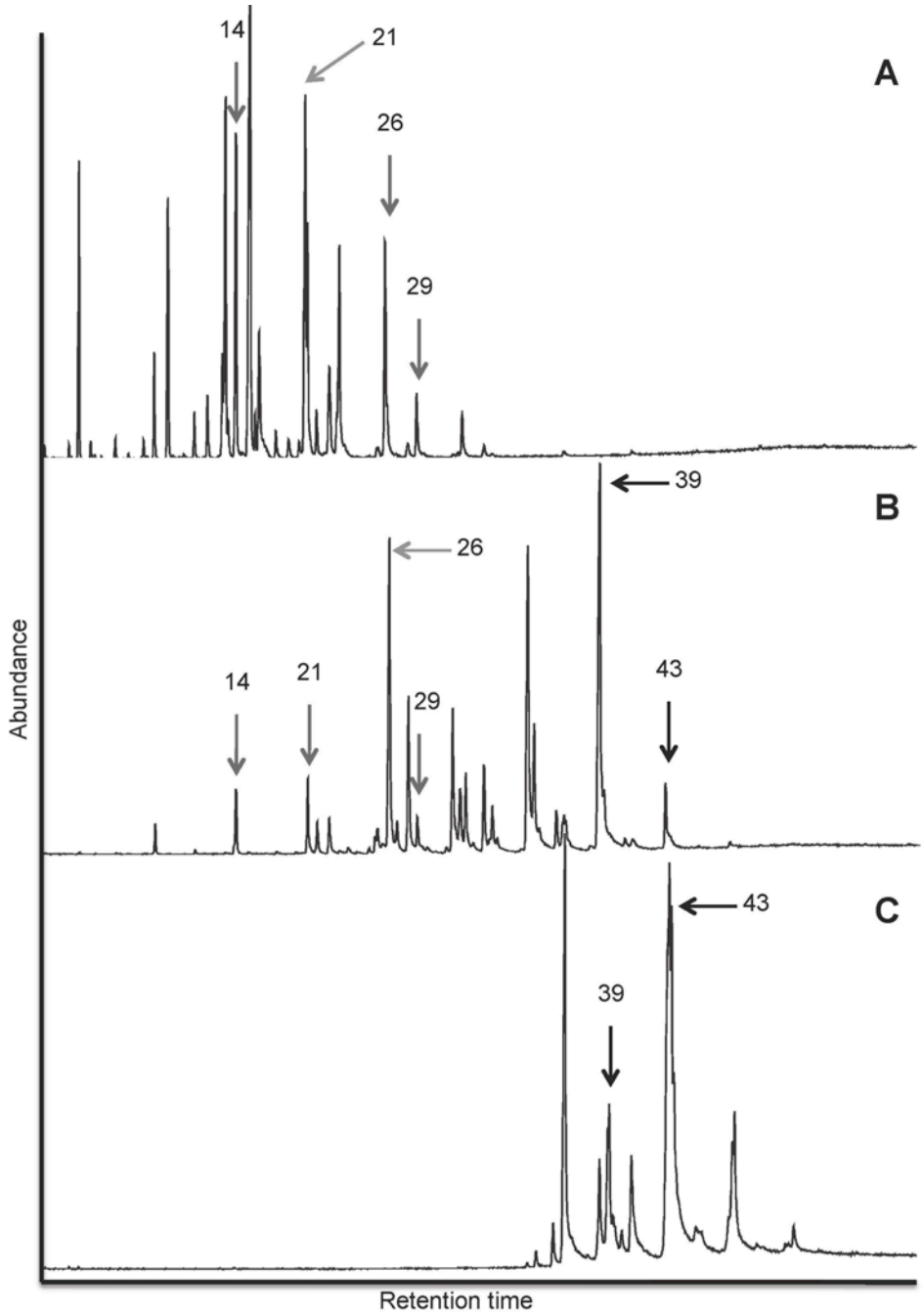


Figure 1. Representative chromatograms of the three chemotypes involved in the parabiotic nests: a) *Cr. levior* Type A, b) *Cr. levior* Type B, c) *Ca. femoratus*. Each peak represents a different hydrocarbon compound, as confirmed by spectral analysis. Compounds shared between species are shown by the arrows, with grey arrows showing peaks shared by only *Cr. levior* Type A and *Cr. levior* Type B, and black arrows being compounds shared between *Cr. levior* Type B and *Ca. femoratus*. Peak numbers refer to compound numbers in Table 2.

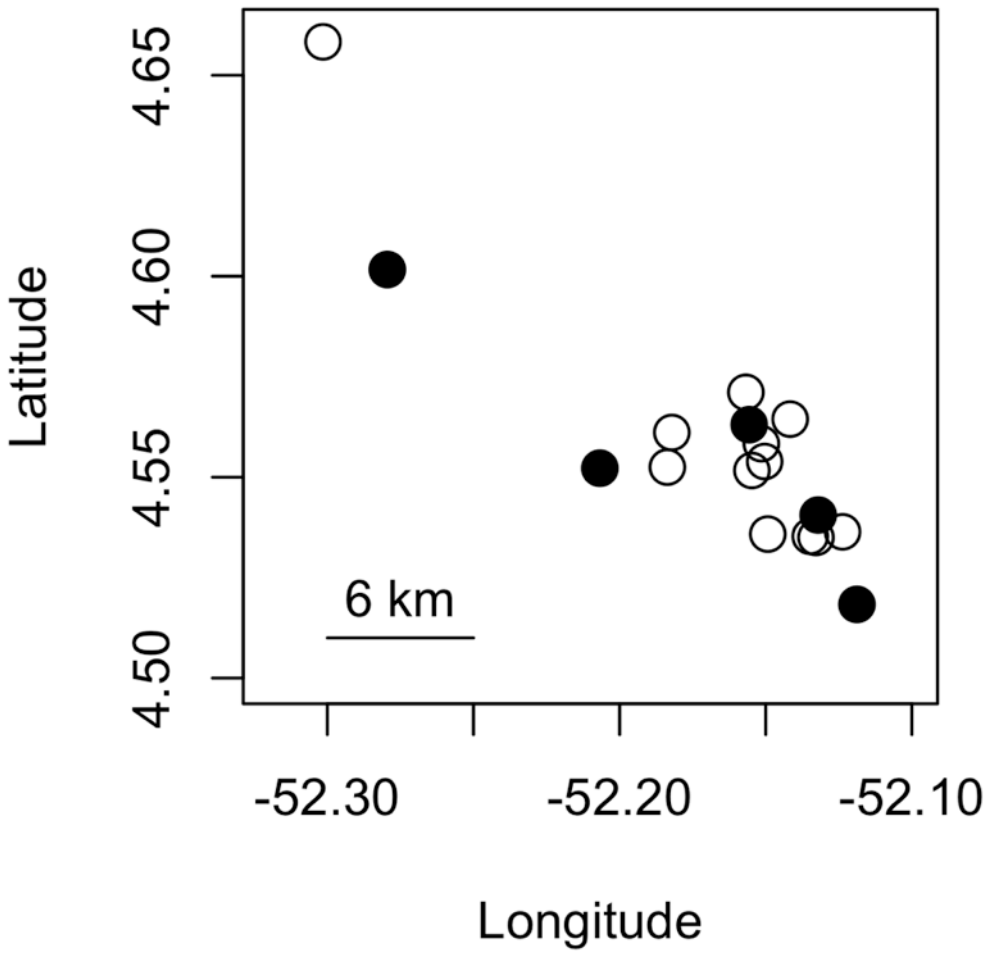


Figure 2. Map of nest locations showing 18 of the nests used in this study. Black circles represent *Cr. levior* Type A, and white circles represent *Cr. levior* Type B nests.

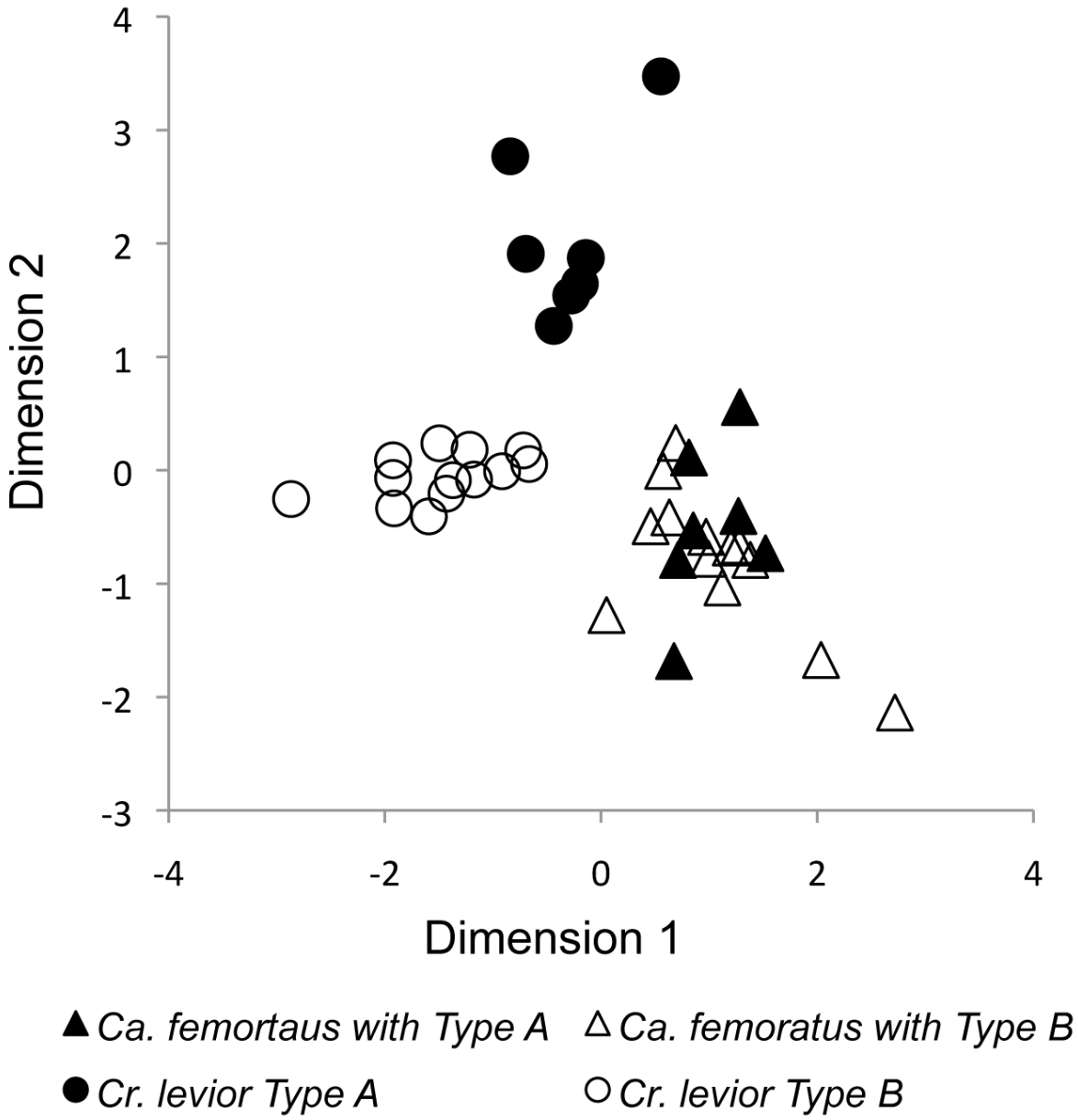


Figure 3. Nonmetric multidimensional scaling plot of the relative proportions of 45 cuticular hydrocarbon peaks from pooled ant profiles. Each shape represents the pooled profile of 30 *Cr. levior* or 5 *Ca. femoratus* worker ants of a different colony (n=20 colonies).

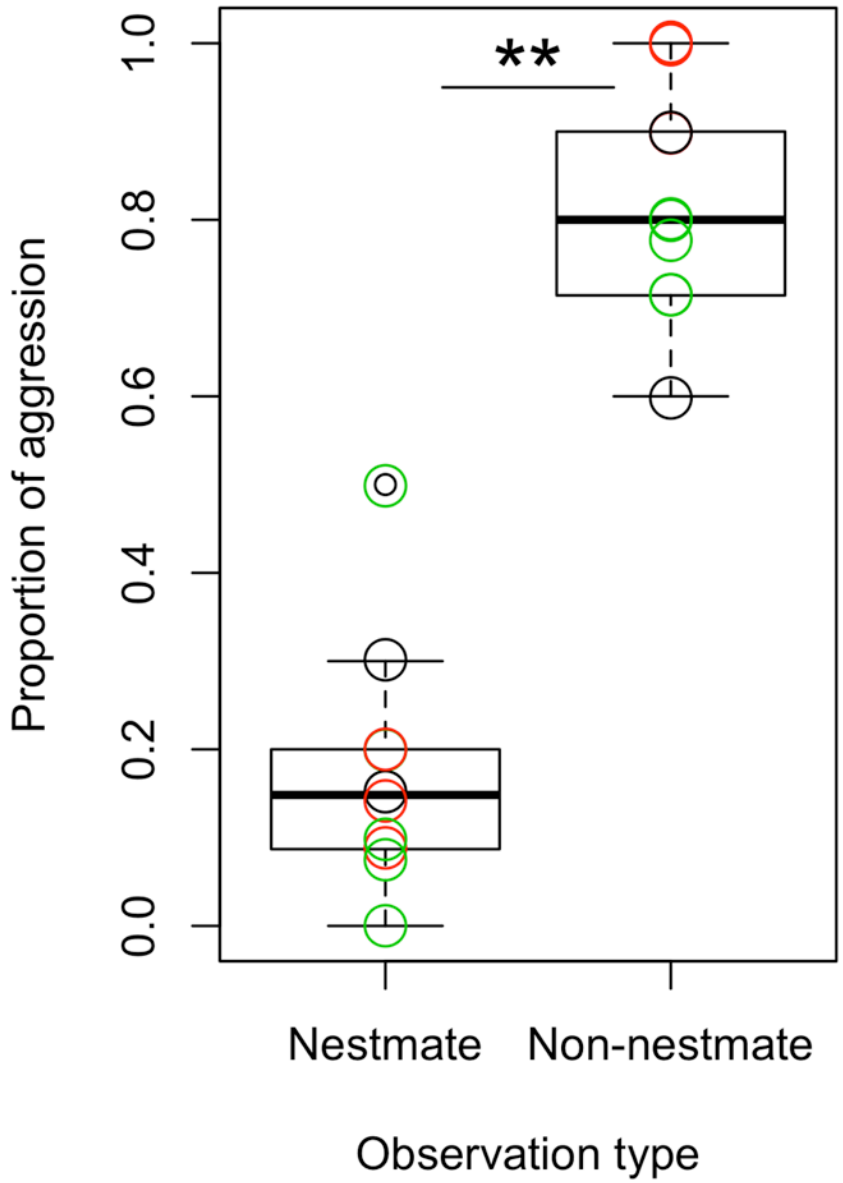


Figure 4. Proportion of aggressive behavior by *Cr. levior* in behavioral assays with nestmate and non-nestmate *Cr. levior* ants. The boxplot shows the mean +/- standard deviation. Black circles are for colony pairs considered within *Cr. levior* Type A combinations (n=2), green circles are for within *Cr. levior* Type B combinations (n=5), and red circles are for between *Cr. levior* Type A and *Cr. levior* Type B combinations (n=3). The asterisks indicates there was significantly more aggression to non-nestmates ($p<0.05$).

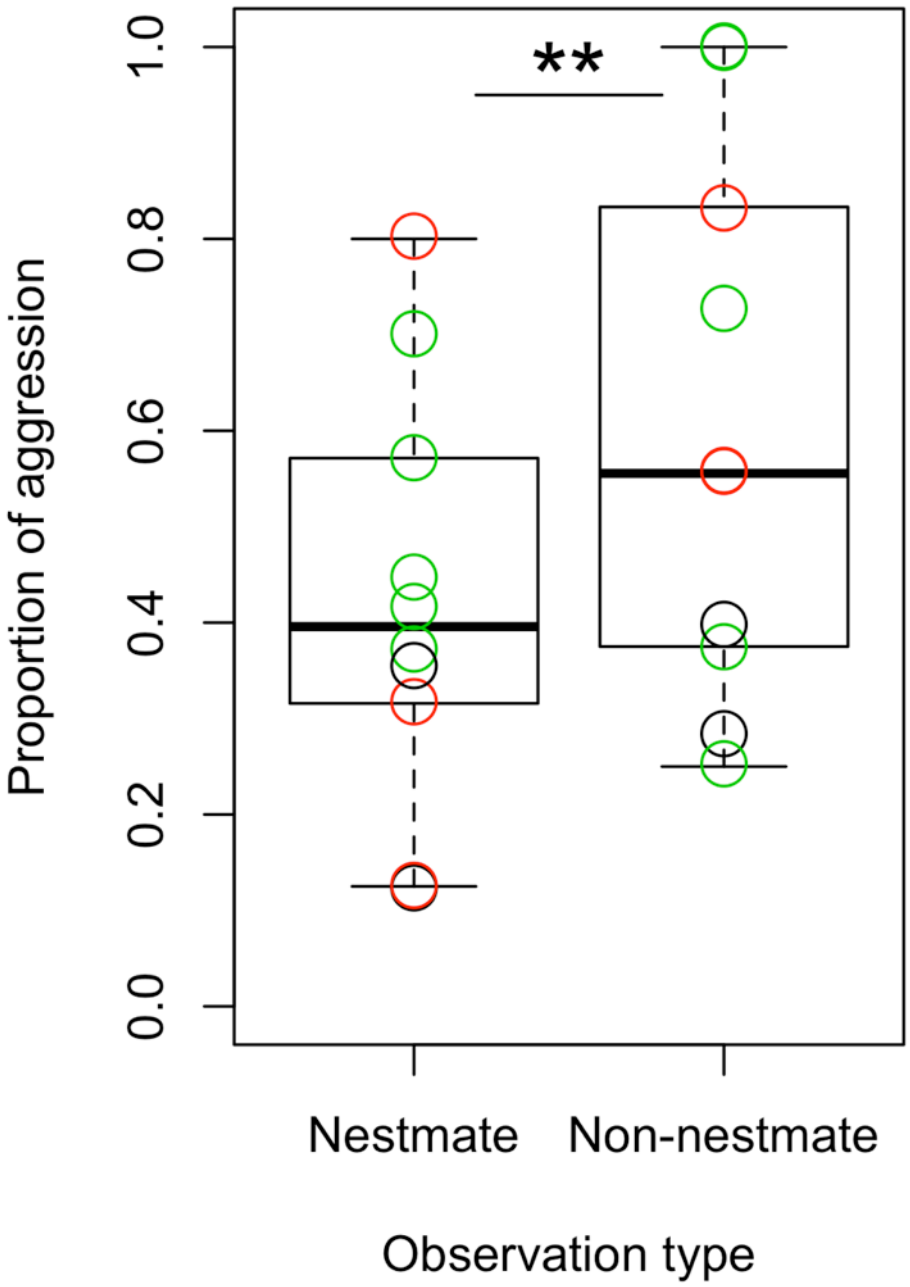


Figure 5. Proportion of aggressive behavior by *Ca. femoratus* in behavioral assays with nestmate and non-nestmate *Ca. femoratus* ants. Although *Ca. femoratus* was only of one chemotype, coloring is as in Figure 4 for consistency. The asterisks indicates there was significantly more aggression to non-nestmates ($p<0.05$).

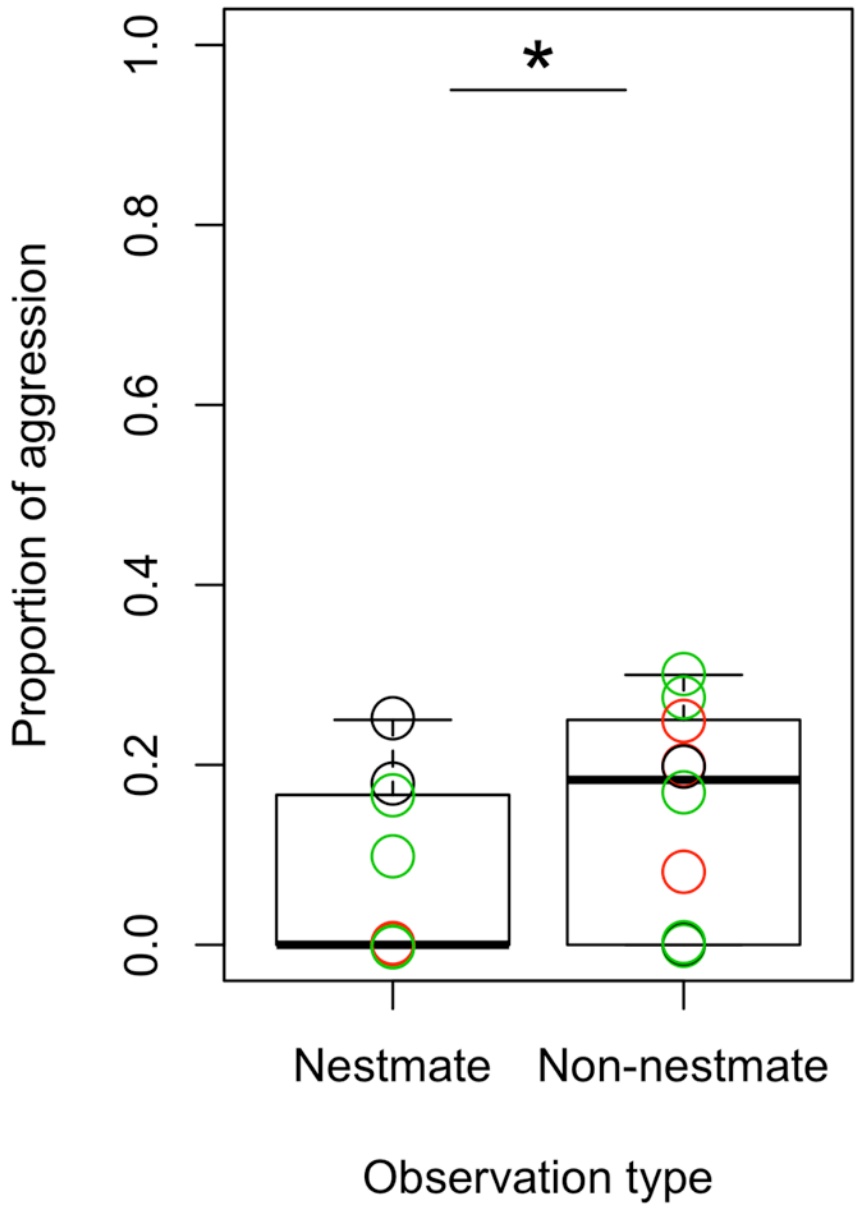


Figure 6. Proportion of aggressive behavior by *Cr. levior* in behavioral assays with nestmate and non-nestmate *Ca. femoratus* ants. Black circles are within *Cr. levior* Type A, green circles are within *Cr. levior* Type B, and grey shapesred circles are for between *Cr. levior* Type A and *Cr. levior* Type B colony pairs. The asterisk indicates there was significantly more aggression to non-nestmates ($p<0.10$).

