Nestmate Recognition and Cuticular Hydrocarbon Profiles in the Ant Formica argentea

Michelle Ochomogo Krasnec
University of Colorado at Boulder, michelle.krasnec@gmail.com

Follow this and additional works at: https://scholar.colorado.edu/ebio_gradetds
Part of the Animal Studies Commons, and the Ecology and Evolutionary Biology Commons

Recommended Citation
https://scholar.colorado.edu/ebio_gradetds/22

This Dissertation is brought to you for free and open access by Ecology & Evolutionary Biology at CU Scholar. It has been accepted for inclusion in Ecology & Evolutionary Biology Graduate Theses & Dissertations by an authorized administrator of CU Scholar. For more information, please contact cuscholaradmin@colorado.edu.
NESTMATE RECOGNITION AND CUTICULAR HYDROCARBON PROFILES IN THE ANT FORMICA ARGENTEA

by

MICHELLE OCHOMOGO KRASNEC

B.S. University of California, Berkeley, 2003

A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy
Department of Ecology and Evolutionary Biology
2012
This thesis entitled: Nestmate recognition and cuticular hydrocarbons in the ant *Formica argentea* written by Michelle Ochomogo Krasnec has been approved for the Department of Ecology and Evolutionary Biology

Committee Members:

________________________________________________________________________________________

Michael D. Breed – Committee Chair

________________________________________________________________________________________

M. Deane Bowers – Committee Member

________________________________________________________________________________________

Alexander Cruz – Committee Member

________________________________________________________________________________________

Darna Dufour – Committee Member

________________________________________________________________________________________

Rebecca Safran – Committee Member

Date__________________

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
Nestmate recognition, the ability to recognize a nestmate from a non-nestmate, is critical to the ecological success of eusocial insects. Although cuticular hydrocarbons are thought to serve as cues in nestmate recognition, little is known about how specific hydrocarbons vary between colonies and which ones are responsible for evoking nestmate recognition behaviors. The aim of my study was, using the ant *Formica argentea*, to investigate cuticular hydrocarbons in great detail by addressing the following questions: (1) What cuticular hydrocarbons are present? (2) Can any one group of compounds statistically classify workers by nest? (3) Which structural classes of hydrocarbons evoke a nestmate recognition response? (4) Do increased differences in cuticular hydrocarbon profiles predict aggression? (5) How do different structural classes of hydrocarbons change with time? After chemical analyses I found that the cuticular hydrocarbon profile of *F. argentea* workers contain a mixture of n-alkanes, alkenes, and methyl-alkanes which stay fairly constant within a nest but vary in relative proportion between nests. Additionally, using the only the C_{29} methyl-alkanes in a hierarchical cluster analysis was enough to correctly classify workers by nest. Behavioral experiments demonstrated that both methyl-alkanes and alkenes increased aggression in workers while n-alkanes did not. Differences in cuticular hydrocarbon profiles between pairs were a good predictor of aggression with differences between particular methyl-alkanes weighing more heavily on the statistical analysis. When kept in a uniform environment the average relative proportions n-alkanes, alkenes, and methyl-alkanes, varied with time, while the relative proportion of the C_{29} methyl-alkanes
remained constant. These finding indicate that all structural classes of cuticular hydrocarbons should not be grouped together or treated equally in nestmate recognition analyses. This study improves our understanding of nestmate recognition and cuticular hydrocarbon profiles in eusocial insects.
# Table of Contents

Abstract ......................................................................................................................................... iii

Table of Contents ...........................................................................................................................v

## Chapter One: Introduction ...........................................................................................................1

Study Species ...................................................................................................................................4
Overview of Dissertation Chapters ..............................................................................................7

## Chapter Two: Eusocial evolution and nestmate recognition systems in social insects ............10

Abstract ......................................................................................................................................11
Introduction ................................................................................................................................12
Recognition theory and phenotype matching .............................................................................15
Neutral substitution and phenotypic variation ............................................................................17
Threshold models for expression of discriminations .................................................................18
Ant chemistry .............................................................................................................................20
*Formica* ants ................................................................................................................................22
Argentine Ants ...........................................................................................................................24
The western honeybee, *Apis mellifera* .......................................................................................26
Social Wasps ................................................................................................................................27
Termites ......................................................................................................................................28
Experimental approaches to recognition studies ........................................................................29
Conclusions ................................................................................................................................30

## Chapter Three: Colony-Specific cuticular hydrocarbon profiles in *Formica argentea* ants 32

Abstract ......................................................................................................................................33
Introduction ................................................................................................................................34
Methods ...................................................................................................................................36
Results .......................................................................................................................................38
Discussion ..................................................................................................................................40
Tables and Figures .....................................................................................................................45