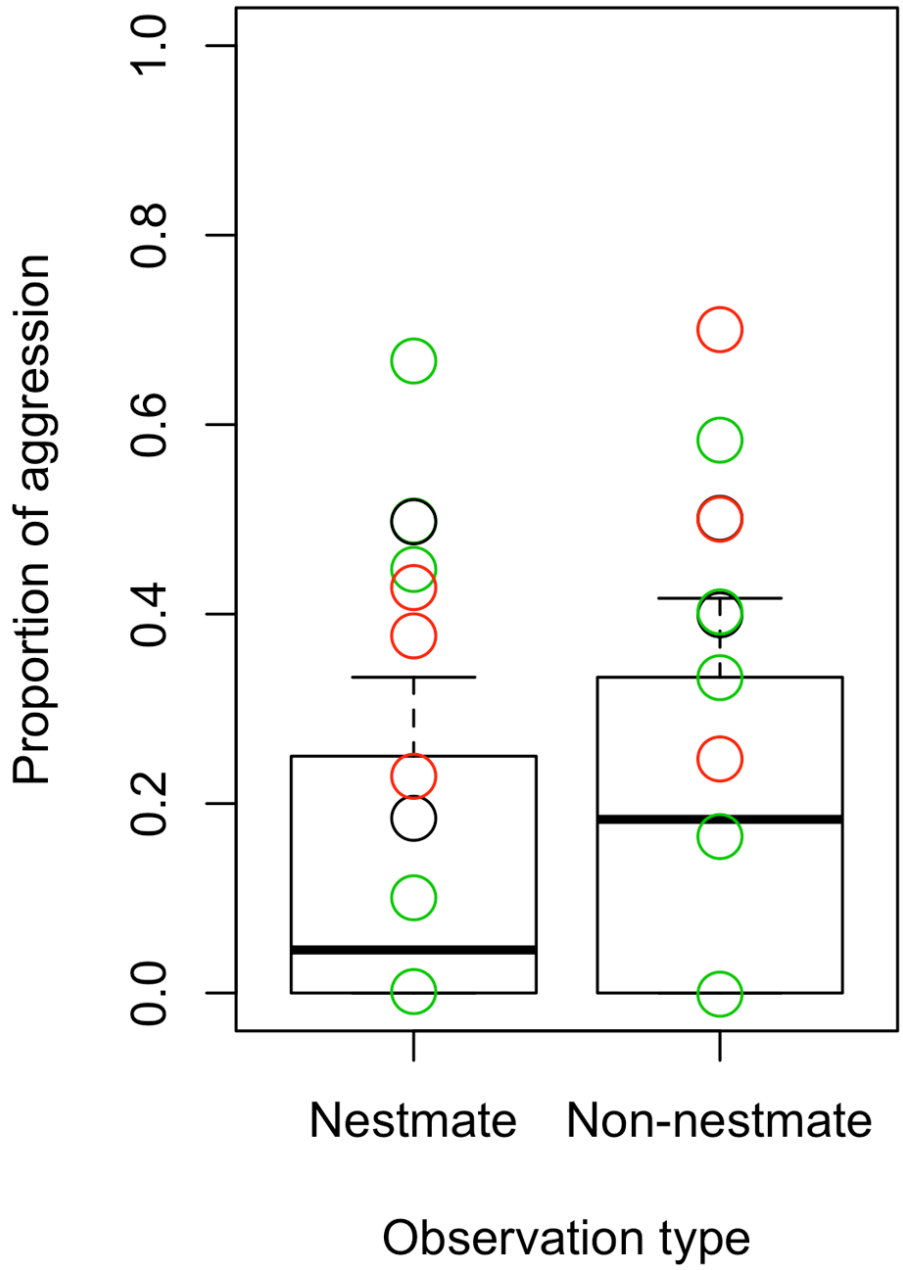


Figure 7. Proportion of aggressive behavior by *Ca. femoratus* in behavioral assays with nestmate and non-nestmate *Cr. levior* ants. Black circles are within *Cr. levior* Type A, green circles are within *Cr. levior* Type B, and grey shapesred circles are for between *Cr. levior* Type A and *Cr. levior* Type B colony pairs. There was not a significant difference in aggression towards non-nestmates.

CHAPTER 2

Social parasites are more likely to share chemical cues than social mutualists

ABSTRACT

Chemical recognition systems are central for maintaining the unity of social insect nests. Colonies form a common chemical signature, called the gestalt odor, which is used to distinguish colony members and non-members. This chemical integration is usually achieved actively through social interactions such as trophallaxis, or passively such as through exposure to common nest material. When colonies are infiltrated by social parasites, the intruder typically uses some form of chemical mimicry. However, it is not always clear how this chemical mimicry is accomplished. Here, we used a three-species nesting symbiosis to test the differences in chemical integration of mutualistic (parabiotic) and parasitic ant species. We find that the parasite (*Solenopsis picea*) obtains chemical cues from both of the two parabiotic host ant species. However, the two parabiotic ants (*Crematogaster levior* and *Camponotus femoratus*) maintain species-specific cues, and do not acquire compounds from the other species. Our findings suggest that there is a fundamental difference in how social mutualists and social parasites use chemicals to integrate themselves into colonies, and point to the importance of social interaction over environmental factors for the formation of the gestalt odor.

INTRODUCTION

Societies depend on reliable recognition systems to communicate membership (Mateo 2002). Membership decisions are based on comparison of identification labels to a recognition template (Lehmann and Perrin 2002, Bos and D'Ettorre 2012, Sturgis and Gordon 2012). Non-members are rejected because their identification labels do not match that of the group, and, non-members can infiltrate a group by acquiring a member-like identification label (Errard and Hefetz 1997, Sledge et al. 2001, Fürst et al. 2012). Most societies consist of related individuals, and so kin-informative cues are often used as recognition labels. In small societies members may be individually remembered and identified (Tibbetts 2002, Sheehan and Tibbetts 2011), but larger societies need more generalized recognition systems, with both generalized labels and recognition templates. In these large groups, identification labels are not individualized, but rather shared between group members. Thus in order to understand the complicated dynamics of society membership, it is crucial to understand how identification labels are acquired and shared.

Amongst the social insects, chemical cues are commonly used as identification labels (Richard and Hunt 2013). These chemical cues are homogenized such that individuals within a colony acquire a common chemical odor (Lenoir 2002, Richard and Hunt 2013). This 'gestalt' odor is then used as the recognition label for the colony (Breed et al. 1985). However, it is unclear how the colony gestalt odor is achieved, and whether the mechanisms for chemical sharing varies between systems. The chemical cues, typically cuticular hydrocarbons, are genetically constrained (Beye et al. 1997), but new cues can be acquired through

social interaction (Vienne et al. 1995), or a shared environmental factor, such as shared nest space (Bos et al. 2011) or diet (Liang and Silverman 2000).

Multi-species nests are a great system to test hypotheses about the formation of identification labels because, unlike single species nests, the relative contributions of genetics and environment can be separated. Cross-fostering experiments are one approach for testing the mechanisms of recognition (Mateo and Holmes 2004). In these experiments, individuals from unrelated genetic backgrounds are mixed in a common environment. This set-up allows us to differentiate the relative genetic and environmental contributions to the recognition system. For example, ants reared in mixed-species nests will readily reject their genetic siblings from a foreign nest (Carlin and Hölldobler 1983). They also become more chemically similar to their heterospecific nestmates (Hoffmann et al. 1992). This means that the recognition template is learned, and that the recognition cues can be shared amongst unrelated individuals.

This flexible recognition system facilitates the formation of large social groups (Brandt et al. 2009a, Drescher et al. 2010), but also makes the group vulnerable to intruders. These social parasites infiltrate a host colony by usurping the recognition system. For example, in the nests of the social parasite *Polyergus rufescens* and their *Formica* hosts, the parasite forms a chemical odor similar to its host, which facilitates social integration (D'Ettorre and Mondy 2002). The parasite has some genetically coded local adaptation to different host species, but still maintains the ability to switch host by social mechanisms, notably abdominal trophallaxis, which allows for plastic chemical camouflage (D'Ettorre and Mondy 2002). Other social parasite systems show a similar combination of genetic adaptation and environmentally or socially mediated chemical plasticity (Lenoir et al. 1997, 2001, Turillazzi et al. 2000, Sledge et al. 2001, Brandt et al. 2005, Bauer 2009, Bauer et al. 2009).

Most research on odor-sharing within multi-species nests has involved either artificial systems (Carlin and Hölldobler 1983, Breed et al. 1985, Hoffmann et al. 1992, Vienne et al. 1992, Errard and Vienne 1994, Errard and Hefetz 1997, Errard et al. 2005), or systems involving parasites (Sledge et al. 2001, Lenoir et al. 2001, Lorenzi and Bagnères 2002, D'Ettorre et al. 2004, Akino and Tsuneoka 2012), with the notable exception of the parabiotic nesting symbioses. Parabiosis is a nesting symbiosis in which two species, generally of different subfamilies, share foraging trails and a common nest, although the brood are kept in separate chambers in what is termed compound nesting (Mann 1912, Wheeler 1921, Weber 1943). These parabiotic associations are typically thought to be mutualisms (Menzel and Blüthgen 2010), with both species benefiting from the relationship. This is unusual given that most nest sharing symbioses in ants are social parasitisms, with one species benefiting at the expense of the other (Buschinger 2009).

Although parabioses are not well studied, examples from SE Asia and the Neotropics provide some insights into how recognition cues are shared. In the SE Asian parabioses, *Camponotus rufifemur* and *Crematogaster modiglianii* share fewer chemical cues than in a typical social parasitism (Menzel et al. 2008b, 2009). Interspecifically tolerated *Camponotus* have unusual cuticular compounds which may be required for the parabiotic relationship (Menzel and Schmitt 2012). In a

different Neotropical parabiosis, there is little cue overlap between *Crematogaster* ants and their multiple partner species (Orivel et al. 1997, Emery and Tsutsui 2013). This contrasts with xenobiotic compound nests, where the host and parasite species share many chemical cues (Chapter 1, Table 1). These findings suggest there may be different chemical integration techniques for social parasites and social mutualists.

Here, we investigate the chemical cues in the parabiotic nests of *Crematogaster levior* and *Camponotus femoratus*, which are found throughout ant-gardens in the Amazonian rainforest. These mixed-species nests occur from French Guiana to Peru, and the association is likely obligate for *Cr. levior* (Longino 2003). Within these mixed species nests, there is also a third player, *Solenopsis picea* which is a social parasite. This 'thief ant' steals food and brood of the parabiotic species (Pacheco 2008). Dissection of the ant-gardens confirms that each of the three species maintains their brood in separate chambers (Emery, personal observation).

How are the colony odors formed in these compound multi-species colonies? Are these patterns different for the social parasite and social mutualists? This is the first study to characterize the chemical ecology within a single nest harboring three species, and therefor the first opportunity to directly contrast the chemical integration of a social parasite and social mutualist.

METHODS

Study sites

Parabiotic nests of *Ca. femoratus, Cr. levior and S. picea* were observed in the lowland Amazonian rainforest of French Guiana near the village of Kaw (3° 30' 43. 19" N, 30° 15' 54"W). Collections were made during two trips in February-March 2010, and in July 2010. These ants nest in arboreal carton ant-gardens, and form polydomous colonies that span several nest units. Colony boundaries were assumed when there was a minimum 100-meter distance between nest units. Only easily accessible nests were used for this experiment (all <3 meters from the ground). The location of each nest was recorded using GPS. Workers were collected alive at the nest using an aspirator and freeze killed before chemical extraction.

Cuticular hydrocarbon extraction

For each parabiotic nest (n=27) we collected 10 individual worker ants per species. Of these collected samples, we analyzed a minimum of 3 samples per nest for *Cr. levior* and 7 samples per nest for *Ca. femoratus*. Both pooled and individual *S. picea* samples were collected from 7 colonies. Each ant or group of ants was submerged in 50-200 µL of hexane for 10 minutes. The ants were removed and stored in 95% EtOH, and the hexane was evaporated for transport back to UCB. Each CHC sample was re-eluted in 200 µL of hexane, and filtered through a 1 cm hexane-rinsed silica column to remove impurities and polar compounds. To ensure complete sample recovery from the column, each was further rinsed with 300 µL of hexane.

The 500 µL sample was blown down under nitrogen gas to concentrate the sample. When necessary, extracts were further concentrated and re-injected for

better resolution of the chromatogram or mass spectra. For *Ca. femoratus* individuals, samples were concentrated to a 60 µL volume with 2 µL injected for analysis. The entire sample was analyzed for individual *Cr. levior* workers. Due to their small size, the individual *S. picea* samples were injected directly without processing through silica. A subset of pooled *S. picea* samples was filtered through silica as above to confirm similarity of the processed and unprocessed extracts. Only profiles of individual workers were used in the analysis, although profiles of groups of workers (3 workers for *Ca. femoratus* and 30 workers for *Cr. levior)* were used to confirm peak identities because the spectra were sometimes of low quality for individual ants.

Cuticular hydrocarbon extract processing

Extracts were analyzed using electron impact-mass spectrometry (70 eV) on an Agilent 5975 C mass selective detector interfaced to an Agilent 7890A gas chromatograph fitted with a DB-5 column (30-m×0.32-mm i.d., Agilent Technologies). Two µL of each sample were injected at 325˚C in splitless mode using helium as a carrier gas, with a flow rate of 54.8 mL/min, and the following temperature program: 100 ˚C hold for 1 min, ramp of 15 ˚C/min to 200 ˚C, and then a 2nd ramp of 2 ˚C/min to 325 ˚C with a hold at 325˚C for 10 min, for a total run time of 80.167 minutes. Each resulting chromatogram was first automatically integrated using Chemstation vE.02.00 (Agilent Technologies), and then manually integrated using ACDC Labs (Advanced Chemistry Development) to ensure consistent integration of smaller peaks. The identity of each compound was verified using both library comparisons and also by manual comparison of the mass spectra diagnostic ions and calculation of Kovats retention indices (Katritzky et al. 2000).

Statistical analysis for chemical data

All peaks eluting after a retention time of 15 minutes (>C20 backbone length) with a relative proportion of at least 0.5% of the total peak area were considered. A total of 43 cuticular hydrocarbon peaks were included in the analysis. The data matrix consisted of relative proportion data for these 43 peaks for a total of 297 individual profiles, with 177 *Ca. femoratus*, 112 *Cr. levior* and 8 *S. picea* samples.

We considered species (*Ca. femoratus*, *Cr. levior* and *S. picea*) as one set of groupings to compare. Because there were two *Cr. levior* chemotypes (Emery and Tsutsui 2013), we also considered individuals from colonies with each *Cr. levior* chemotype (eg: *Ca. femoratus* nesting with *Cr. levior* Type A, and *Ca. femoratus* nesting with *Cr. levior* type B) as another set of groupings to compare. To confirm the chemotype comparisons, we also repeated the analysis with three smaller matrices consisting only of samples from 1) *Ca. femoratus*, 2) *Cr. levior* Type A and 3) *Cr. levior* Type B. We also tested for an effect of colony identity using these single-species data matrices, to test the gestalt hypothesis that ants within a same colony are more chemically similar than ants from different colonies.

We compared the relative proportion data matrices using two-dimensional plots made by nonmetric multidimensional scaling. We set a maximum of 100 iterations to reach the minimum stress plateau for two dimensions. Each species, chemotype, and colony was compared with a permutation MANOVA (10 000

permutations) on the Bray-Curtis dissimilarity matrix, as implemented with the ADONIS function in the *vegan* and *ecodist* packages in R (Dixon and Dixon 2003, Goslee and Urban 2007). We used R v 2.14.0 for all statistical analysis (R Development Core Team 2011).

RESULTS

Consistent with our previously published data (Emery and Tsutsui 2013), we found two chemotypes of *Cr. levior* in our population in French Guiana (hereafter referred to as *Cr. levior* Type A and *Cr. levior* Type B) (Figure 1a,b). We found 11 nests of *Cr. levior* Type A and 16 nests of *Cr. levior* Type B, with both chemotypes occurring sympatrically throughout the population. Within each nest, we only found a single *Cr. levior* chemotype.

We found that *Ca. femoratus* had a distinct profile of mainly long-chained branched alkenes and alkadienes, and the two parabiotic species shared few chemical cues (Figure 1c). However, the social parasite *S. picea*, shared some chemical cues with both of the parabiotic species, including the most abundant chemical compounds from *Ca. femoratus* (Figure 1d, Table 1).

The NMDS required 10 iterations to obtain a minimal stress of 0.15, which represents a good fit of the scaling to our data (R^2=0.93). Species, chemotype and colony were all significant factors in the perMANOVA, indicating that individual profiles cluster according to each of these factors (Table 2). However, for the full dataset the most important of these factors was species (R^2 =0.58), followed by the species-chemotype interaction (R^2=0.11). Consistent with the gestalt hypothesis, ants from the same nest cluster together, indicating a higher chemical similarity within nests than between nests after accounting for species and chemotype (ADONIS, F=4.11, R^2=0.06, p<0.01, with the terms species, chemotype and colony added sequentially).

When analyzed separately in a single-species matrix, the *Cr. levior* individual profiles were different for each chemotype (ADONIS, $F_{1,114}$=342.12, R^2=0.60, p<0.01) and colony ($F_{25, 114}$=5.71, R^2=0.25, p<0.01) . The same was true of the *Ca. femoratus* profiles, however the factor of *Cr. levior* chemotype was not as strongly correlated with the distance matrix ($F_{1,176}$=5.54, R^2=0.02, p<0.01) as colony ($F_{24,176}$= 6.22, R^2=0.49, p<0.01). Visually it was clear that *Ca. femoratus* profiles did not cluster according to their associations with *Cr. levior* chemotypes (Figure 2). All *Ca. femoratus* ants cluster together regardless of whether they were nesting with *Cr. levior* Type A or *Cr. levior* Type B, indicating that all *Ca. femoratus* ants have a single similar chemotype across the population. The low number of *S. picea* samples did not allow for this single-species comparison to include colony level comparisons, but there was no clustering according to *Cr. levior* chemotype ($F_{1,7}$=1.31, R^2=0.18, p=0.29).

DISCUSSION

Our investigation of ants living in Amazonian ant-gardens is the first to examine the chemical phenotypes of both mutualistic and parasitic species living together in a single nest. It is uncommon to find three species sharing a nest, since most nest-sharing symbioses are between a single social parasite species and a single host species. Our results highlight important differences between the chemical integration patterns of social mutualists and social parasites.

We recovered two sympatric chemical types of *Cr. levior* in our population in French Guiana. The chemical profiles of individual *Cr. levior* ants confirm the patterns previously observed from pooled samples (Emery and Tsutsui 2013), and support a chemical separation between ants of *Cr. levior* Type A and *Cr. levior* Type B. These morphologically and ecologically indistinguishable ants have unexpectedly divergent chemotypes. For example, a chemical comparison of sympatric *Formica* ants found that each species has a distinct set of cuticular hydrocarbons, but all species had a similar homologous series of alkanes, alkenes and monomethylalkanes (Martin et al. 2008b). None of the compounds common to both *Cr. levior* Type A and Type B are part of such a homologous series, and each *Cr. levior* chemotype shares more in common with other *Crematogaster* species than the opposite *Cr. levior* chemotype (Emery and Tsutsui, in prep).

Despite these differences, within each single-chemotype nest, *Cr. levior* ants form a common intraspecific odor. Likewise, *Ca. femoratus* ants from the same nest are more chemically similar to one another than to *Ca. femoratus* ants from other nests. The greater chemical similarity within nests than between nests supports the gestalt hypothesis that ants form a common colony odor. However, this common odor does not extend to the interspecific colony level. Unlike the shared single-species colony odors, *Ca. femoratus* does not share chemical cues with their parabiotic *Cr. levior* nesting partners. There was no separation of *Ca. femoratus* ants that were found nesting with different *Cr. levior* chemotypes.

This result is surprising, because in parabiotic nests there should be many opportunities for ants to passively acquire chemical cues. Chemical cues could be passively acquired from a shared diet (Liang and Silverman 2000), or the common nesting environment (Bos et al. 2011). In controlled experiments, *Camponotus aethiops* ants acquired HCs from nest soil of foreign colonies (Bos et al. 2011). In these soil-transfer experiments, the CHC profiles of ants from two colonies converged after only 24 hours of exposure to soil from the foreign chemotype (Bos et al. 2011). Likewise, the social parasite wasp *Polistes sulcifer* spreads its hydrocarbons on the nest material to encourage its acceptance into a host colony (Sledge et al. 2001). Host wasps pick up these hydrocarbons from the shared nest and incorporate them into their own profiles and recognition templates (Turillazzi et al. 2000). Why then do *Cr. levior* and *Ca. femoratus* share few chemical cues, despite sharing a nest?

In our parabiotic ants, many of the nest's chambers (both with and without brood) host only one species of ant. It is possible the segregated nest results in few opportunities for passive transfer of hydrocarbons. Notably, certain classes of compounds, such as straight chain alkanes, were transferred less frequently to the

soil than methyl-branched alkanes in these experiments (Sledge et al. 2001, Bos et al. 2011). This suggests some compound-specific limitations in terms of passive transfer. It is possible that the methyl-branched alkenes and dienes of *Ca. femoratus* may be less likely to transfer to the surrounding environment, perhaps because they may be less volatile (Menzel and Schmitt 2011). The unique properties of these unusually long hydrocarbons would be a fruitful avenue for future research.

In addition to passive transfer, ants may acquire heterospecific cues through active biosynthesis and transfer to the cuticle (Vienne et al. 1995, van Zweden et al. 2010, Bos et al. 2011). Within species, ants may be able to genetically upregulate the biosynthesis of certain compounds to match the colony odor (Martin et al. 2012). However, in experiments with two species in artificially mixed nests, ants could not biosynthesize the compounds of the other species (Vienne et al. 1995). Genetic limitations likely play an important role in the ability to chemically integrate, a possible reason most social parasites are closely related to their hosts (Huang and Dornhaus 2008).

Nonetheless, in these artificial mixed nest experiments, the species had no evolutionary history of living together, which is not the case for our study system. Although the history and specificity of the parabiotic association is unclear, there are signs that interspecific tolerance has been a selective pressure for at least *Ca. femoratus* (Menzel and Schmitt 2012). The unusual methylbranched alkenes and alkadienes of these ants are only found on other interspecifically tolerated *Camponotus* species (Menzel and Schmitt 2012), and only amongst three genera of ants (Martin and Drijfhout 2009a). One of these ants, *Nothomyrmecia macrops*, is from a basal ant lineage, which suggests all ants have the basic genomic machinery to produce these compounds (Martin and Drijfhout 2009a). Given this possibility to evolve chemical mimicry by biosynthesis, the lack of an interspecific chemical gestalt suggests that unlike social parasitism, parabiosis does not select for a mixing of species cues.

A third way to mix chemical cues, in addition to passive transfer and biosynthesis, is through active transfer. Socially mediated transfer, particularly through trophallaxis (Vienne et al. 1995), helps accelerate the homogenization of chemical cues in single species nests (Lenoir et al. 2001b). Social parasites also use allogrooming and trophallaxis to mix cues with their hosts (Beeren 2011). In controlled pair-wise assays, we only observed three instances of interspecific trophallaxis, and it seemed unlikely that the small *Cr. levior* could solicit trophallaxis from the larger *Ca. femoratus*. The lack of an interspecific gestalt suggests that the parabiotic ants may interact much less than previously assumed, and highlights the importance of social interactions in the formation of a common colony odor.

To examine the role that social interactions may have in achieving chemical similarity, it is useful to contrast the mutualistic interaction of these two parabiotic species with patterns seen for the parasitic interaction with *S. picea*. As predicted, the social parasite appears to be more chemically integrated than the other ants. The social parasite shares more cues with both species than the mutualists share with one another, including the unusual long-chained hydrocarbons of *Ca. femoratus*.

However, despite having more cues in common with its nesting partners, *S. picea* is not chemically different when nesting with *Cr. levior* Type A or Type B, which suggests a limited chemical specificity. This finding supports the hypothesis that *S. picea* is a generalist social parasite (Pacheco 2008), and that it is limited in its integration ability by having multiple host species. For example, the social parasite *Harpagoxenus sublaevis* has two chemically dissimilar *Leptothorax* hosts (Bauer et al. 2009). This slave-making ant faces divergent selection pressures, and is unable to genetically match both hosts simultaneously. As a result, *H. sublaevis* genetically produces only some of the cues from one host, and uses social means to acquire the other host's cues (Bauer et al. 2009).

Dissection of the ant-garden nest confirms that the brood of all three species is kept in separate chambers. This compound nesting contrasts with the mixed nesting seen in many social parasites where the brood of the host and parasite ants co-occur in the same chamber (Lenoir et al. 2001a). This mixing of the brood facilitates chemical cue transfer by increasing contact between young ants. Due to the observed compound nesting, it is unlikely that *S. picea* has any increased opportunities for passively acquiring CHCs from the mixing of brood. However, with known habits of stealing food from its hosts, *S. picea* workers could be spending more time in other parts of the nest occupied by its host species, and thereby be more likely to passively acquire heterospecific chemical cues.

Could *S. picea* also be actively acquiring CHC cues? Previous isotopic work has confirmed that *S. picea* has a high δN^{15} ratio, indicating it is eating at a higher trophic level than expected due to its size (Davidson 2005). Such an effect could be due either to consumption of prey captured by its host parabiotic ants, or by consuming the parabiotic ants themselves. If the latter is happening in ant-garden nests, this could be a route through which *S. picea* acquires interspecific hydrocarbons. We recommend tracing a stable isotope pulse through the ant-garden to confirm the trophic relationships between the ants.

In conclusion, we have found multiple chemotypes in the multi-species Amazonian ant-garden nests. Unlike single species nests or socially parasitized nests where a common chemical odor is achieved, none of the three species in these nests achieve an interspecific gestalt odor. We hypothesize that the compound nesting of our three species, in addition to their large size differences, limits heterospecific chemical cue sharing. However, the social parasite *S. picea* shares more cues in common with the other ants than *Cr. levior* and *Ca. femoratus* share with one another. Our findings highlight the importance of social interaction in the formation of the chemical gestalt, and we suggest using similar multi-species nests to further investigate the gestalt processes underlying chemical recognition systems.

TABLES

Table 1. Summary of average abundance of the 43 most abundant peaks from the individual chemical profiles of ants found in ant-garden nests. The percentages indicate the average relative proportion of each compound +/- SD. The bolded compounds show the 5 most abundant compounds on each ant's profile.

# in Figure	# in Chapter 1, Table 2	Retention time (min)	Class of compound	Compound ID	*Ca. femoratus*	*Cr. levior Type A*	*Cr. levior Type B*	*S. picea*
1	*1*	15.3	straight	C23		0.7 +/- 1.3		**11.3 +/- 11.9**
2		16.9	single methyl	3-me C23			0.1 +/- 0.3	4.7 +/- 2.8
3		17.2	straight	C24		0.3 +/- 1.3	1.4 +/- 5.9	1.9 +/- 2.2
4		19.1	unsaturated	unsat C25		0.3 +/- 1.3	0.2 +/- 0.6	**30.1 +/- 16.5**
5	3	19.9	straight	C25		**12.8 +/- 8.2**	0.4 +/- 1.9	**8.6 +/- 6.4**
6	*4*	20.6	single methyl	mix of 11me and 13me C25		0.4 +/- 1.7	0.1 +/- 0.3	2.1 +/- 1.5
7		21.8	single methyl	3-me C25				**8.7 +/- 5.8**
8	*5*	22.5	straight	C26		0.7 +/- 1.1	0.1 +/- 0.4	0.1 +/- 0.3
9		24.7	unsaturated	unsat C27			0.1 +/- 0.3	2.3 +/- 2.1
10	7	25.2	straight	C27		5.8 +/- 3.8	1.5 +/- 2.4	2.2 +/- 2
11	8	26.1	single methyl	mix of 11me and 13me C27		3.3 +/- 2.7		3.2 +/- 2.2
12		26.9	multimethyl	unidentified				0.9 +/- 1.2
13	*10*	27.3	single methyl	3-me C27		0.3 +/- 1	0.4 +/- 2.3	3.7 +/- 6.7
14	*11*	28.1	straight	C28		0.5 +/- 2.1	0.3 +/- 0.6	
15	12	29	single methyl	mix of 10me, 11me, 12me, 13me, 14me and 15me C28		0.2 +/- 0.6		
16	13	30.4	unsaturated	unsat C29		**12.1 +/- 8.6**	0.1 +/- 0.4	2.9 +/- 7.4
17	14	31	straight	C29		**8.7 +/- 8.5**	**7.6 +/- 7**	0.5 +/- 0.7
18	15	32.0	single methyl	mix of 7me, 9me, 11me, 13me, and 15me C29		**22.5 +/- 9.2**	0.1 +/- 0.3	0.4 +/- 0.8
19	16	32.4	multimethyl	11,13 dime C29		2 +/- 2.9	1 +/- 2	
20	17	32.8	unsaturated	unsat C30		1.5 +/- 2		
21		33.8		unidentified		0.3 +/- 0.8	0.2 +/- 0.6	
22	*20*	34.8	single methyl	mix of 12me, 13me, 14me, and 15me C30		0.4 +/- 0.8		
23	21	36.0	unsaturated	unsat C31		**15.5 +/- 10.5**	1.6 +/- 1.5	**7.1 +/- 17**
24	22	36.7	straight	C31	0.1 +/- 0.5	0.5 +/- 1.5	2.3 +/- 2	
25	23	37.9	single methyl	mix of 7me, 9me, 11me, 13me, 15me, and 17me C31		4.5 +/- 4.7	0.4 +/- 0.9	
26	24	38.3	multimethyl	unidentified		1.7 +/- 2.6	1.3 +/- 2.7	
27	25	41.1	unsaturated	C33 diene			**13.9 +/- 13.3**	
28	26	42.0	unsaturated	C33 alkene		2.6 +/- 3	**22.6 +/- 8.4**	
29	27	42.6	straight	C33			1.2 +/- 1.2	
30	28	43.4	single methyl	mix of 11me, 13me, 15me, and 17me C33	0.1 +/- 0.8		5.2 +/- 2.6	
31	29	44	multimethyl	unidentified		1 +/- 2.7	0.5 +/- 0.8	
32	30	47.0	unsaturated	unsat C35	0.1 +/- 0.6	0.2 +/- 0.6	**17.5 +/- 8**	
33	31	48.9	single methyl	mix of 11me, 13me, 15me, and 17me C35			3.8 +/- 2.2	
34	32	49.5	multimethyl	13,15,20,22 tetrame C34			0.4 +/- 0.8	
35	33 and 34	52.8	unsaturated	unsat C37	1 +/- 4.9		**8 +/- 6.4**	
36	35	53.7	single methyl	mix of 10me, 13me, 15me, 17me, and 19me C37	1.4 +/- 2		0.1 +/- 0.2	
37	38	54.9	multimethyl	13, 15 dime C38	**20.4 +/- 8.1**	0.4 +/- 1.6	1.3 +/- 2	5 +/- 7
38	39	57.4	unsaturated	unsat C39	**11.7 +/- 4.8**		4.7 +/- 7.1	2.4 +/- 4.5
39	40	58.3	single methyl	mix of 11me, 13me, 15me, 17me, and 19me C39	0.9 +/- 1.5		0.2 +/- 0.5	
40	41	59.9	multimethyl	unidentified	**4.8 +/- 2.7**	0.1 +/- 0.3	0.4 +/- 2.4	0.9 +/- 2.4
41	43	63.1	unsaturated	C41 diene	**50.3 +/- 9.8**	0.4 +/- 2	0.7 +/- 1.7	1 +/- 1.9
42	44	66.3	unsaturated	C43 diene	**7.8 +/- 3.3**			
43	45	71	unsaturated	C45 diene	0.1 +/- 0.3		0.1 +/- 0.4	

For comparison with Chapter 1:Table 2, which summarized the results from pooled profiles only, the matching compound numbers are shown with the compounds that were highlighted in Chapter 1:Figure 1 underlined.

Table 2. Summary of results from the permMANOVA using the function adonis. Factors were added sequentially in the order they are shown in the table. All the factors were significantly correlated with the distance matrix, although the R^2 values indicate that species and the species:chemotype interaction are the most important factors.

Factor	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Species	2	50.149	25.0745	460.9	0.57846	0.001	***
Cr. levior chemotype	1	6.578	6.5776	120.9	0.07587	0.001	***
Colony	25	5.591	0.2236	4.11	0.06449	0.001	***
Species:chemotype interaction	2	9.741	4.8707	89.53	0.11237	0.001	***
Residuals	269	14.635	0.0544	0.16881			
Total	299	86.693	1				

FIGURES

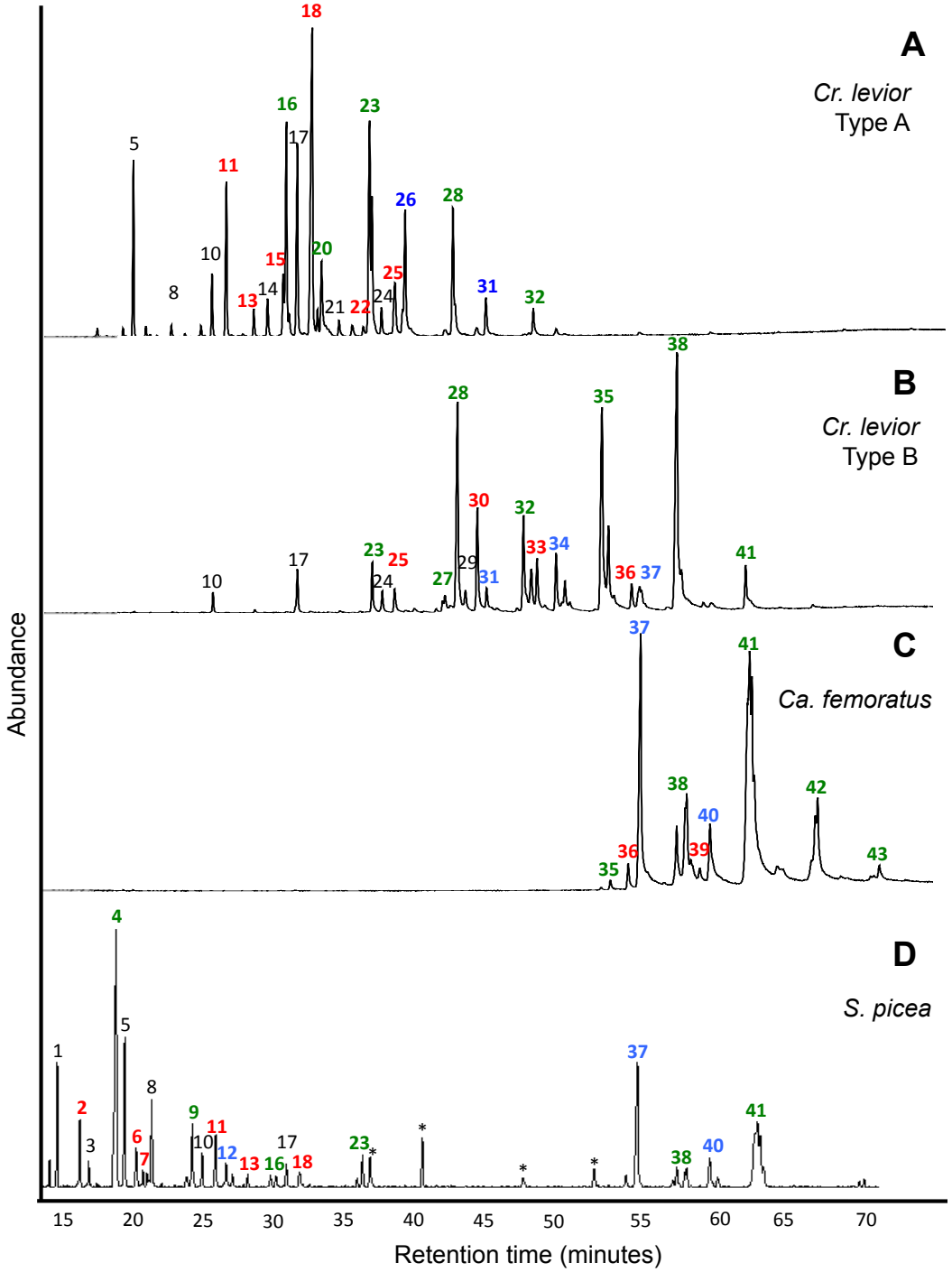


Figure 1. Representative chromatograms of the four chemotypes of ants found in ant-garden nests: a) *Cr. levior* Type A, b) *Cr. levior* Type B, c) *Ca. femoratus,* d) *S. picea*. Each peak represents a different hydrocarbon compound, as confirmed by spectral analysis. Each peak is numbered according to the compound numbers in Table 1, with colors according to compound class (black=straight chain alkane, red=single methyl branch, blue=multimethyl branch, green=alkene and alkadienes). Asterisks denote non-hydrocarbon peaks.

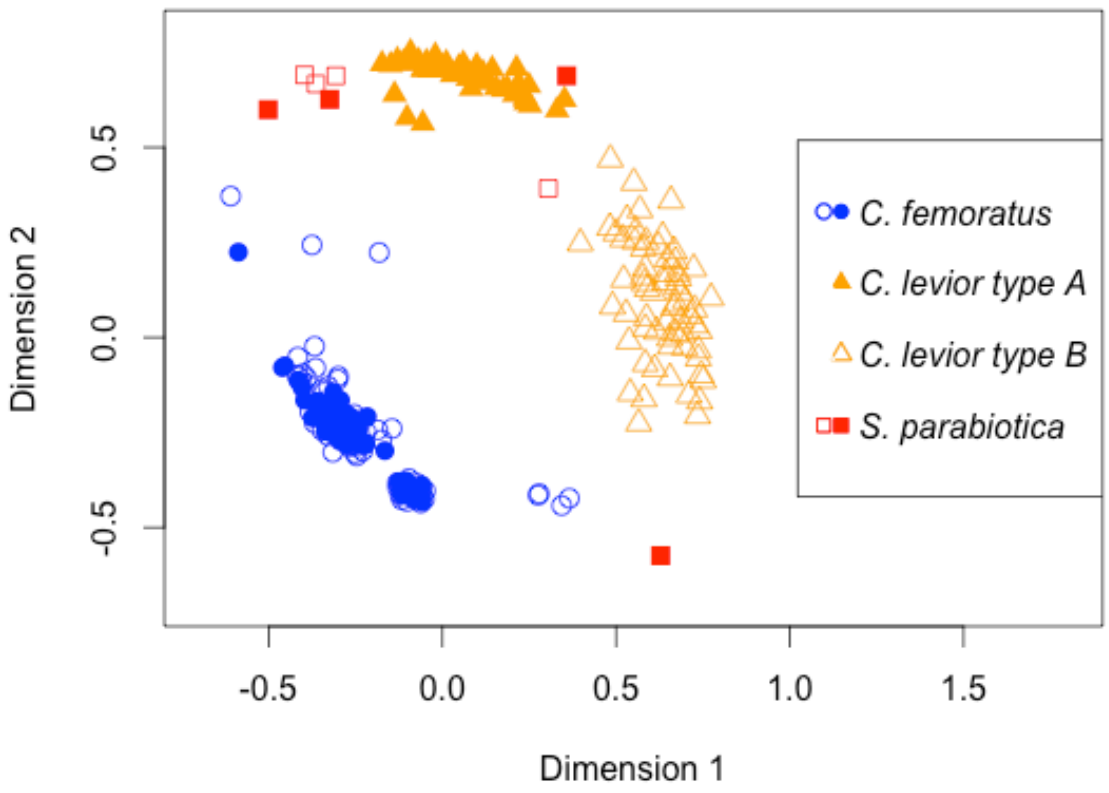


Figure 2. Nonmetric multidimensional scaling plot of the relative proportions of 43 cuticular hydrocarbon peaks from individual ant profiles. Each shape represents the profile of an individual ant. Filled shapes are ants found in colonies with *Cr. levior* Type A, and open shapes are ants found in colonies with *Cr. levior* Type B. The blue circles are *Ca. femoratus*, orange triangles are *Cr. levior*, and red squares are *S. picea*. A total of 297 profiles are shown here from a total of 27 different colonies. The relative positions of shapes to one another gives an indication of their chemical similarity, with more similar profiles clustering together.

CHAPTER 3

The genetic basis of chemical identity in parabiotic ants

ABSTRACT

Plastic traits are often mediated by a combination of genetic and environmental factors. The relative roles of genetics (nature) and environment (nurture) are particularly obscured when related individuals share common surroundings. In social insect colonies, sisters are reared together and achieve a common set of chemical cues, but it is not clear how nature and nurture interact to achieve this complex and plastic phenotype. Here we use a unique nesting symbiosis to look at the relative effects of genetics and a shared nest on ant chemotypes. Using the individual chemical profiles of *Crematogaster levior* and *Camponotus femoratus* ants, we compare these chemotypes to genotypic information from both nuclear microsatellite loci and mitochondrial co-1. For both species, there is a correlation between chemical phenotypes and genotypes. In *Ca. femoratus*, there are positive correlations between genetic distances and both chemical and geographic distances of colony pairs. The genetic basis for chemotype includes a correlation between some alleles and the proportion of straight-chain alkanes in *Ca. femoratus* profiles. Likewise, there are correlations between chemical phenotypes and genotypes of *Cr. levior* ants, with a strong genetic distinction between the two *Cr. levior* chemotypes. There is no geographic partitioning of either chemical or genetic differences, which supports the observation of sympatry of the *Cr. levior* Type A and Type B. We find correlations between several alleles and the proportion of most chemical compounds. Specifically, there appear to be opposing genetic trends for the alkane and methyl branched compounds that dominate *Cr. levior* Type A profiles, and the unsaturated alkenes and alkadienes that typify *Cr. levior* Type B profiles. Together this evidence supports the hypothesis that *Cr. levior* Type A and Type B are genetically distinct and potentially different cryptic species. In both parabiotic ants, there is a clear genetic basis for the cuticular hydrocarbon phenotype used in recognition.

INTRODUCTION

A central theme in the study of evolutionary biology is the relative contribution of environment and genetics to the phenotype. Although many traits are strictly genetically determined, some are plastic within the environmental context (Pigliucci 2001, Agrawal 2001, Alpert and Simms 2002, Miner et al. 2005, Johnson and Tricker 2010). Not only can multiple loci contribute to these phenotypes, but also environmental factors can directly and indirectly influence trait values. The gene and environment interaction is particularly important in determining the outcome for complex traits (Fordyce 2006, Callahan et al. 2008, Johnson and Tricker 2010, Molet et al. 2012).

Amongst the insects, cuticular hydrocarbons (CHCs) are a complex phenotype of central importance for survival. These waxy substances are found on the exoskeletons of most terrestrial arthropods, providing a water-proofing barrier that helps prevent desiccation and infection (Blomquist et al. 1987). These compounds have also been co-opted for use in communication amongst many

insects. For example, fruit flies use long chain cuticular hydrocarbons to identify mates of the correct species and sex (Ferveur 2005). Because of their importance in mate recognition, CHCs are under strong selection in *Drosophila* (Ferveur and Jallon 1996, Coyne et al. 1999, Takahashi et al. 2001).

Selection acts on phenotypes, but evolution can only occur if those phenotypes are heritable. Cuticular hydrocarbons can evolve because they are a phenotype largely under genetic control (Coyne et al. 1999, Dallerac et al. 2000). Hydrocarbons are synthesized in the oenocytes, which are secretory cells in the fat body near the dermal layer (Wigglesworth 1970). Fatty acyl-CoAs are produced by fatty acid synthetases, desaturated by desaturases, elongated into fatty acid chains by elongases and then decarboxylated into hydrocarbons by cytochrome-P450s (Blomquist 2010). Methyl branches are added through incorporation of amino acid derived CoAs during elongation (Chase et al. 1990). The identity and transcriptional regulation of these various classes of genes, along with the amino acids available for methyl branching, determine the types of hydrocarbons an organism can produce.

There are three main modifications that can alter the structure of CHC compounds. First, chain-length can be modified, ranging from very short chains of a couple carbons to compounds with a backbone of 48 or more carbons (Akino 2006, Menzel and Schmitt 2012). Second, functional groups, such as methyl branches, can be added in varying numbers and placements. Amongst insects, most methyl-branched compounds contain a single methyl branch with external and internal placements along the chain (Blomquist and Bagnères 2010). However, multi-methyl branched hydrocarbons are not uncommon and CHCs may contain up to 6 methyl branches (Fletcher et al. 2003). Third, double bonds can also be added in varying numbers and placements. Typically these unsaturated CHCs are alkenes and alkadienes, but trienes and tetraenes can also be produced (Howard and Blomquist 2005). The combination of chain length, methyl branch number and placement, and double bond number and placement results in hundreds of potential hydrocarbon compounds.

The ants are one group of insects that have developed a particular diversity of communicative uses for this rich array of possible CHCs. Although CHCs are a genetic product, they can be shared amongst individuals through social interactions or a shared environment (Weddle et al. 2012), which make them ideal for communication in these colonial insects. Cuticular hydrocarbons are used to identify reproductive individuals (Lommelen et al. 2006, Holman et al. 2010b, 2010a), job status (Wagner et al. 2001, Martin and Drijfhout 2009b), and colony membership (Martin et al. 2008a, Guerrieri et al. 2009, Van Wilgenburg et al. 2010). Some of these signaling purposes, such as fertility signaling, require honest signals that are unmodified by environmental influences (Heinze and d'Ettorre 2009, Zweden 2010, Holman 2012). Other processes, such as nestmate recognition, require the CHC cues to be mixed enough amongst nestmates to create a generalized recognition phenotype (van Zweden et al. 2010). These conflicting selection pressures make ant CHCs an interesting phenotype to examine from the nature versus nurture perspective.

To date, few studies have looked at the genetic basis and heritability of hydrocarbons in ants. From meta-analysis, it is clear that there is little phylogenetic

signal amongst these quickly evolving traits (Martin and Drijfhout 2009a, van Wilgenburg et al. 2011). However, one consistent pattern seems to be a potential trade-off in the production of methyl branched and unsaturated compounds (Antonialli et al. 2008, Berville and Hefetz 2013). Amongst the seven published ant genomes, we know that ants possess a large diversity of hydrocarbon synthesis genes such as elongases and CP450s (Bonasio et al. 2010, Smith et al. 2011a, 2011b), and hydrocarbon perception genes, such as chemosensory proteins (Kulmuni et al. 2013). Recent work has shown that there are ant specific diversifications of desaturases, one class of gene related to hydrocarbon production (Badouin et al. 2013).

Despite the genetic potential for generating a range of CHCs, it is difficult to discern the relative roles of genetics and environment because ants can readily pick up hydrocarbons from environmental influences such as diet (Liang and Silverman 2000) and nesting material (Bos et al. 2011). Unlike model organisms such as *Drosophila*, it is nearly impossible to mate ants, and thereby impossible to create controlled genetic lines. A common alternative approach to study the genetic basis of hydrocarbons is to use a cross-fostering nesting design (Carlin and Hölldobler 1983). For example, ants from different chemotypes are cross-fostered to look at which hydrocarbons are transferred among workers from different patrilines (van Zweden et al. 2009, 2010). Another approach can involve feeding radio-labeled hydrocarbon precursors to differentiate the synthesized and acquired hydrocarbons (Vienne et al. 1995). These studies indicate that the linear alkanes are likely less heritable than methyl branched compounds, but patterns vary across taxa (van Zweden et al. 2010, Martin et al. 2012b).

We can also exploit natural cross-fostering experiments, because many ants have evolved to live in multi-species nests. In most multiple species nests, one species is a social parasite on the other species, taking advantage of the social benefits of their hosts, such as nest maintenance, foraging and brood care. These social parasites integrate into the nest through a diversity of genetically and environmentally determined chemistry (Lenoir et al. 2001). For example, the social parasite *Harpagoxenus sublaevis* is unable to simultaneously synthesize the hydrocarbons needed match its two chemically disparate *Leptothorax* hosts (Bauer et al. 2009). As a result, some of its hydrocarbons are passively acquired from its host to help its chemical camouflage. Growing evidence suggests that multi-species nesting is a strong selection pressure for chemical cues (Martin et al. 2011, Menzel and Schmitt 2012, Menzel et al. 2013).

One unique nesting symbiosis is parabiosis, where multiple species share a common nest and foraging trails in a seemingly mutualistic association (Swain 1980, Menzel and Blüthgen 2010). In South America, the ant-garden nests of the Amazon are shared by the two species *Cr. levior* and *Ca. femoratus*. We have previously described the behavioral and chemical basis for the recognition systems in these unique nests (Emery and Tsutsui 2013). Both ants have unique CHC profiles, sharing very few cues in common, despite sharing a nest. One of these ants, *Cr. levior*, is found in two very distinctive chemotypes, hereafter referred to as *Cr. levior* Type A and *Cr. levior* Type B. Both species maintain efficient conspecific recognition systems, by rejecting non-nestmates, likely through evaluating odors that are shared

amongst the colony. For *Cr. levior,* conspecific recognition broadly correlates with patterns of chemotype, and they may also be able to differentiate nestmate and non-nestmate *Ca. femoratus* ants.

Here, we explore the genetic basis of chemotype in these parabiotic ants. The common colony odor achieved by each species (Chapter 2) is truly a marvel given the large environmental heterogeneity in these nests. First, multiple partner chemotypes are found throughout the population. Second, all the colonies are polydomous, with each colony consisting of multiple nest units. Third, polygyny, or the presence of multiple reproductive queens within a single colony, is high for both species, reaching into the hundreds. These multi-nest, multi-queen and multi-species colonies present the opportunity to assess whether a genetic signature for chemotype can be maintained in spite of so many sources of potential variation.

To explore the genetic basis of chemotype in these ants, we use microsatellite loci and mitochondrial sequencing. The resultant genotypes are compared to the chemical dataset from individuals collected in Chapter 2. We also explore whether there is spatial genetic and chemical structure. For both species of ants, we explore the interaction of genotype and chemotype, and for *Cr. levior* we specifically consider whether the two chemotypes are genetically distinct.

METHODS

Study sites and geographic distribution of colonies

Ants were collected from parabiotic ant-garden nests of *Ca. femoratus* and *Cr. levior* in February-March and July 2010, in French Guiana near the village of Kaw (4° 29′ 12″ N, 52° 2′ 13″ W), and near Petit Saut research station (5° 3′45″N, 53° 2′ 46″W). Each nest was marked with a Garmin portable GPS unit, and these coordinates were used to determine geographic distances between colony pairs. Ants were aspirated directly off the nest surface, and kept alive in clean fluon-coated boxes for behavioral assays. Ants that were used for chemical analysis were freeze-killed after live collection. The same ants used for chemical sampling were preserved in 95% ethanol for later genetic extraction.

Cuticular hydarocarbon extraction and processing

Details of the hydrocarbon extraction and processing methods have been described elsewhere (Chapters 1 and 2). The chemical data from individual ants (Chapter 2) was used to compute chemical distances.

Morphological identification of Crematogaster

Two or three ants per colony were point mounted and examined under a microscope, and identified as *Cr. levior* according to the descriptions in (Longino 2003). *Crematogaster* expert Jack Longino, also examined these ants, without prior knowledge of chemotype. None were identified as *Cr. carinata*, and no morphological distinctions could be found between the *Cr. levior* Type A and Type B ants.

DNA Extraction

Individual ants were cut at the petiole, and the head and thorax were extracted using the standard protocol of the QiaGen DNEasy Blood and Tissue kit (Qiagen Group). In brief, the tissue was ground using plastic pestles, and cells were lysed by mixing with 20 µL proteinase-K and 180 µL buffer in a 56˚C water bath overnight. The resultant liquid was extracted with various buffers as per kit specifications, and the DNA was eluted into a 200 µL volume. Gasters were preserved in 95% EtOH for cases where re-extraction was necessary. We extracted approximately 10 individuals per species per nest (26 nests, n=239 total workers of *Ca. femoratus*, n=229 total workers for *Cr. levior*).

Microsatellite genotyping (SSRs)

We used the microsatellite primers described in for *Cr. levior* (Booth et al. 2009) and for *Ca. femoratus* (Booth et al. 2008). There were 3 previously developed loci (CL23, CL24, CL34) that could not be amplified for our populations. One locus (CF-38) could not reliably be amplified and one locus (CL-22) was non-variable, and so were not included in the analysis. In total, we collected information for 5 loci for *Cr. levior* and 8 loci for *Ca. femoratus*. When necessary, PCR recipes and annealing temperatures were re-optimized for our populations, with conditions outlined in Table 1. All PCR reactions were done in 10 µL total volumes, with 0.7 µL of DNA template, 1 µL of 5X buffer, 0.33 µL of 2.5 mM dNTPs, 0.05 µL of 5U/µL TAQ, with primer and Mg^{2+} volumes as specified in Table 1, and water added for volume. Thermocycler temperature programs were: 95°C for 5 min, 36 cycles of (95°C for 30 sec, annealing temperature for 30 sec, 72˚C for 30 sec), then final elongation hold at 72°C for 5 min. All products were stored in the fridge at 4°C until ready for fragment analysis.

Fragment analysis of amplified products was done at the UCB Sequencing Facility using an Applied Biosystems 3730XL DNA Analyzer and the DS-33 dye set (Applied Biosystems). Alleles were identified using the free program Peak Scanner v 1.0 (Applied Biosystems). All ambiguous or homozygote loci were reamplified to double-check allele identity. Allele sizing was systematically done with consideration of the recommendations in (Selkoe and Toonen 2006).

Mitochondrial DNA sequencing

Using the universal primers HC02198 and LC01490 (Folmer et al. 1994) we sequenced the barcoding region of mitochondrial CO-1 aiming for at least 1 individual per species per nest (n=25 sequences from 23 nests for *Ca. femoratus* and n=41 sequences from 26 nests for *Cr. levior*). PCR reactions were done in 10 µL total volumes, with 0.7 µL of DNA template, 2 µL of 5X buffer, 0.8 µL of 2.5 mM dNTPs, 0.08 uL of 5U/µL TAQ, 0.8 µL of each primer, 1.2 µL of 2.5 mM Mg^{2+}, and water added for volume. Thermocycler temperature programs were: 95°C for 5 min, 36 cycles of (95°C for 30 sec, 58˚C for 30 sec, 72˚C for 30 sec), then final elongation hold at 72°C for 5 min. All products were stored in the freezer at -20°C until ready for sequencing. Sequencing was done at the UCB Sequencing Facility. All sequences were manually trimmed and aligned using the software Geneious v. 6 (Kearse et al. 2012).

Statistical analysis

Computing pairwise colony differences for geography

Geographic distances between nests were computed using the GPS coordinates for each nest, and reported to the nearest meter. We assume that the distance 'as the crow flies' is biologically relevant for these species since queens are thought to disperse by air, and there were no obvious landscape features separating any of the observed colonies in the main sampling site near Kaw. Two nests (colonies P2 and P3) did not have accurate GPS coordinates due to mechanical issues in the field, and so were excluded from the geographic analysis. Although there was one colony (colony 958) from more than 100 km away, this colony had a chemotype nearly identical to the main site's *Cr. levior* Type B and was not genetically differentiable for either *Ca. femoratus* or *Cr. levior* (see Structure analysis in Results), so we included it in all analyses. However, we also confirmed that this distance outlier was not impacting our results by repeating all of the described geographic tests without this nest. All distances were log transformed due to the non-normal distribution of geographic distances in our study (ranging from 100 m to 100 km between colonies). A matrix of distances between colonies was generated using GenAlex. A map of the studied colonies can be found in Chapter 1, Figure 2.

Pairwise colony differences for chemistry

The chemical data was analyzed as previously described (see Chapter 2) by integrating the area under each peak to get a relative proportion of each compound for each profile. These relative proportions were analyzed using non-metric multidimensional scaling, and the chemical distances for individual profiles were extracted from the resultant Brays-Curtis dissimilarity matrix. We computed distance matrices for four partitions of the samples: 1) *Ca. femoratus*, 2) all *Cr. levior*, 3) only *Cr. levior* Type A, and 4) only *Cr. levior* Type B). All chemical distance matrices were computed using the package *ecodist*, implemented in R v. 3.0.2 (Goslee and Urban 2007, R Development Core Team 2011).

The average colony-level chemical similarity was calculated by averaging the values for each possible pairing of individual profiles for that colony pair, and the chemical variation was estimated by computing the standard error. Since we also sampled pooled individuals (see Chapter 1), we verified these average distance values by also computing the inter-colony chemical distances from pooled profile comparisons. We compared the dissimilarity matrices from averaged individual profiles and pooled profiles using a Mantel test. The pooled and individual chemical profiles were significantly correlated for both *Cr. levior* (Mantel, $r=0.74$, $p=0.00$) and for *Ca. femoratus* (Mantel, $r=0.92$, $p=0.02$), as expected, so we used the averaged values from individual profiles for all further comparisons.

Genetic analyses and pairwise colony differences for genetics

The genotype data were analyzed using the program GenAlEx v 6.5 (Peakall and Smouse 1996). The genetic data were converted between formats using the program Convert (Glaubitz 2004). For each locus we calculated basic genetic variables (number of alleles, allele frequencies, expected and observed

heterozygosities), and averaged these across loci for each nest. Using Genepop on the web (Raymond and Rousset 1995, Rousset 2008), we used a Fisher's exact test to confirm linkage equilibrium of all loci. Using Kingroup v.2 (Konovalov et al. 2004) we estimated the relatedness of individuals within each nest. All relatedness measures were strongly correlated, so we used the widely reported relatedness coefficient r (Queller and Goodnight 1989), which performs well with genetic parameters similar to ours (Van de Casteele et al. 2001).

We used Genepop to calculate the adjusted pairwise genetic differentiation among groups based on allele size, Rho_{ST}, (an F_{ST} derivative that assumes a stepwise mutation model which is suitable for microsatellites Michalakis and Excoffier 1996), with colonies being the defined groups. We also used SMOGD (Crawford 2010) to calculate the actual genetic differentiation (Jost's D, Jost 2008). Like the chemical data, the *Ca. femoratus* and *Cr. levior* genetic data were analyzed separately, and then the *Cr. levior* data was further subdivided into data matrices containing only information from colonies of *Cr. levior* Type A, and of *Cr. levior* Type B. The two resultant matrices from the two genetic distance measures (Rho_{ST} and D) were used in the Mantel tests comparing geographic and chemical distances of colonies.

We did an analysis of population subdivision for both species of ants, using Structure v. 2.3.4 (Pritchard et al. 2000) to calculated Bayesian genetic clusters (K). We looked for a range of K from 2-26, and ran 3 replicates per K value, with an initial burn-in of 5,000 and 100,000 MCMC runs, assuming population admixture and all other parameters as default. We then used StructureHarvester (Earl and vonHoldt 2012) to summarize the runs and infer the most likely K by looking at the maximum ΔK, or the greatest change in the log probability between successive K values (as per Evanno et al. 2005). We performed similar analyses using BAPS6 (Corander et al. 2004, Corander and Marttinen 2006) to confirm the cross-platform consistency of our results. In BAPS6 we used the most likely K to analyze mixture of individuals within colonies, and then tested for admixture by assuming at least 5 individuals per population, doing 50,000 iterations with 10,000 iterations from 10 reference individuals per population.

We used an analysis of molecular variance (AMOVA), based on F_{ST} to determine the source of genetic variation for each species. For both *Cr. levior* and *Ca. femoratus,* we considered variation within and between 1) individuals, and 2) colonies, and for *Cr. levior* we also considered 3) chemotype. The AMOVA analysis was computed using 10,000 permutations in GenAlEx.

Phylogenetic methods

To build the phylogenetic tree we used an alignment of 683 basepairs from 285 sequences, which included other *Crematogaster* and *Camponotus* species from Genbank, and some *Ca. femoratus* and *Cr. levior* samples from other study populations in Peru, with one population sampled near Iquitos, and another sampled near Madre de Dios, courtesy of Elsa Youngsteadt, (the same population as in Booth et al. 2008, 2009). We used jModelTest v. 2.1.4 to determine the best-fit model of evolution to be GTR + I + G (generalized time reversible with an assumed invariant gamma distribution of DNA substitution rates). Using this model, we estimated phylogenetic relationships by Bayesian analysis for 1 million generations

with a burn-in of 100,000 and sampling every 200 generations, implemented in MrBayes v. 3.2.2 (Huelsenbeck and Ronquist 2001). The resultant consensus tree was used to extract patristic genetic distances between haplotypes. All Bayesian analyses were computed using plug-ins for Geneious v 6 (Kearse et al. 2012).

Multivariate analyses of geography, chemistry and genetics

We compared the pairwise geographic (log of distance), chemical (Brays Curtis dissimilarity) and genetic (Rho_{ST}, and Jost's D) distance matrices using Mantel tests, with 10,000 permutations, as implemented in GenAlEX.

There were 48 *Cr. levior* individuals (20 of Type A, 28 of Type B), and 99 *Ca. femoratus* individuals that had both their chemistry and microsatellite genetics sampled, and for which we could directly correlate genetic and chemical details. In order to directly compare the genotypes and chemical phenotypes of these ants, we used multiple regression and Kendall's Tau to assess relationships between the three main principle components from an analysis of genetic distance (based on F_{ST}), and the overall proportion of the chemical profile belonging to four distinct chemical compound classes (straight chain alkanes, single methyl branches, multiple methyl branches, and unsaturated hydrocarbons).

RESULTS

Overall SSR genetic traits and patterns

None of the loci for either species were in linkage disequilibrium and are assumed to be independent genetic markers (Fisher's exact tests, all $p>0.3$). A summary of relevant genetic parameters for each locus is in Table 1, and for each colony is shown in Table 2. Overall, we found similar allelic diversity to the Peruvian population examined in (Booth et al. 2008, 2009), with 6-22 alleles per locus for *Cr. levior*, and 3-24 alleles per locus for *Ca. femoratus*. Since the Peruvian sampling was from only 56 individuals for *Ca. femoratus*, and 28 individuals for *Cr. levior*, it is not surprising that overall we found a greater number of alleles. However, we found lower values of observed and expected heterozygosity for both species, with a large number of *Cr. levior* colonies deviating from Hardy-Weinburg equilibrium at several loci.

Population genetic structure of Cr. levior

The Structure analysis revealed an optimal value of K=3 for *Cr. levior*, with the maximum $\Delta K=71.2$, supported by similar results in BAPS (Figure 1). In general, the structure analyses separated the nests of *Cr. levior* Type A and *Cr. levior* Type B, with one cluster consisting of exclusively *Cr. levior* Type A, and two clusters within *Cr. levior* Type B. One colony (11) did not cluster with the other Type A colonies, but this is likely due to low sample size (n=2 individuals) and potential null alleles. The two genetic partitions within *Cr. levior* Type B ants are consistent with the higher average observed heterozygosity amongst Type B ants (0.34 for Type A compared to 0.46 for Type B). The AMOVA analysis indicated that the genetic variation was partitioned with 44% within individuals, 22% among individuals, 22% among colonies, and 12% among chemotypes ($p<0.01$). When considering only the *Cr.*

levior Type A partition, results were similar with 45% within individuals, 33% among individuals and 22% among colonies ($p<0.01$). For *Cr. levior* Type B ants, there was 53% of the variation within individuals, 21% among individuals, and 26% among colonies ($p<0.01$).

Phylogenetic relationships amongst Cr. levior

There were two clear haplotype groups amongst the sampled population, which matched exactly to the chemotype separation, with *Cr. levior* Type A and *Cr. levior* Type B ants belonging to two well supported clades (Figure 2). All *Cr. levior* Type B ants shared a common haplotype, whereas three potential haplotypes were found amongst Type A ants. Interestingly, each chemotype is most closely related to a subset of Peruvian ants than to its sympatric congeners of the opposite chemotype. The two haplotypes are on average 6.2% genetically distinct.

Population genetic structure of Ca. femoratus

For *Ca. femoratus*, the optimal K was 1 or 2, as evidenced by the low change in ΔK for most values of K (Figure 3). Three colonies (colonies 23, 44, P1) consistently clustered as genetically distinct under conditions where $K\geq2$. The AMOVA analysis indicated that the genetic variation was partitioned with 73% within individuals, 13% among individuals, and 14% among colonies ($p<0.01$).

Phylogenetic relationships amongst Ca. femoratus

Of the three genetically distinct colonies identified in the structure analysis, two (23 and P1) were sequenced at CO-1. In agreement with the structure analysis, these ants had different haplotypes from other *Ca. femoratus* in the population. In contrast to *Cr. levior*, *Ca. femoratus* from French Guiana formed a single clade. However there were at least 4 haplotypes amongst *Ca. femoratus* from French Guiana, with no clear haplotype differences based on *Cr. levior* nestmate chemotype (Figure 4).

Relationships between geographic, genetic and chemical distances of Cr. levior

The various Mantel tests are summarized in Table 3 and Figure 4. There was no evidence of spatial genetic structure for *Cr. levior* when considering all ants. Although there appears to be potential spatial chemical patterning, this effect disappears when excluding the geographic outlier. This finding is consistent with our observation of complete sympatry of the two chemotypes across the population. When partitioning the *Cr. levior* ants by chemotype, the *Cr. levior* Type B ants showed evidence of genetic spatial partitioning, but only when the geographic outlier was included. Likewise, there was potential spatial chemical partitioning, indicating higher chemical similarity amongst colonies that were more physically distant, but only when the geographic outlier was excluded.

There was no indication of a correlation between genetic and chemical similarity in pairwise comparisons considering all *Cr. levior* ants, but when broken down by chemotype, the *Cr. levior* Type B ants showed correlated genetic and chemical features. This relationship was negative, indicating higher chemical similarity amongst colonies that were more genetically similar. Despite insignificant

Mantel tests comparing the chemical and genetic distances between colonies, the strong differentiation of genotypes in the genetic structure analysis (Figure 1) shows a broad genetic difference between the two *Cr. levior* chemotypes.

The genetic basis for chemotype was further confirmed using the subset of individuals with both genetic and chemical sampling. In the multivariate analysis, three of the compound classes were significantly correlated with the first principle component (Figure 5). The straight chain (Kendall's τ =0.24, p=0.02 with PC1) and single methyl branched compounds (Kendall's τ =0.41 , p<0.01 with PC1) were consistently correlated at all PCs and in opposite directions to the unsaturated hydrocarbons (Kendall's τ =-0.39 , p<0.01 with PC1). This pattern likely reflects the large differences in chemical composition of *Cr. levior* Type A profiles (dominated by single methyl branched alkanes), and *Cr. levior* Type B profiles (dominated by alkenes and alkadienes).

Relationships between geographic, genetic and chemical distances of Ca. femoratus

In contrast to *Cr. levior*, there were patterns of both spatial genetic and chemical structure for *Ca. femoratus* (Figure 4). Genetic differentiation increased with increased geographic distance, and chemical dissimilarity also increased with increasing distance. There was also a significant positive correlation between the genetic and chemical similarity matrices, indicating a potential genetic signature to the chemical patterns of *Ca. femoratus* in the study population.

In the multivariate analysis of individuals with both genetic and chemical sampling, only the proportion of straight chain alkanes was significantly correlated with the genetic principle components (Kendall's τ =0.24, p<0.01 with PC1, Kendall's τ =-0.30, p<0.01 with PC2). Due to the co-eluting nature of many of the unsaturated compounds in the *Ca. femoratus* profile, we can only analyze few peaks with little variation, and we predict that we are largely underestimating the true correlations of genotype and chemotype.

DISCUSSION

There was significant genetic structure in our population for both *Cr. levior* and *Ca. femoratus*. These patterns were correlated with both chemical variation and spatial structure, and each species is discussed in turn.

For *Cr. levior*, there are multiple lines of evidence supporting the genetic basis of the two chemotypes. First, the population structure analysis maps perfectly to chemotype differences, with *Cr. levior* Type A nests and *Cr. levior* Type B nests clearly differentiated. Second, when considering only *Cr. levior* Type B nests there is a correlation between pairwise genetic and chemical similarity. Third, when directly comparing the chemotype and genotype of a subset of individuals, there were strong correlations in genetic and chemical traits, particularly as relates to the chemotype differences. Fourth, mitochondrial haplotypes were strongly divergent and divided by chemotype. Given the proven genetic basis for cuticular hydrocarbon phenotypes in wild populations of other insects (Brandt et al. 2009b, Krasnec and Breed 2013), and the lack of spatial chemical structure, we are confident that the chemical

differences of *Cr. levior* reflect genetic differentiation, and not an environmental effect.

The *Cr. levior* Type A and Type B ants are behaviorally and morphologically indistinguishable, and ecologically they share very similar niches. We suspect that *Cr. levior* ants of different chemotypes are in the process of incipient speciation or are different species, given the strong genetic partitioning amongst the chemotypes, despite their living in geographic and ecological sympatry. One agent that could be driving this differentiation is a reproductive manipulator, such as *Wolbachia* (Sharon et al. 2010). Such reproductive manipulators act as selective pressures on recognition phenotypes by reinforcing the rejection of ants carrying different endosymbiont strains (Jaenike et al. 2006). These endosymbionts can also produce mitochondrial sweeps, inflating the genetic differences between strain-carriers in a similar fashion to the genetic differentiation of *Cr. levior* chemotypes at co-1. We have identified two different *Wolbachia* strains carried by the different *Cr. levior* chemotypes, consistent with this predicted scenario (Emery and Tsutsui, in prep).

Amongst the *Cr. levior* ants, there are several signals that the genotypes and associated chemotype are undergoing selection. There were several colonies that showed consistent deviations from Hardy-Weinberg equilibrium, including when the analysis was redone with only a single-chemotype dataset. There is also a heterozygote deficit amongst all loci and colonies of *Cr. levior* ants, a common signature of population subdivision. When considering only single chemotype partitions of the *Cr. levior* data, this effect was lessened. Further, the strong rejection of worker ants of the opposite chemotype (Emery and Tsutsui 2013) could reinforce differentiation if reproductive individuals of the two chemotypes are also rejecting one another. Further tests of mating interactions of *Cr. levior* ants would be an interesting avenue to confirm any pre-zygotic reproductive isolation.

For *Ca. femoratus* there was genetic and chemical isolation by distance, indicating that nests that were more physically distant from one another where also more genetically and chemically dissimilar. This pattern suggests a potential role of budding for colony reproduction, a behavior where queens and a subset of workers walk to a new nest site instead of dispersing in a mating flight (Vargo and Porter 1989, Drescher et al. 2010). Virtually nothing is known of the reproductive behaviors of either of these ants. We did observe a single queen aggregation of *Ca. femoratus* at a nest in late March, but after several hours of agitated aggregation on the nest surface, no queens flew off, and no males arrived or matings were observed. This observation, coupled with the genetic isolation by distance, physogastric queens, and polydomous and polygynous nesting habit suggest that these ants are likely dispersing via colony fission, or budding.

There was a significantly positive relationship between chemical and genetic similarity of *Ca. femoratus*, with more genetically distant ants being more chemically dissimilar. Although most ants had profiles consisting of 5 main distinguishable peaks (from C38-C45 in length), the ants from the three most distinctive colonies (23, 44, and P1) all had a larger proportion of their profiles composed of other smaller compounds (from C31-37). These smaller compounds are also found on *Cr. levior* Type B ants, and so without this genetic information, we may have assumed that all these compounds were passively acquired from their conspecific nestmates.

Our findings highlight the need to remain impartial as to the source of an individual's chemical phenotype, because all insects share the common genetic architecture necessary to produce all possible compound classes (Blomquist 2010).

The lack of genetic differentiation amongst *Ca. femoratus* nesting with different *Cr. levior* types is consistent with a previously reported lack of chemical differentiation in these ants (Emery and Tsutsui 2013). Together these findings suggest that *Ca. femoratus* ants have not co-diverged with their *Cr. levior* nestmates, and suggests that the ants do not disperse simultaneously. If the *Cr. levior* chemotypes are indeed different species, we might expect that each chemotype interacts differently with *Ca. femoratus*. Further behavioral studies of these parabiotic nests could reveal differences in the costs and benefits provided to *Ca. femoratus* by *Cr. levior* of different chemotypes, and reveal much about the nature of their interaction. We anticipate that these parabiotic ants could easily become a model system for studying the effects of interspecific nesting on cooperative phenotypes such as common chemical cues.

Table 1. Characteristics of the 13 microsatellite DNA loci used in this study. The first three columns show PCR recipes and annealing temperatures, which have been modified from the original conditions outlined in Booth et al. 2009 a,b. We have included for comparison the traits reported from the population in Madre de Dios, South Peru, and for this study from the population in French Guiana. Reported traits are number of alleles observed (N_A), average expected (H_E), and observed (H_O) heterozygosities, range of PCR product sizes in (bp), and conformance to Hardy–Weinberg equilibrium (HWE test). For our population the information shown is the average of n=26 nests, with the HWE test column showing the number of nests with significant deviations from HWE.

	Name	uL of 25 mM Mg2+	uL of 10 mM Primer	Annealing temperature	N_A	H_E	H_O	Size (bp)	HWE Test	N_A	H_E	H_O	Size (bp)	HWE Test
					From Booth et al. 2009- South Peru					From this study- French Guiana				
Cr. levior loci	CL-4	0.66	1.23	45	5	0.788	0.521	346-356	n.s	6	0.240	0.216	321-341	3/26
	CL-12	0.33	0.62	50	8	0.841	0.555	236-258	*	12	0.495	0.372	216-240	12/26
	CL-26	0.33	2.88	55	11	0.845	0.785	206-230	*	15	0.526	0.549	179-213	6/26
	CL-31	0.33	0.49	52	14	0.909	0.689	290-362	*	22	0.611	0.525	268-318	6/26
	CL-37	0.33	0.41	52	5	0.715	0.519	163-173	n.s	6	0.403	0.370	140-150	3/26
Ca. femoratus loci	CF-2	0.49	0.62	58	6	0.595	0.518	216-238	*	9	0.612	0.634	194-220	4/26
	CF-3	0.49	0.49	58	10	0.875	0.714	192-214	n.s	14	0.704	0.737	147-189	2/26
	CF-7	0.33	3.29	50	3	0.531	0.375	148-152	n.s	3	0.463	0.456	124-128	0/26
	CF-10	0.49	0.8	52	16	0.858	0.714	192-228	*	24	0.732	0.770	163-215	2/26
	CF-13	0.33	0.33	60	5	0.73	0.464	226-252	n.s	13	0.650	0.590	202-232	0/26
	CF-35	0.33	0.41	55	2	0.378	0.286	240-242	n.s	3	0.152	0.185	221-225	0/25
	CF-37	0.33	0.82	55	17	0.661	0.661	165-207	*	16	0.658	0.680	143-185	2/26
	CF-38	0.58	0.62	50	2	0.339	0.339	237-239	n.s	3	0.197	0.142	216-220	2/26

Table 2. Genetic characteristics of the 26 colonies in this study with details for *Cr. levior* (top panel) and *Ca. femoratus* (bottom panel). Reported traits are number of alleles observed (N_A), number of effective alleles (NE), average expected (H_E), and observed (H_O) heterozygosities, average within colony relatedness, and the probability of sampled individuals belong to each of the Structure partitions.

Crematogaster levior

Nest	Chemotype	Sample size	N_A	N_E	H_E	H_O	Relatedness	% of ind. In Structure partition 1	% of ind. In Structure partition 2	% of ind. In Structure partition 3
5	a	9	3.8 +/- 0.9	2.6 +/- 0.7	0.50	0.23	0.18	**0.92**	0.02	0.06
11	a	2	1.6 +/- 0.4	1.5 +/- 0.3	0.23	0.40	NA	0.36	0.03	**0.61**
12	a	10	3.6 +/- 0.9	2.4 +/- 0.6	0.49	0.36	0.13	**0.86**	0.12	0.02
17	a	10	3 +/- 0.7	2.2 +/- 0.4	0.45	0.34	0.23	**0.79**	0.03	0.18
28	a	8	3.8 +/- 1.2	2.5 +/- 0.6	0.49	0.43	0.14	**0.90**	0.04	0.07
38	a	10	2.6 +/- 0.5	1.7 +/- 0.3	0.34	0.31	0.03	**0.97**	0.01	0.03
46	a	9	3.2 +/- 0.8	2.5 +/- 0.6	0.49	0.31	0.22	**0.97**	0.02	0.01
48	a	9	2 +/- 0.4	1.3 +/- 0.2	0.19	0.16	0.00	**0.98**	0.01	0.01
50	a	10	2.8 +/- 0.7	2.3 +/- 0.4	0.47	0.39	0.14	**0.85**	0.03	0.12
P2	a	10	2.4 +/- 0.4	1.9 +/- 0.3	0.41	0.52	0.05	**0.95**	0.01	0.04
P3	a	4	2.2 +/- 0.4	1.8 +/- 0.3	0.36	0.30	0.09	**0.88**	0.10	0.02
1	b	9	2.6 +/- 0.4	1.8 +/- 0.2	0.42	0.33	0.22	0.12	**0.65**	0.24
6	b	9	2.4 +/- 0.2	2.1 +/- 0.3	0.47	0.52	0.06	0.01	**0.97**	0.02
14	b	7	3.2 +/- 0.2	2.6 +/- 0.2	0.60	0.71	0.08	0.01	0.41	**0.58**
22	b	10	2.8 +/- 0.6	2 +/- 0.3	0.45	0.43	0.04	0.01	**0.97**	0.02
23	b	10	5 +/- 0.5	3.5 +/- 0.5	0.68	0.40	0.34	0.03	0.32	**0.65**
27	b	10	3.4 +/- 0.2	2.1 +/- 0.2	0.51	0.61	0.02	0.02	**0.96**	0.02
30	b	9	2.6 +/- 0.5	2.3 +/- 0.4	0.48	0.51	0.07	0.01	**0.96**	0.04
31	b	8	2.4 +/- 0.4	2.1 +/- 0.3	0.46	0.42	0.15	0.01	**0.86**	0.13
36	b	14	5.4 +/- 1.3	3.4 +/- 0.7	0.66	0.42	0.35	0.01	0.46	0.53
37	b	9	1.8 +/- 0.2	1.6 +/- 0.2	0.34	0.36	0.01	0.01	**0.99**	0.01
40	b	10	4.2 +/- 1	2.9 +/- 0.7	0.60	0.59	0.16	0.03	0.52	0.46
41	b	10	2.8 +/- 0.7	2.4 +/- 0.6	0.43	0.31	0.10	0.01	0.04	**0.95**
44	b	8	3.6 +/- 0.8	2.4 +/- 0.6	0.48	0.44	0.25	0.49	0.46	0.06
958	b	10	1.8 +/- 0.2	1.5 +/- 0.2	0.28	0.32	0.01	0.10	0.01	**0.89**
P1	b	8	3.2 +/- 0.4	2.4 +/- 0.3	0.56	0.51	0.22	0.01	0.02	**0.96**
Average for Type A			2.82	2.06	0.40	0.34	0.12	**0.86**	0.04	0.10
Average for Type B			3.15	2.34	0.49	0.46	0.14	0.06	**0.57**	**0.37**

Camponotus femoratus

Nest	Chemotype	Sample size	N_A	N_E	H_E	H_O	Relatedness	% of ind. In Structure partition 1	% of ind. In Structure partition 2
5	a	10	4 +/- 0.7	2.7 +/- 0.5	0.54	0.52	0.30	**0.99**	0.01
11	a	4	3.4 +/- 0.7	2.8 +/- 0.6	0.50	0.54	0.26	**0.99**	0.01
12	a	11	3.6 +/- 0.5	2.5 +/- 0.2	0.56	0.63	0.23	**0.99**	0.01
17	a	7	4.4 +/- 0.7	3.5 +/- 0.5	0.66	0.73	0.61	**0.99**	0.01
28	a	10	4.8 +/- 0.8	3.3 +/- 0.5	0.61	0.67	0.39	**0.99**	0.01
38	a	9	3.6 +/- 0.7	2.6 +/- 0.5	0.49	0.50	0.22	**0.99**	0.01
46	a	10	3.1 +/- 0.4	2.6 +/- 0.4	0.53	0.55	0.23	**0.99**	0.01
48	a	10	3 +/- 0.5	2.4 +/- 0.4	0.48	0.48	0.17	**0.98**	0.03
50	a	9	4.4 +/- 0.9	3.1 +/- 0.7	0.57	0.55	0.30	**0.96**	0.04
P2	a	8	3.8 +/- 0.6	2.7 +/- 0.4	0.52	0.48	0.38	**0.99**	0.02
P3	a	8	3.8 +/- 0.8	2.6 +/- 0.4	0.53	0.42	0.24	**0.96**	0.04
1	b	11	5.1 +/- 1	3.5 +/- 0.7	0.57	0.53	0.42	**0.63**	0.37
6	b	10	4 +/- 0.8	2.8 +/- 0.5	0.50	0.51	0.19	**0.99**	0.01
14	b	10	3.6 +/- 0.6	2.6 +/- 0.5	0.50	0.43	0.24	**0.95**	0.05
22	b	10	3.8 +/- 0.6	2.7 +/- 0.5	0.51	0.53	0.32	**0.99**	0.01
23	b	9	3.8 +/- 0.7	2.6 +/- 0.4	0.52	0.53	0.19	0.01	**0.99**
27	b	10	2.4 +/- 0.5	2 +/- 0.4	0.44	0.35	0.16	**0.99**	0.01
30	b	7	3.9 +/- 0.7	2.6 +/- 0.5	0.51	0.49	0.25	**0.99**	0.01
31	b	9	3.8 +/- 0.6	2.7 +/- 0.5	0.55	0.53	0.25	**0.99**	0.01
36	b	10	4.6 +/- 0.9	3.7 +/- 0.8	0.61	0.68	0.39	**0.99**	0.01
37	b	10	3.6 +/- 0.7	2.8 +/- 0.5	0.51	0.56	0.24	**0.99**	0.01
40	b	10	4.1 +/- 0.7	2.4 +/- 0.3	0.51	0.47	0.27	**0.99**	0.01
41	b	10	4.3 +/- 0.7	3.1 +/- 0.5	0.59	0.50	0.43	**0.99**	0.01
44	b	9	2.4 +/- 0.6	2 +/- 0.3	0.39	0.47	0.00	0.00	**1.00**
958	b	10	3.6 +/- 0.5	2.4 +/- 0.3	0.51	0.44	0.21	**0.95**	0.05
P1	b	8	2.1 +/- 0.4	1.9 +/- 0.3	0.35	0.55	0.00	0.01	**0.99**

Table 4. Summary of Mantel tests of correlations between geographic, chemical, and genetic distance matrices. For the first section, genetic isolation by distance, the results compare two types of genetic distance matrices (Rho_{ST} and Jost's D), and the geographic distance matrix (log(1+distance)) for all colony pairs, and then for all colony pairs excluding the geographic outlier, colony 958. The second section, chemical isolation by distance does likewise but using the average Bray-Curtis chemical dissimilarity between colonies. The third section, genetic basis of chemical differences, compares the two types of genetic distance matrices and the BC chemical dissimilarity matrix. The pink cells indicate significant p-values, and the orange cells indicate a positive relationship between matrices, while the blue cells indicate a negative relationship.

	Ca. femoratus		All *Cr. levior* ants		Only Cr. levior Type A		Only *Cr. levior* Type B	
Genetic isolation by distance	r	p	r	p	r	p	r	p
RhoST	0.42	0.01	0.07	0.28	0.27	0.17	0.05	0.31
Jost's D	0.30	0.08	0.00	0.52	0.24	0.17	0.25	0.03
Excluding geographic outlier (958)								
RhoST	0.38	0.01	0.10	0.22			0.03	0.36
Jost's D	0.40	0.01	-0.04	0.33			0.04	0.38
Chemical isolation by distance								
Average chemical dissimilarity	0.18	0.05	-0.41	0.05	-0.14	0.31	-0.19	0.13
Excluding geographic outlier (958)								
Average chemical dissimilarity	0.23	0.04	-0.07	0.13			-0.27	0.04
Genetic basis of chemical differences								
RhoST	0.45	0.01	-0.15	0.69	-0.19	0.19	-0.32	0.01
Jost's D	0.49	0.05	-0.31	0.19	0.15	0.22	0.03	0.37

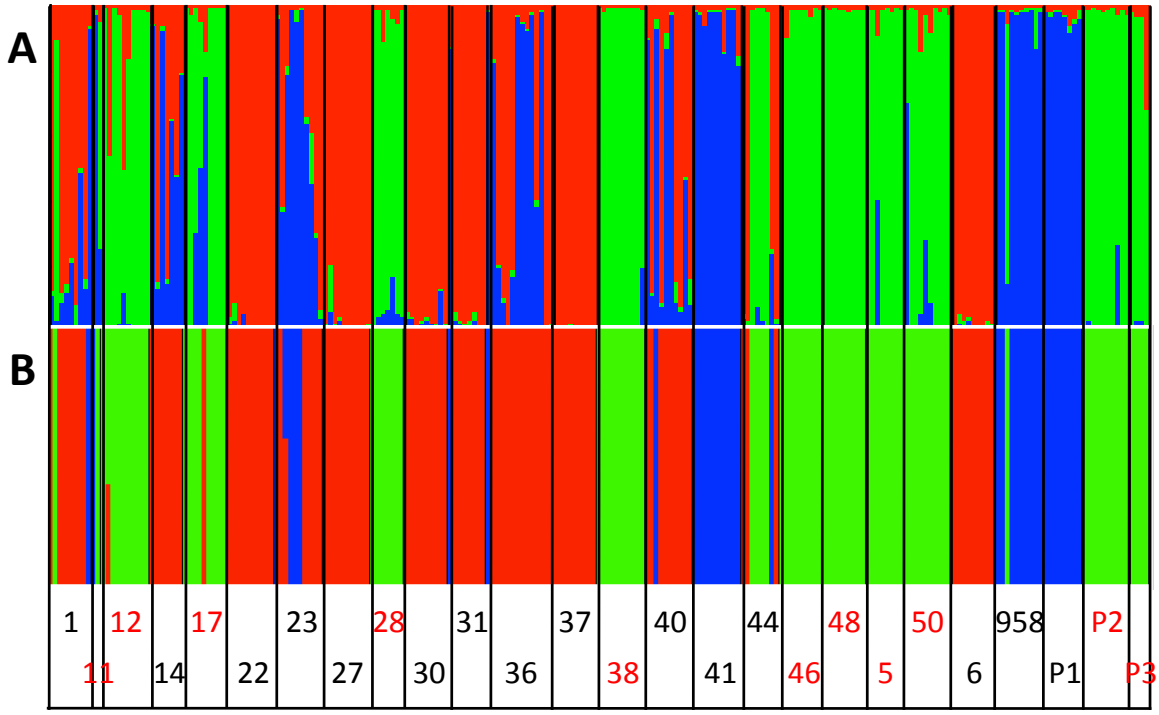


Figure 1. Results of a) Structure analysis, and b) BAPS analysis for *Cr. levior* genetic samples, assuming K=3. Each color represents a different population partition estimated from the analysis. Each column represents one individual, with the color showing the probability of each individual belonging to each partition. Black lines divide each colony. The red colony names represent colonies of *Cr. levior* Type A and the black colony names represent colonies of *Cr. levior* Type B.

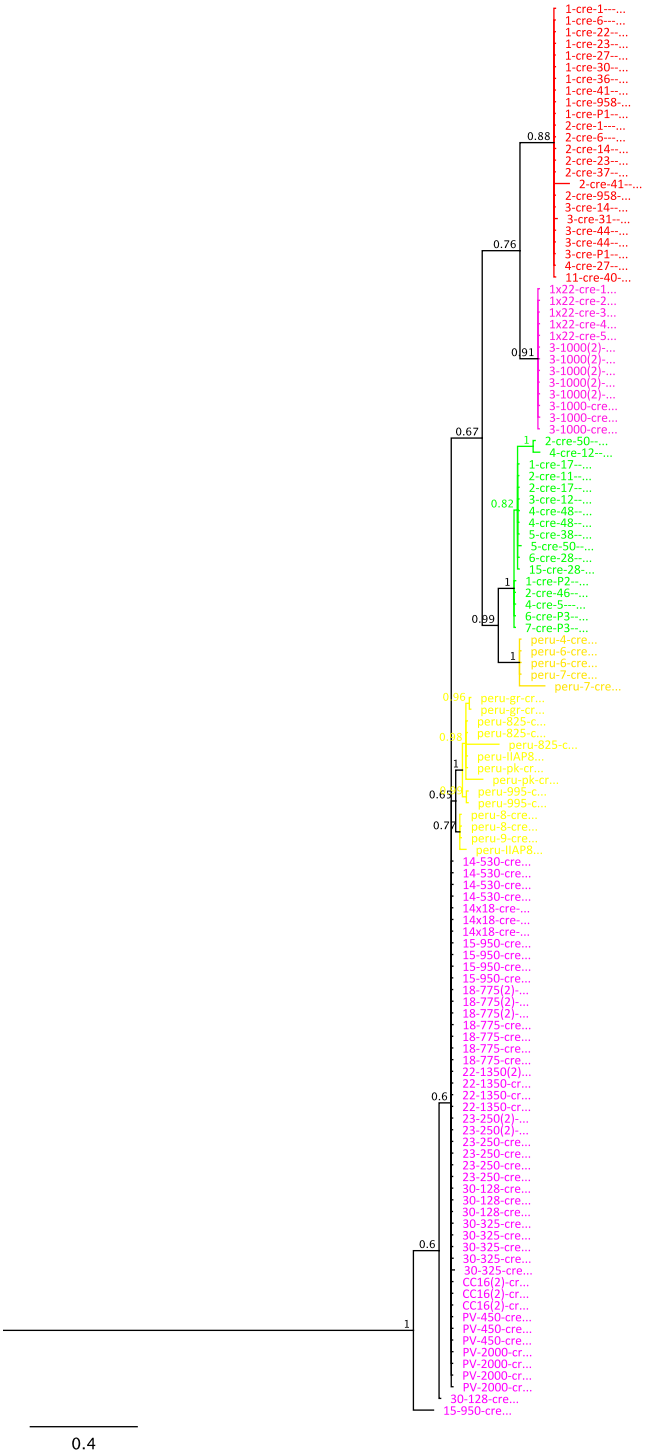


Figure 2. Consensus tree from Bayesian analysis showing the relationships between *Cr. levior* ants, using the information from 683 bp of mitochondrial co-1. The values above nodes represent node support in the form of posterior probability values. Tips are labeled with each sample name, with the nest name in the final part of the identifier. The pink and yellow individuals are from the Peruvian populations, green individuals are from Type A nests, and red individuals are from Type B nests.

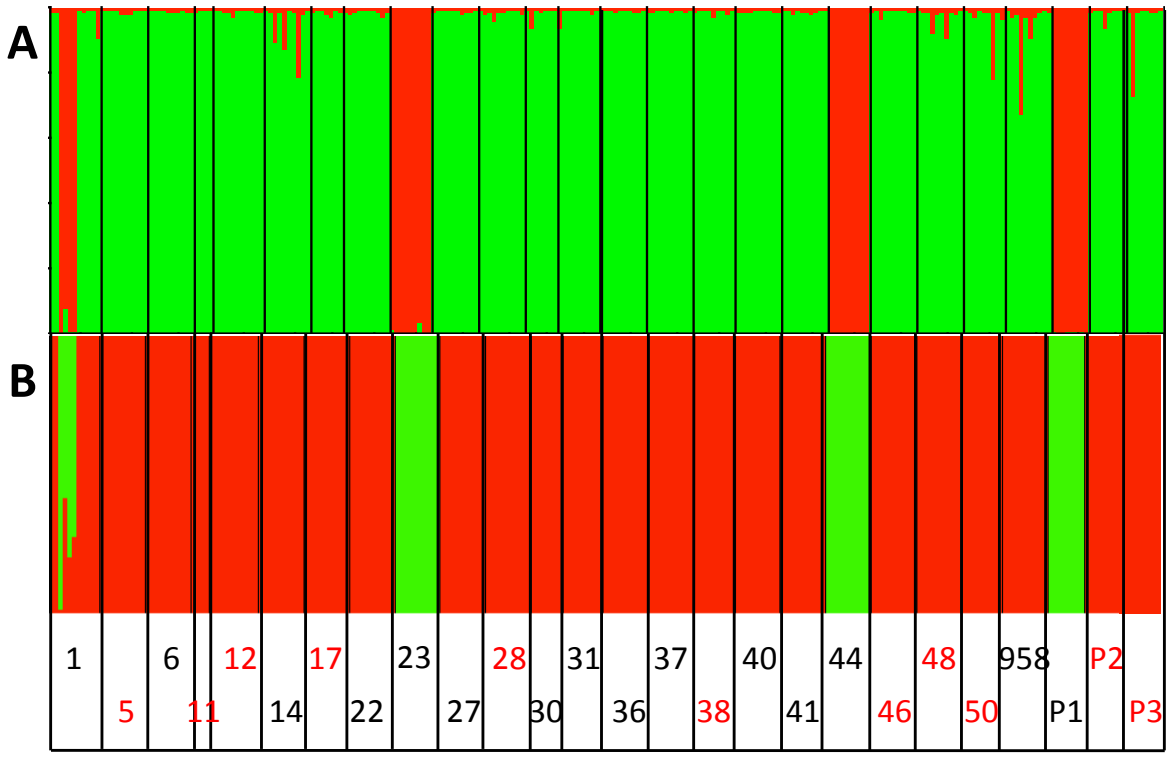


Figure 3. Results of a) Structure analysis, and b) BAPS analysis for *Ca. femoratus* genetic samples, assuming K=2. Each color represents a different population partition estimated from the analysis. Each column represents one individual, with the color showing the probability of each individual belonging to each partition. Black lines divide each colony. The red colony names represent colonies nesting with *Cr. levior* Type A and the black colony names represent colonies nesting with *Cr. levior* Type B.

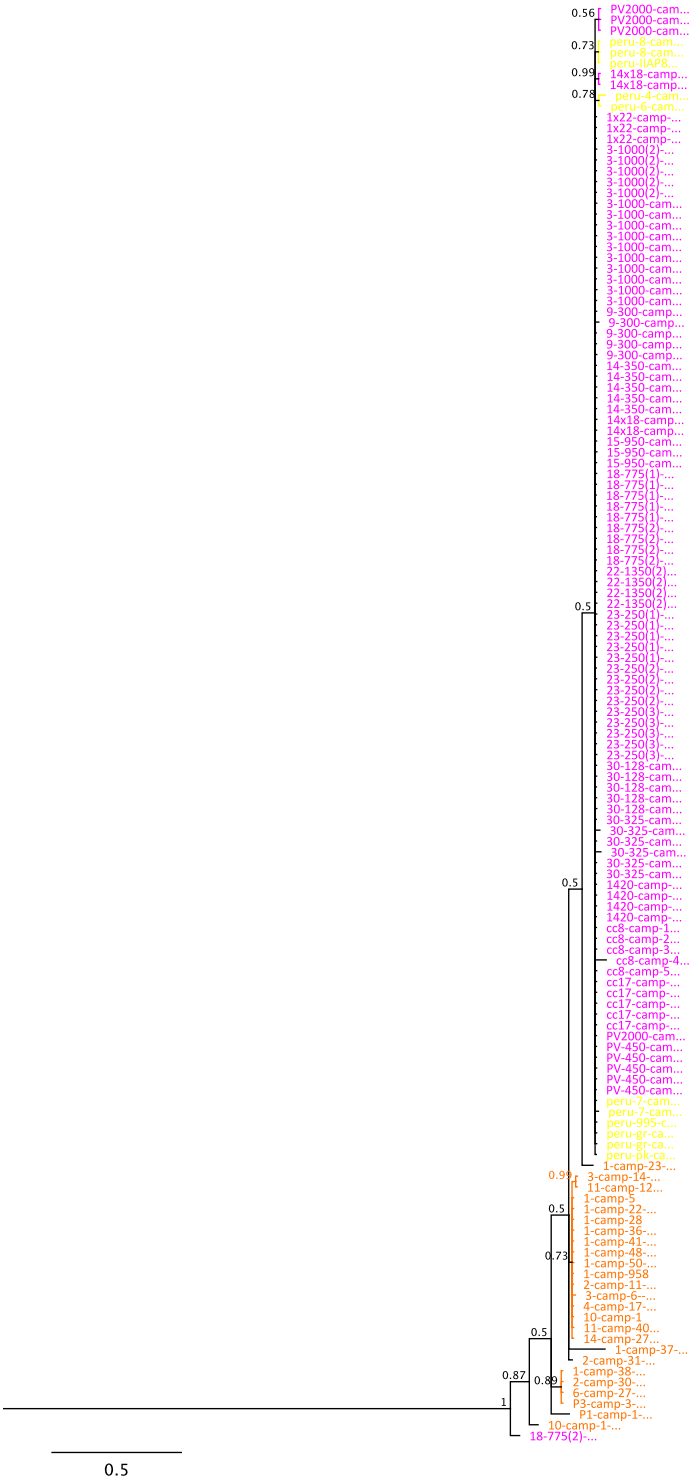
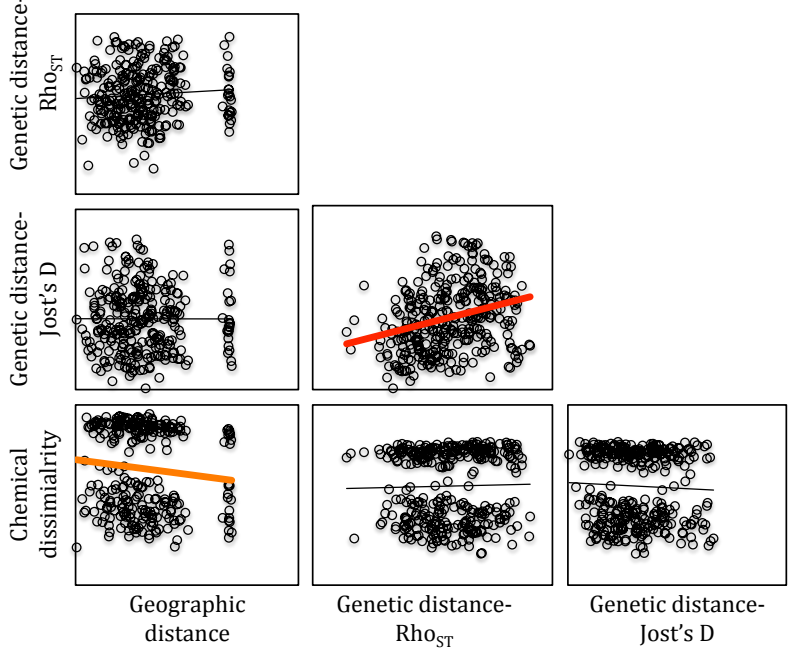


Figure 4. Consensus tree from Bayesian analysis showing the relationships between *Ca. femoratus* ants, using the information from 683 bp of mitochondrial co-1. The values above nodes represent node support in the form of posterior probability values. Tips are labeled with each sample name, with the nest name in the final part of the identifier. The pink and yellow individuals are from the Peruvian populations, and orange individuals are from French Guiana.

a) *Crematogaster levior*



b) *Camponotus femoratus*

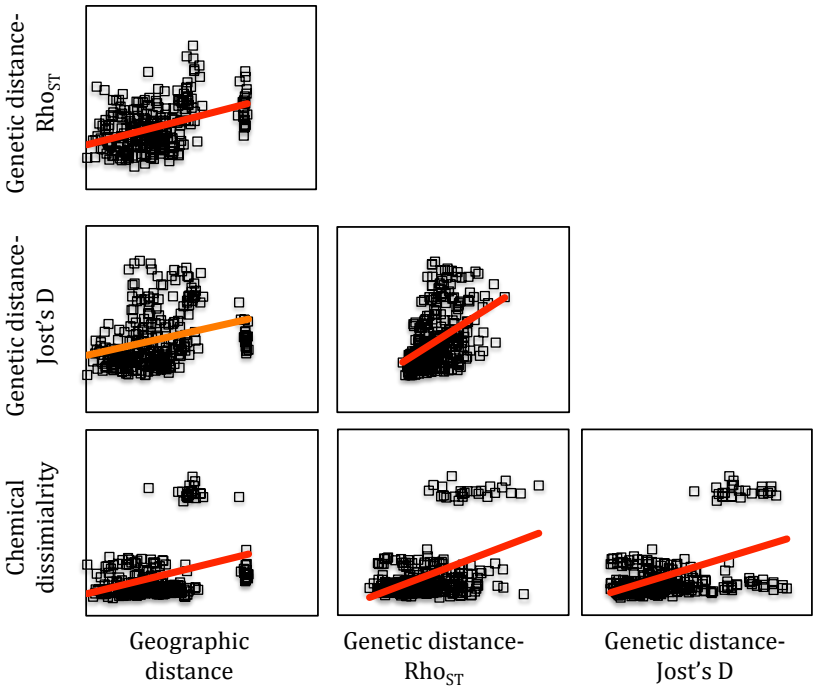


Figure 5. Pairwise correlations between geographic, chemical and genetic distances, as tested using Mantel tests summarized in Table 4. Significant correlations are shown by a colored best fit line, with orange lines showing conditional outcomes and red lines showing consistent outcomes.

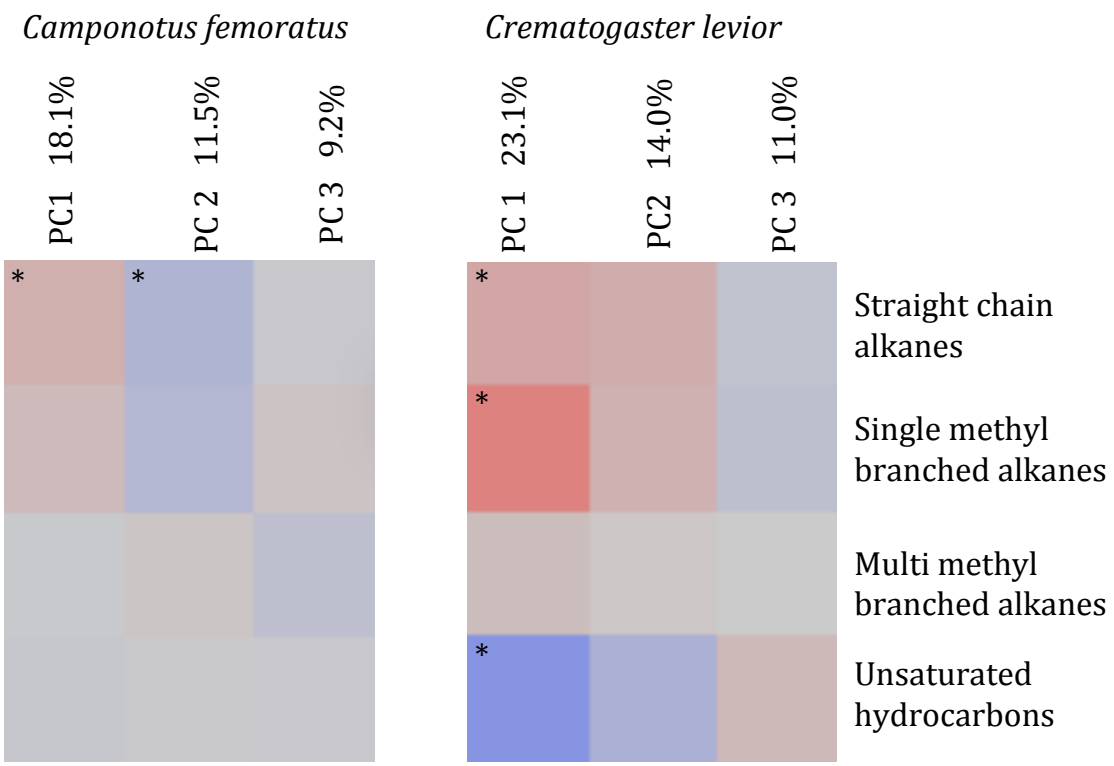


Figure 6. Heatmap showing the relationships between chemical compound classes and genetics. The x-axis shows the first three components of genetic variation from a distance based Principle Components Analysis. The percentage of variation explained by each principle component (PC) is shown in the figure, with 38.8% of the total *Ca. femoratus* genetic variation represented and 48.1% of the total *Cr. levior* genetic variation represented. The y-axis shows the four main classes of hydrocarbons found on ant's profiles. The color of the square indicates whether the relationship between genetic and chemical features is positive (red) or negative (blue), with the intensity of the color showing the strength of the relationship. The stars denote comparisons that were significant with a Kendall's Tau test.

CHAPTER 4

Ants recognize broad chemical and genetic differences in parabiotic nests

ABSTRACT

Recognition processes allow societies to remain exclusively composed of members. Usually these social groups consist of related individuals who cooperate towards the goals of foraging and rearing offspring. Social insects, such as ants, form enormous cooperative societies, and recognition processes are a central mechanism that maintains the integrity of these colonies. In one unusual relationship called parabiosis, two ant species share a nest and foraging trails in interspecific cooperation. We have previously shown that ants within these nests recognize nestmates and defend against conspecific and heterospecific non-nestmates, but it is unclear whether these recognition patterns follow similar patterns to single species nests. Here**,** we attempt to resolve the relationships between geography, chemistry, genetics, and recognition behaviors of the parabiotic ants *Crematogaster levior* and *Camponotus femoratus*. In other systems, cuticular chemistry plays an important role in determining the outcome of recognition assays, with increased chemical dissimilarity usually resulting in increased aggression. Similarly, more genetically distant non-nestmates are expected to be subject to more aggression. We find that for *Ca. femoratus*, conspecific recognition relies more on genetic differences than on chemical differences. This suggests kin-informative chemical cues exist that we have not measured. In contrast, *Cr. levior* conspecific recognition is more strongly related to chemical differences, but mainly at the level of chemotypes. For *Ca. femoratus*, interspecific aggression is correlated with the geographic relationships of nests, in combination with *Cr. levior* chemistry and the genetics of their companion *Ca. femoratus* ants. However, we failed to find evidence of interspecific nestmate recognition by *Ca. femoratus*, so it is unclear whether interspecific cues are used in nestmate recognition by this species. For *Cr. levior* ants, we did find a pattern of more aggression towards heterospecific non-nestmates than nestmates. The aggression towards non-nestmates was correlated with geographic distance and a combination of *Ca. femoratus* genetic and chemical differences. This pattern was similar to the relationships with intraspecific recognition in *Ca. femoratus,* suggesting that both ant species rely on some as-yet unmeasured genetically informative *Ca. femoratus* cues. These results highlight the importance of considering all levels of chemical and genetic differentiation when assessing nestmate recognition patterns. Both species of parabiotic ant uses chemical and associated genetic information to assess nest membership. The fully functioning recognition systems in parabiotic nests likely maintain the relationship by excluding exploiters of this unique cooperative relationship.

INTRODUCTION

Altruism is one of the greatest ecological innovations, facilitating the success of social organisms across taxa. Social organisms, such as humans and ants, live in cooperative groups where labor is divided between group members. The integrity of these social groups depends on the ability to exclude intruders who would parasitize the benefits of cooperative living. Reliable recognition systems allow

these social parasites to be excluded and for beneficial behaviors to be targeted towards related individuals (Bos and D'Ettorre 2012, Sturgis and Gordon 2012). In most cases a reliable system for identifying kin is useful for maintaining altruistic benefits in evolutionary time (Holmes 2004). However, if kin recognition systems are too precise, nepotism can result in the destruction of diverse cooperative groups (van Zweden et al. 2009).

Amongst the ants, these recognition systems rely on a combination of genetically and environmentally derived cues to assess nest membership. These cues are most commonly chemical, and are often cuticular hydrocarbons (CHCs), waxy substances found on the cuticle of most terrestrial arthropods. A common cuticular chemistry is achieved through social interactions, such as trophallaxis, which allows ants to exchange and homogenize chemical phenotypes (Lenoir 2002). This common colony odor is used as a mental template to compare with when encountering foreign individuals (Obin and Meer 1989, Errard et al. 2005, Newey 2011). Generally, we predict to see a greater increase in aggression with decreasing chemical similarity. However, not all of the chemical traits are equally important to recognition, and the relative contributions of genetic and environmental factors to these patterns are also unclear (Martin et al. 2008a, van Zweden et al. 2009).

As a genetic product, cuticular hydrocarbons are more similar between related individuals, and there is often a trend of increasing chemical similarity with higher genetic similarity. Some ability to assess relatedness is an implied condition of Hamilton's rule for altruistic behavior (Hamilton 1963). It is unclear whether genetic relatedness can be assessed directly by insects, but it is probable that due to predicted correlations of chemical and genetic distance, ants only need to assess chemical similarity (Schmidt et al. 2010). However, relying completely on chemical similarity to assess nest membership opens the door for social parasites who can chemically camouflage by acquiring cues from the environment (Lenoir et al. 2001). This vulnerability to social parasites could select for more direct ways to assess relatedness and thereby group membership (Martin et al. 2011).

In addition, learning strongly impacts recognition decisions, with both encounter frequency and social environment playing roles in the learning of colony membership. For example, amongst *Iridomyrmex purpureus* ants, familiarity with neighbours resulted in increased aggression to non-nestmates found nearby (Van Wilgenburg 2007). Time and again, geography has been found to be an important factor in determining the aggressive behaviors in terrestrially nesting species, with varying patterns. The distance between nests is related to the encounter rates between the colonies, with workers from nearby nests encountering one another in the foraging area more frequently. There are two predictions of aggressive behaviors in relation to the distance between colony pairs. The dear enemy effect predicts that individuals will be less aggressive to commonly encountered conspecifics (Van Wilgenburg 2007). Under this prediction we expect to see more aggression with greater distance. The opposite pattern can also occur, with encounter frequency increasing aggression, resulting in neighbors showing a higher amount of aggression to one another than to ants from further away (Sanada-Morimura 2003).

All three factors of distance, chemistry and genetics can be correlated with one another in several ways that impact behavior differently. For example, the correlation of genetic relatedness and geographic distance depends on dispersal of queens and males in that species and population. It is unclear what variables contribute most to the behavioral response, since multiple patterns have been found in different systems (Table 1). Our review of the recognition literature suggests a publication bias of significant results. There is also strong evidence of confirmation bias in recognition studies not performed blind, which includes around 70% of published studies (van Wilgenburg and Elgar 2013). Another reason for the varied relationships between chemistry, genetics, geography and behavior is the choice of traits, and the scale of their measurement, as well as the statistical methods employed.

Further, there are technological constraints that limit our ability to infer what levels of detail ants perceive when evaluating recognition cues. For example, our chemical instrumentation, such as the Gas Chromatograph-Mass Spectrometer, is both inferior to ants at detecting methyl branch placement, since internally branched compounds co-elute as single peaks, and superior at detecting chain length differences, which ants do not easily differentiate (Van Wilgenburg et al. 2012). We also typically sample a very small number of loci when evaluating genetic patterns. Another problem is the use of incorrect measure of differentiation, such as F_{ST}, to infer genetic distance (Jost 2008). For such reasons, a cautionary approach should be taken when considering the interactions of genetics, chemistry and aggressive behavior, to avoid Type II errors.

We used a unique nesting symbiosis to explore the combined effects of distance, chemistry and genetics on aggressive ant behaviors. In the lowland Amazon rainforest, arboreal ant garden nests are abundant, and the ants living with them are behaviorally dominant (Davidson 1988). Most ant gardens are occupied by the parabiotic partners *Cr. levior* and *Ca. femoratus*, who live in seeming harmony in a likely interspecific mutualism (Swain 1980, Vantaux et al. 2007). Each colony consists of several nest units, a nesting habit known as polydomy, which increases the likelihood of within-colony chemical diversity. Coupled with high polygyny, with up to hundreds of queens per colony for both species, there is a large potential for high genetic and high chemical variation within each colony. The potential for within-species diversity is further enhanced by the coexistence of at least two species in each colony. The two species share their polydomous arboreal nests, but brood of each species is kept in separate chambers in compound nesting. Both ants share common arboreal and terrestrial foraging trails. Given that ants can pick up hydrocarbon cues from the surrounding nest environment (Bos et al. 2011), parabiosis presents the potential for unparalleled within-colony genetic and chemical diversity. How then do recognition systems remain intact in such diverse colonies?

In previous work, we have shown that there are two *Cr. levior* chemotypes within our study population in French Guiana, hereafter referred to as *Cr. levior* Type A and *Cr. levior* Type B. These chemotypes are sympatric throughout our study population but within each nest there is only a single *Cr. levior* chemotype. The *Ca. femoratus* parabiotic partners do not appear to distinguish between *Cr. levior* Type

A and *Cr. levior* Type B despite their chemical dissimilarity. However, *Cr. levior* ants are more aggressive to non-nestmates of a different chemotype, and may be able to distinguish *Ca. femoratus* nestmates and non-nestmates. Both species show some degree of intraspecific recognition behavior, and *Cr. levior* may be able to detect interspecific nestmates. However, there are further details about the pattern and mechanism of these recognition behaviors, such as the role of genetics, which are unresolved.

By combining the previous behavioral dataset with additional behavioral data from six other colony pairings, we look at the relative contributions of geography, genetics and chemistry to the aggressive behaviors of parabiotic ants. We consider two scales of chemical and genetic detail. We use the common approaches of chemical dissimilarity and genetic distance to measure continuous variation in chemistry and genetics. We also consider categorical differences in chemotype and population genetic structure. This is the first study to look at the effects of these variables in a polydomous, arboreal nesting ant, and one of few studies looking at interspecific recognition in nest-sharing symbiosis. The patterns observed in this unique nesting symbiosis will contribute to our understanding of recognition in not only intraspecific but also interspecific cooperative societies.

METHODS

Recognition behaviors

In Chapter 1 we used 10 independent colony pairs from 20 nests to examine the recognition behaviors of *Cr. levior* and *Ca. femoratus* (n=613 behavioral observations). In addition to these 10 colony pairs, 6 of the 20 colonies (22, 23, 28, 36, 37, 44) were also paired in behavioral assays with a second nest, making for a total of 16 colony combinations from 26 sampled nests. Assays for these additional colony pairings were done similarly to those described in Chapter 1. The total expanded dataset consisted of n= 869 behavioral observations (607 observations from the Chapter 1 dataset, and 262 additional observations). We repeated the behavioural analyses outlined in Chapter 1 with our extended dataset, with similar results.

We used the proportion of assays with aggressive behavior as the response variable in nestmate comparisons (n=26 nests), and in non-nestmate comparisons (n=16 colony pairs). We defined the presence of aggression as any behavioral score 2-5, including mandible flares, biting, stinging/spraying and sustained fighting. For the interspecific comparisons, we were able to distinguish between the aggressive responses of *Cr. levior* and *Ca. femoratus* ants, giving each an independent behavioral score. For the intraspecific comparisons, we could not follow individual ants, so there was a single behavioral score representing the highest level of aggression shown by either ant.

There were in total two types of comparisons (nestmate or non-nestmate) for four types of behavioral observations (intraspecific *Ca. femoratus*, interspecific *Ca. femoratus*, interspecific *Cr. levior*, and intraspecific for *Cr. levior*), for a total of 8 sets of response variables to consider.

Geographic, chemical and genetic variables

For nestmate comparisons, we considered three measures of chemical and genetic variability: 1) the average relatedness of individuals in a nest (r as per (Queller and Goodnight 1989)), 2) the standard deviation of chemical distances of individuals within a colony (chemical variance), and 3) the average inter-individual chemical dissimilarity of individuals (chemical distance). Both the chemical variance and chemical distances were extracted from the species-specific Bray-Curtis chemical dissimilarity matrices.

For the non-nestmate comparisons, the independent variables we considered were four measures of distance: 1) geographic distance, 2) Rho_{ST} as a genetic measure of distance, 3) Jost's D as a genetic measure of distance, and 4) the average Bray-Curtis chemical dissimilarity of individuals. Jost's D is a more accurate measure of genetic differentiation than Rho_{ST} (Jost 2008), but since almost all recognition studies to date use F_{ST} and related measures, we included it in our analyses. We used the various distance matrices and colony summaries used in Chapter 3 to extract relevant values for our analysis. We used the proportion of aggression from all interactions as our response variable instead of the presence/absence of aggression for individual assays (as done in Chapter 1) because the genetic, chemical and geographic distances are colony-level traits.

Statistical analysis

Relationships with recognition behavior

We used generalized linear mixed models (GLMMs) with a quasibinomial distribution and logit link function to test for overall effects of genetic and chemical distance. First, we included all nestmate combinations (n=26) and non-nestmate combinations (n=16) in the same dataset. We used the proportion of aggressive behavior as the response variable, and whether the values were for a nestmate or non-nestmate assay as a random effect. The fixed effects were genetic distance (as measured by F_{ST}), and chemical distance (as measured by the Bray-Curtis dissimilarity matrix), and we also tested for interactions.

Next, we looked separately at the behavior within nestmate and non-nestmate comparisons. For the nestmate combinations (n=26), we were interested in whether an increased genetic or chemical variation within a nest results in increased aggressive interactions. We tested for relationships between the proportion of aggressive behaviors, and two chemical variables (the average chemical differences of individuals, and the variance of individual's chemotypes), and one genetic variable (the average relatedness of individuals in a nest). We used generalized linear models (GLMs) with a quasibinomial distribution and logit link function, with proportion of aggression as the response variable and the three variables of interest as fixed effects with fully factorial interaction.

For the non-nestmate comparisons (n=16), we were interested in whether genetic, chemical or geographic distances were important predictors of aggressive behavior. We tested for relationships between the proportion of aggressive behaviors, and four factors: 1) the geographic distance between colonies, 2) Rho_{ST}

as a genetic measure of distance, 3) Jost's D as a genetic measure of distance, and 4) the average Bray-Curtis chemical dissimilarity of individuals. First, we used all colony combinations and tested the genetic and chemical effects, with each genetic measure tested separately. Then we used a smaller set of colony pairs (n=14) and included geographic effects. For intraspecific *Ca. femoratus* and *Cr. levior* behavior we used only parameters measured for those species. For the interspecific behavior, we used parameters for both species. We allowed interactions between factors of the same species. In all cases, models were compared by model reduction with ANOVAs.

We also tested for more general behavioral responses with a Wilcoxon signed rank test, using the proportion of aggressive behavior as the response variable, and the categorical chemical variables 'within' or 'between' *Cr. levior* chemotype, and 'within' or 'between' population partitions, as defined in the Structure analysis of Chapter 3, with 3 partitions considered for *Cr. levior* and 2 partitions considered for *Ca. femoratus*. We could also make direct comparisons between the behaviour of 5 individual nests towards non-nestmates of similar or different *Cr. levior* chemotypes. We used a one-way matched-pairs t-test to test whether ants were more aggressive to non-nestmates of a different chemotype than to non-nestmates of the same chemotype.

All of these statistical tests were implemented in R v. 3.0.2 (R Development Core Team 2011).

RESULTS

Nestmate recognition behaviors

As expected, and similarly to our previously reported behavioral results (Chapter 1, Emery and Tsutsui 2013), there was significantly more aggression in non-nestmate comparisons that in nestmate comparisons. When accounting for comparison type (whether nestmate or non-nestmate) in the GLMMs, no other effects were significant, suggesting that the nestmate vs non-nestmate distinction supersedes other differences.

Interactions of geographic, genetic and chemical distances on nestmate behaviors

There was a large variation in aggression in nestmate comparisons, with some colonies showing relatively high levels of aggression towards nestmates. This result is not uncommon to recognition studies performed blind (van Wilgenburg and Elgar 2013). When considering only the nestmate behavioral comparisons, there was no significant correlation between the proportion of aggression and either the chemical or genetic similarity of individuals within the colony for either species (Figure 1). This pattern was true for all 4 combinations of behavioral comparisons and variables.

Interactions of geographic, genetic and chemical distances on non-nestmate behaviors

In the matched pairs t-tests, *Ca. femoratus* ants were not more aggressive to non-nestmates from colonies with a different *Cr. levior* chemotype (towards *Ca. femoratus* t-ratio=1.30, dF=4, one-tailed p=0.13; towards *Cr. levior* t-ratio=0.49,

dF=3, one-tailed p=0.33). However, *Cr. levior* ants were more aggressive to non-nestmate *Cr. levior* workers of a different chemotype (t-ratio=2.46, dF=4, one-tailed p=0.03) (Figure 2), which is consistent with findings from the GLM analysis of Chapter 1 and GLM reanalysis of the expanded dataset.

Ca. femoratus conspecific behavior

When considering the proportion of aggressive behaviors in non-nestmate comparisons, and the pairwise geographic, chemical and genetic differences, the best fit model included only geographic distance and Jost's D as a measure of genetic differentiation (Figure 3a). Likewise, pairings that involved ants from different genetic partitions (n=6 between) had more aggression than pairings of colonies from the same genetic partition (n=10 within) (Figure 3b) (Wilcoxon, X^2=10.8, dF=1, p<0.01). When we compared the proportion of aggressive behavior within (n=11) and between (n=5) *Cr. levior* chemotypes, we found no significant trend for *Ca. femoratus* intraspecific behavior (Wilcoxon, X^2=0.21, dF=1, p=0.65).

Cr. levior conspecific behavior

None of the four factors were significant predictors of aggressive behavior (Figure 4a). When considering the genetic partitions, there was no relationship between *Cr. levior* behaviors shown to non-nestmates from similar (within, n=8) or different (between, n=8) genetic partitions (Wilcoxon, X^2=0.91, dF=1, p=0.34). However, when assuming only K=2 genetic partitions, the comparison is significant because the two partitions map perfectly to the chemotype distinctions, and there was more aggression by *Cr. levior* in 'between chemotype' non-nestmate pairings (Figure 4b) (Wilcoxon, X^2=5.50, dF=1, p=0.02). This result is consistent with all previous findings comparing *Cr. levior* aggression and chemotype.

Ca. femoratus interspecific behavior

When considering the aggressive behavior of *Ca. femoratus* in pairings with non-nestmate *Cr. levior*, the best fit model included geographic distance, *Cr. levior* chemical distance, and Jost's D as a measure of *Ca. femoratus* genetic distance (Figure 5 and b). However, when considering the categorical distinctions of *Cr. levior* genetic (Wilcoxon, X^2=2.84, dF=1, p=0.09), *Ca. femoratus* genetic (Wilcoxon, X^2=0.08, dF=1, p=0.77) and *Cr. levior* chemical differences (Wilcoxon, X^2=2.29, dF=1, p=0.13), none of the comparisons were significant (Figure 5c).

Cr. levior interspecific behavior

When considering the aggressive behavior of *Cr. levior* in pairings with non-nestmate *Ca. femoratus*, the best-fit model included geographic distance, *Cr. levior* chemical distance, both measures of *Ca. femoratus* genetic distance and *Ca. femoratus* chemistry (Figure 6 and b). The model also included an interaction between *Ca. femoratus* chemistry and genetics (p=0.05). Categorically, neither *Cr. levior* chemotype (Wilcoxon, X^2=0.14, dF=1, p=0.71), or *Cr. levior* genotype (Wilcoxon, X^2=1.48, dF=1, p=0.22), or *Ca. femoratus* genotype (Wilcoxon, X^2=0.5, dF=1, p=0.48) showed relationships with aggressive behavior (Figure 6c).

DISCUSSION

Our study is the first ever to investigate the chemical, geographic and genetic basis of recognition behaviors in an ant-ant nesting mutualism. Other recognition studies in paraboitic nests have found that *Crematogaster modiglianii* nesting with two chemotypes of *Camponotus rufiffemur* was able to distinguish non-nestmate chemotype, but not within-chemotype differences (Menzel et al. 2009). Similarly to the behavior of *Cr. levior* our study, *Ca. rufifemur* was also more aggressive in conspecific non-nestmate comparisons that involved ants of different chemotypes. We found that both *Cr. levior* and *Ca. femoratus* are more aggressive to non-nestmates of different chemotypes and genotypes than to non-nestmates of a similar type. These combined findings support a recognition ability based on chemotype differences, which are correlated with genetic population structure (Chapter 3).

For *Ca. femoratus*, there was a significant effect of geography and genetics on conspecific non-nestmate aggression. In particular, colony pairs that involved ants from different genetic populations were more aggressive to one another. Despite this increase in aggression to unrelated *Ca. femoratus*, there was no clear chemical basis for aggression. The unsaturated compounds of *Ca. femoratus* profiles co-elute regardless of branching patterns and double bond placements. This technological limitation of GC-MS conceals much of the true chemical variation that may exist on these ants. There was also a pattern of increasing aggression by *Cr. levior* in relation to *Ca. femoratus* genetic partitions. These findings suggest that both ants may be using as-of-yet unaccounted for genetic cues to assess non-nestmate *Ca. femoratus*.

For *Cr. levior*, there were no correlations between continuous genetic and chemical traits, likely due to the high aggression in most non-nestmate conspecific pairs of *Cr. levior*. However, both species showed some variation in aggression towards non-nestmates, and rejection of non-nestmates was no absolute. For the purposes of nestmate recognition, an ability to perceive large differences in chemical or genetic background, such as species identity, is more important than evaluating small-scale differences in chemotype or genotype. Although using such broad recognition rules may lead to recognition errors that allow non-nestmates entry, the collective responses of ants in a colony are sufficient to deter intruders, in spite of these errors (Johnson et al. 2011). Both species are likely able to exclude conspecific non-nestmates, despite some acceptance errors by individuals.

Since we did not sequence or chemically sample the specific individuals involved in the behavioral observations, we cannot completely reject the hypothesis that intracolony aggression is related to more specific chemical or genetic parameters. This caveat needs to be considered for all recognition studies, which often falsely imply direct measurement of recognition through behavioral assays or measurement of colony characteristics. Recognition is a complex cognitive process, with behavior as the outcome. Advances in electrophysiology and neuro-imaging techniques allow more direct assessment of this cognition, but thus far have mixed outcomes and limited explanatory power for general recognition trends (Ozaki et al. 2005).

Variation within populations should also be considered when building these chemical and genetic distance matrices, to avoid skewing distance metrics by sampling a bimodal distribution. For example, in Chapter 3, although we were unable to recover a correlation in genetic and chemical distance matrices when considering *all Cr. levior* ants, there was a significant correlation for matrices of only *Cr. levior* Type B ants. Before rejecting a chemical or genetic component of recognition, researchers should consider broad population patterns in the interpretation of their results.

In summary, we have shown that parabiotic ants maintain functioning recognition systems despite the high potential for chemical and genetic variability in their polygynous, polydomous and polyspecies nests. These recognition systems rely on both chemical and genetic differences to differentiate nestmates and non-nestmates. This study emphasizes the need to consider both broad and fine-scale patterns of differentiation when evaluating nestmate recognition processes. In future, we recommend recognition studies consider several scales of differentiation to avoid spuriously rejecting the chemical and genetic basis of recognition in their system.

Table 1. A summary of 28 previous studies investigating the relationships between nestmate recognition behavior, geography, genetics, and chemistry. Cases where there was a positive relationship with factors and the level of aggressive behavior are highlighted and green, whereas studies that found no effect are in pink. If the particular factor was not investigated, it is blank.

		How does aggressive behavior relate to			Are chemical traits	
Genus	Species	genetic differences?	chemical differences?	geographic distance?	related to genetic traits?	Citation
Dolichoderinae						
Linepithema	*humile*	Positive relationship	Positive relationship	Positive relationship		Blight 2012
Linepithema	*humile*	Positive relationship	Positive relationship		Yes	van Wilgenburg et al. 2009
Linepithema	*humile*	No effect	No effect	No effect	Yes	Vogel et al. 2008
Formicinae						
Anoplolepis	*gracilipes*	Positive relationship	Positive relationship		Yes	Drescher et al. 2010
Anoplolepis	*gracilipes*	Positive relationship		Positive relationship		Gruber et al. 2012
Camponotus	*aethiops*		No effect			Bos et al. 2011
Camponotus	*yamaokai*	No effect		No effect		Satoh and Hirota 2005
Formica	*acquilonia*		Positive relationship			Sorvari et al. 2008
Formica	*exsecta*	No effect	Positive relationship	No effect	No	Martin et al. 2012
Formica	*exsecta*	No effect	No effect	No effect	No	Martin et al. 2009
Formica	*fusca*	No effect			No	Helantera et al. 2011
Formica	*paralugubris*	Positive relationship		Positive relationship		Holzer et al. 2006
Formica	*polyctena*	Positive relationship		No effect		Beye et al. 1997
Formica	*pratensis*	Positive relationship		Positive relationship		Beye et al. 1998
Formica	*seleysi*	No effect				Rosset et al. 2007
Lasius	*flavus*	No effect		No effect		Steinmeyer et al. 2012
Oecophylla	*smaragdina*			Positive relationship		Newey et al. 2010
Plagiolepis	*pygmaea*	No effect		Positive relationship		Thurin and Aron 2007
Myrmicinae						
Atta	*laevigata*	No effect	Positive relationship			Whitehouse and Jaffe 1995
Cataulacus	*mckeyi*	Positive relationship	Positive relationship	Positive relationship	Yes	Debout et al. 2003
Crematogaster	*pygmaea*	Positive relationship	No effect	Positive relationship	No	Hamidi 2012
Leptothorax	*lichtensteini*	Positive relationship				Provost 1991
Monomorium	*pharaonis*	No effect	No effect	No effect	No	Schmidt et al. 2010
Myrmica	*rubra*	Positive relationship	Positive relationship	Positive relationship	Yes	Furst et al. 2012
Pheidole	*megacephala*	Positive relationship	No effect	Positive relationship	No	Fournier et al. 2012
Temnothorax	*spp.*	No effect	Positive relationship			Foitzik 2007
Myrmeciinae						
Myrmecia	*nigriceps*			No effect		van Wilgenburg et al. 2007
Pseudomyrmecinae						
Pseudomyrmex	*pallidus*	Positive relationship				Starks et al. 1998

(Provost 1991, Whitehouse and Jaffe 1995, Beye et al. 1997, 1998, Starks et al. 1998, Debout et al. 2003, Satoh and Hirota 2005, Holzer et al. 2006, Rosset et al. 2007, Foitzik et al. 2007, van Wilgenburg et al. 2007, Sorvari et al. 2007, Thurin and Aron 2008, Brandt et al. 2009b, Vogel et al. 2009, Martin et al. 2009, 2012a, Schmidt et al. 2010, Drescher et al. 2010, Newey et al. 2010, Bos et al. 2011, Helantera et al. 2011, Hamidi et al. 2012, Gruber et al. 2012, Steinmeyer et al. 2012, Fürst et al. 2012, Blight et al. 2012)

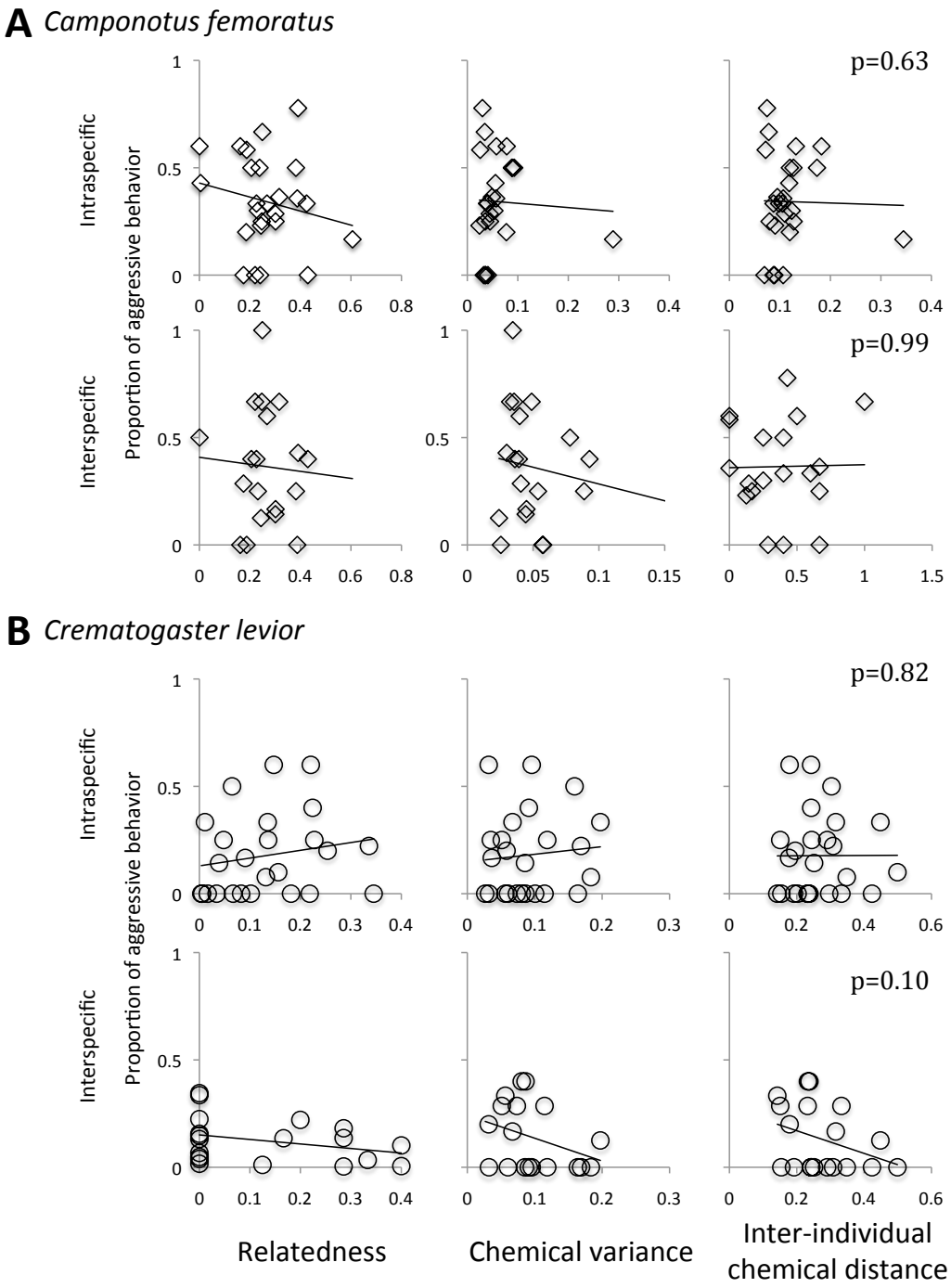


Figure 1. Summary of aggressive behavior in nestmate comparisons, in relation to genetic relatedness, chemical variance and average inter-individual chemical distance. The top six graphs show comparisons involving a) *Ca. femoratus* and the bottom show comparisons involving b) *Cr. levior.* The lines indicate the relationship between variables, but none of the relationships are significant, as determined by the GLM analysis. The p-values indicate the comparison of the full model with all effects, to a null model.

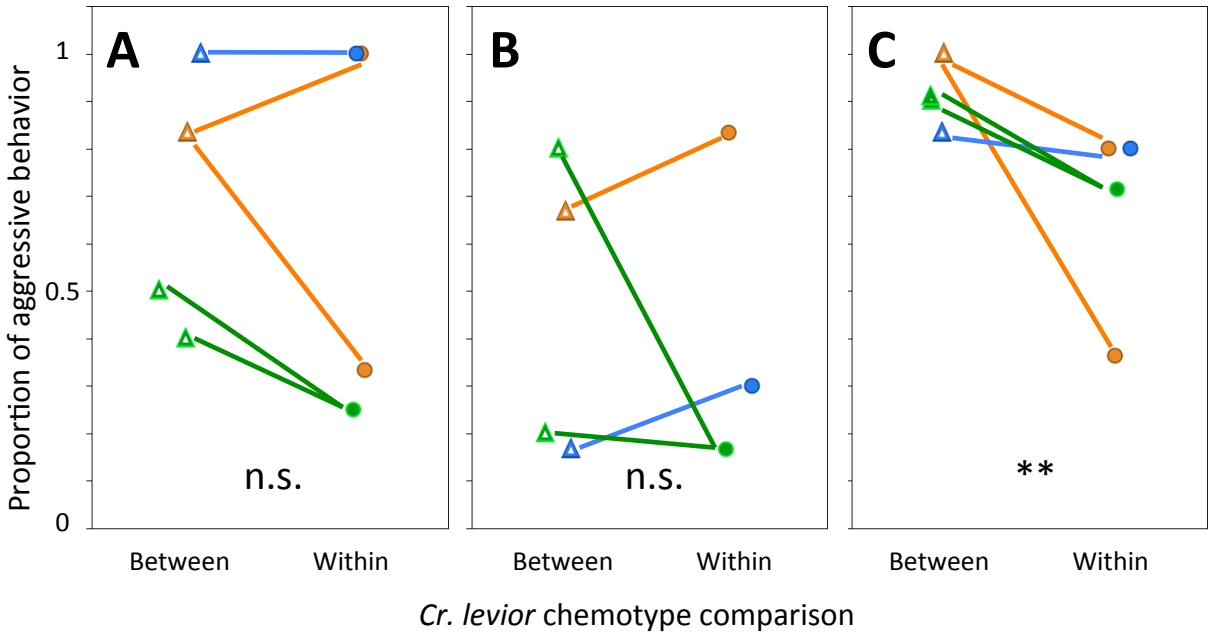


Figure 2. Proportion of aggressive behavior in a) *Ca. femoratus* conspecific, b) interspecific and c) *Cr. levior* conspecific non-nestmate comparisons. The triangles represent between chemotype combinations (one nest of Type A facing one nest of Type B), and the circles represent combinations between two nests of the same chemotype. The lines link the pairings that had a colony in common, to show the relative change in aggressive interactions when facing non-nestmates of a similar chemotype and non-nestmates of a different chemotype. There was only a significant difference for the *Cr. levior* conspecific comparisons.

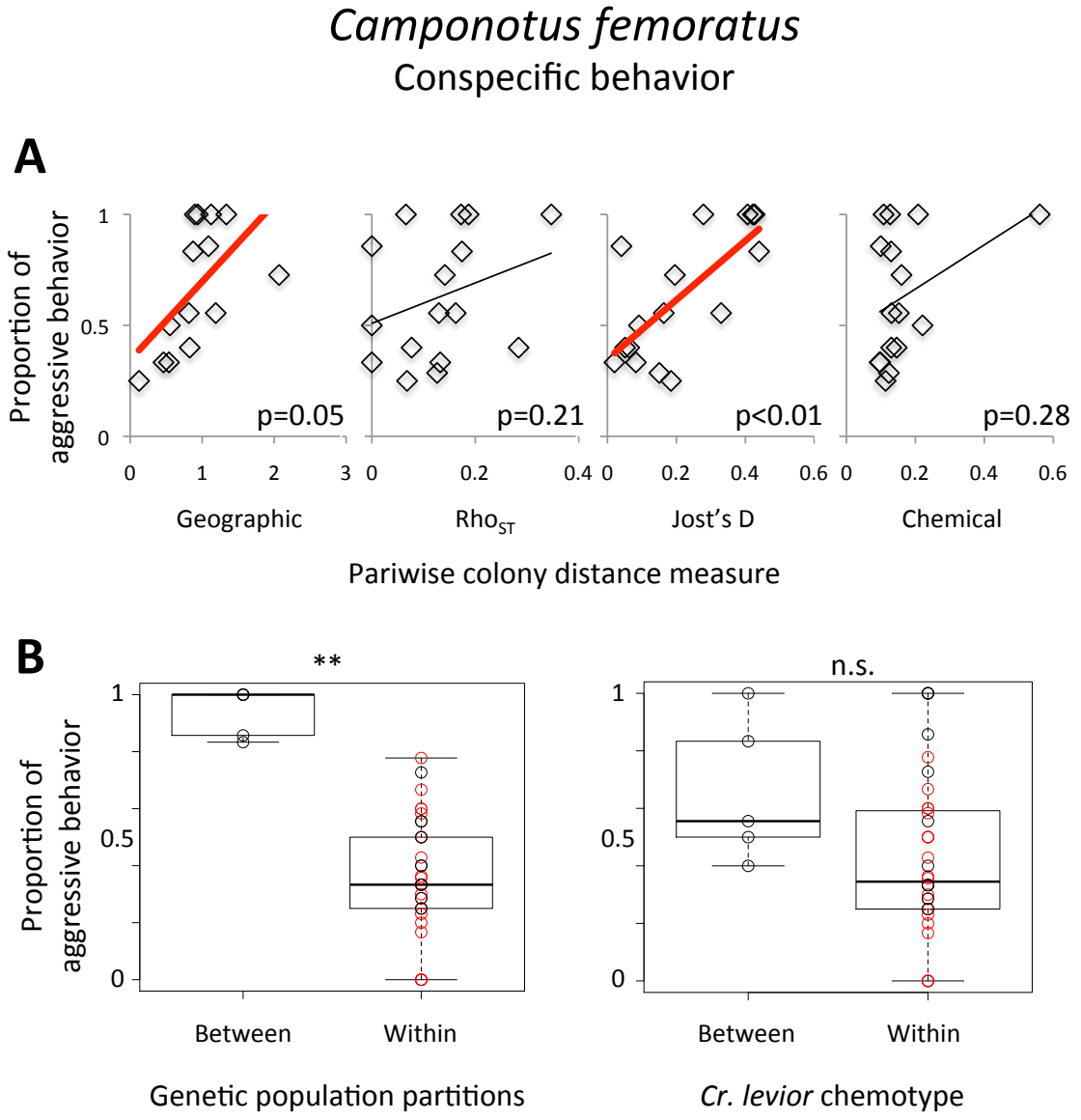


Figure 3. Summary of *Ca. femoratus* conspecific behavior with a focus on non-nestmate comparisons. The proportion of aggressive behavior is shown in relation to a) continuous measures of pairwise distance and b) discrete measures describing the different colony combinations. The black shapes show the behavior towards non-nestmates (n=16 colony combinations), and the red shapes show the behavior towards nestmates (n=26 colonies). The red lines and p-values in a) show the factors that were significant predictors of aggressive behavior in GLM analysis, and in b) the asterisk shows the significant, and n.s. the non-significant Wilcoxon tests between categories.

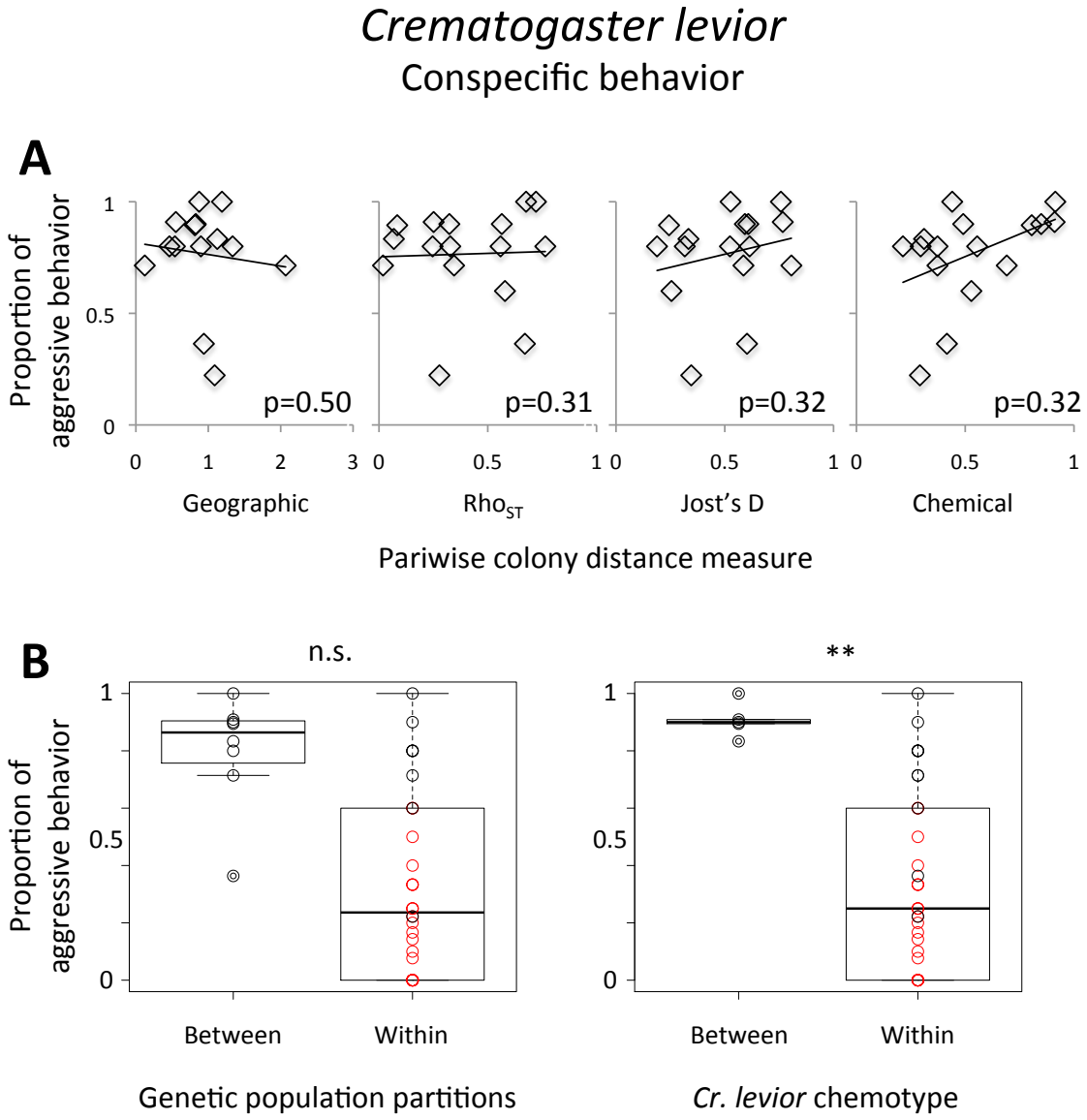


Figure 4. Summary of *Cr. levior* conspecific behavior with a focus on non-nestmate comparisons. The proportion of aggressive behavior is shown in relation to a) continuous measures of pairwise distance and b) discrete measures describing the different colony combinations. The black shapes show the behavior towards non-nestmates (n=16 colony combinations), and the red shapes show the behavior towards nestmates (n=26 colonies). None of the factors in a) were significant predictors of aggressive behavior in GLM analysis, and in b) the asterisk shows the significant, and n.s. the non-significant Wilcoxon tests between categories.

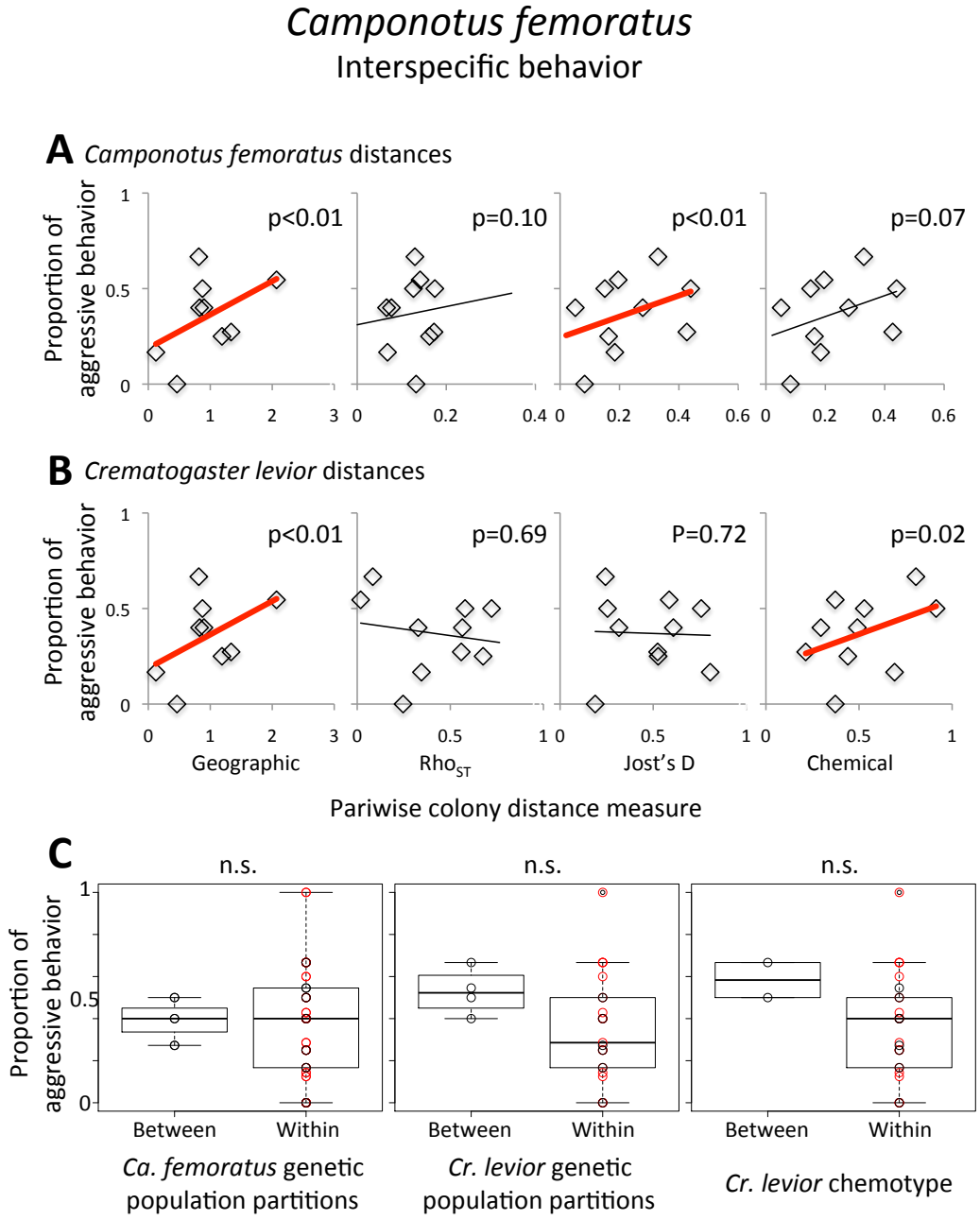


Figure 5. Summary of *Ca. femoratus* interspecific behavior with a focus on non-nestmate comparisons. The proportion of aggressive behavior is shown in relation to a) continuous measures of pairwise distance for *Ca. femoratus* individuals, b) continuous measures of pairwise distance for *Cr. levior* individuals, and b) discrete measures describing the different colony combinations. The black shapes show the behavior towards non-nestmates (n=16 colony combinations), and the red shapes show the behavior towards nestmates (n=26 colonies). The red lines and p-values in a) and b) show the factors that were significant predictors of aggressive behavior in GLM analysis, and in c) none of the Wilcoxon tests between categories were significant.

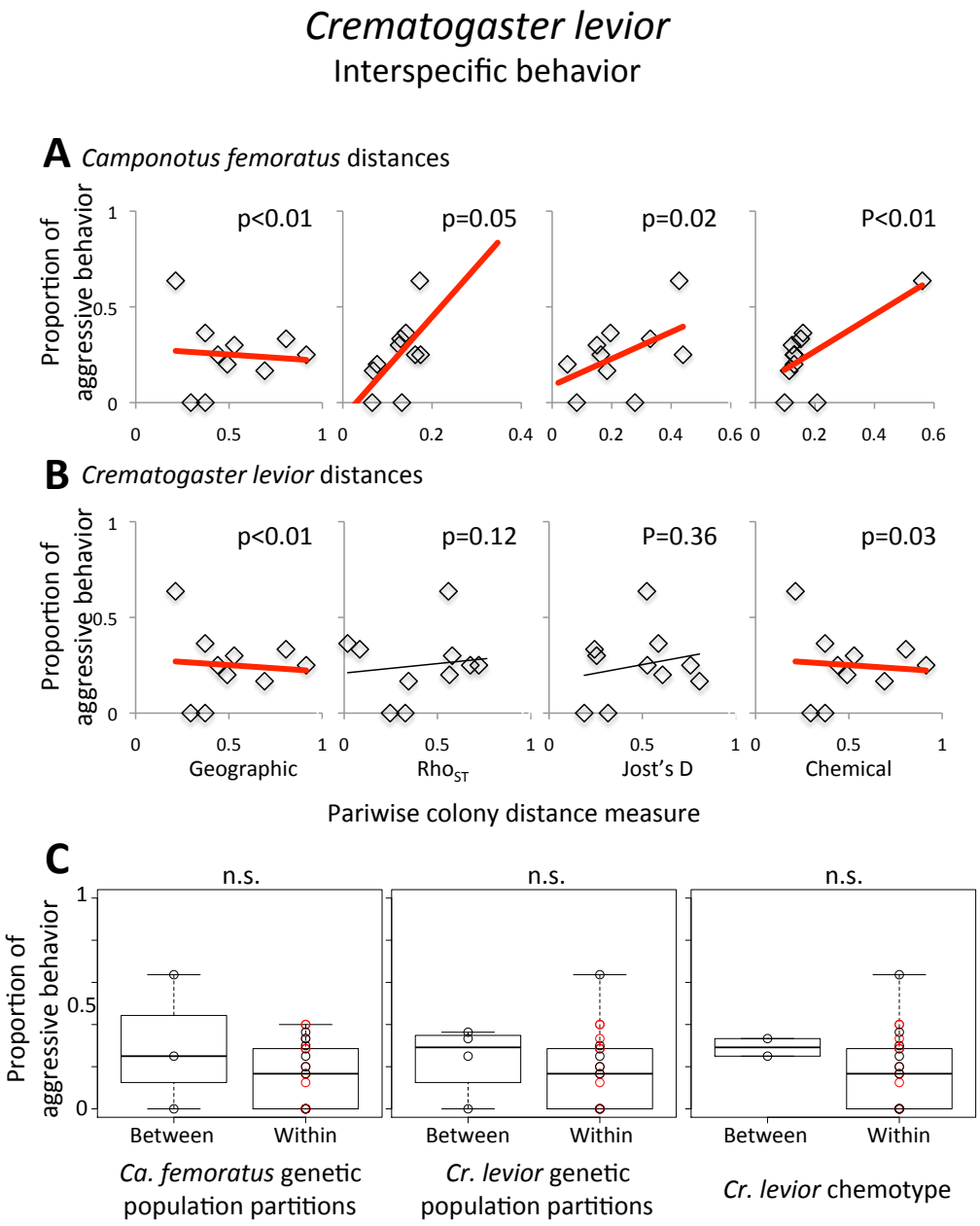


Figure 6. Summary of *Cr. levior* interspecific behavior with a focus on non-nestmate comparisons. The proportion of aggressive behavior is shown in relation to a) continuous measures of pairwise distance for *Ca. femoratus* individuals, b) continuous measures of pairwise distance for *Cr. levior* individuals, and b) discrete measures describing the different colony combinations. The black shapes show the behavior towards non-nestmates (n=16 colony combinations), and the red shapes show the behavior towards nestmates (n=26 colonies).). The red lines and p-values in a) and b) show the factors that were significant predictors of aggressive behavior in GLM analysis, and in c) none of the Wilcoxon tests between categories were significant.

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