Chapter Four: Recognition cues and perception thresholds to changes in cuticular hydrocarbon profiles in *Formica argentea* .............................................................................................................49

Abstract ......................................................................................................................................50
Introduction ................................................................................................................................51
Methods ......................................................................................................................................54
Results ........................................................................................................................................57
Discussion ..................................................................................................................................58
Figures ........................................................................................................................................62

Chapter Five: Differences in cuticular hydrocarbon profiles and their effects on the behavior of *Formica argentea* .........................................................................................................................68

Abstract ......................................................................................................................................69
Introduction ................................................................................................................................70
Methods ......................................................................................................................................73
Results ........................................................................................................................................76
Discussion ..................................................................................................................................77
Tables .........................................................................................................................................80

Chapter Six: Temporal variation of cuticular hydrocarbons in *Formica argentea* workers: differences among structural classes ..................................................................................................................81

Abstract ......................................................................................................................................82
Introduction ................................................................................................................................83
Methods ......................................................................................................................................85
Results ........................................................................................................................................88
Chapter 1:

General Background and Overview

Michelle O. Krasnec

Department of Ecology and Evolutionary Biology

The University of Colorado, Boulder

Boulder, Colorado, USA 80309-0334
The aim of my dissertation is to use the model system *Formica argentea* to gain insights into social recognition by investigating cuticular hydrocarbon profiles and nestmate recognition in great detail. I aim to understand which structural classes of hydrocarbons are important in providing a colony-specific profile, which compounds act as nestmate recognition cues and influence aggression, and how these compounds can vary with time. The ultimate goal of this study is to achieve an understanding of cooperation and social recognition in eusocial insects.

Cooperation has been of interest to evolutionary biologists since before Darwin and is the central evolutionary force favoring social behavior. Social recognition, the ability to identify social group members, is a key mechanism underlying cooperation. For individuals to cooperate they must have the capability to recognize those with whom they are cooperating. Social recognition relies on phenotypic variation among animals within populations; this variation provides cues for social discriminations. Many animals can use more than one sensory mode in recognition. For example, recognition cues can be visual, as in humans, auditory, as in birds, crickets, and frogs (Gerhardt 1978; Ligon 1991; Pollack and Hoy 1979) or olfactory, as in some mammals and insects (Breed 1998a; Halprin 1991). The importance of social recognition in social structures makes studies of this phenomenon significant. Investigations focusing on mechanisms, such as social recognition, that are important in eusocial insects can provide particularly important insights into the evolution of social recognition.

In this study I focus on social recognition in an ant species in the context of nestmate recognition. Nestmate recognition, the ability to discriminate a nestmate from a non-nestmate, is a vital component of eusocial life that facilitates cooperation. Although the cuticle primarily functions to protect insects from desiccation and infection, cuticular chemicals play key roles in communication in many insects (Blomquist and Dillwith 1985; Hadley 1985; Howard and
Blomquist 1982; Lockey 1988; Sugumaran 1996). In nearly all species of social insects, cuticular hydrocarbons play an important role in nestmate recognition (Breed 1998a; Dani et al. 2001; Ruther et al. 2002; Thomas et al. 1999; Wagner et al. 2000). Eusocial insects detect cuticular hydrocarbons through antennal contact; chemical information is received and then processed for nestmate recognition (Le Moli et al. 1983).

While the role of cuticular compounds in social recognition is well established, our understanding of how variation in surface chemicals is interpreted by an individual is much less well developed (Howard 1993). Crozier and Dix (1979) proposed a "Gestalt Model" which describes one well-supported idea of how nestmate recognition occurs. In this model, individuals from the same nest share a set of recognition cues to form a common nest odor. The collective profile of the cue is considered a template which can be learned by colony members as the shared odor of the nest. The learned template is then compared to the detected hydrocarbon profile in social discriminations. If the detected hydrocarbons are different from the nest template, and the visiting insects’ phenotype does not match that of individual it encounters, it will be considered an intruder and be prevented from entering the nest (Lacy and Sherman 1983; Sherman et al. 1997). The use of a learned template is sometimes referred to as phenotypic matching (Lacy and Sherman 1983; Sherman et al. 1997). Phenotypic matching facilitates cooperation in animals and may be the key to understanding cooperation in social insects.

In the case of social insects, phenotypic diversity in cues is critical in using phenotypic matching for nestmate recognition. There must be ample cue phenotypes available in the population so that each nest is different enough from one another to allow distinction, however maintaining too many cue phenotypes may be costly (Breed and Buchwald 2008). Cue diversity
greatly influences the expression of nestmate recognition and warrants close examination in studies of social recognition.

Study Species

*Background*

The ecological success of ants is mainly due to their complex social organization (Hölldobler and Wilson 1990). They can be the leading predator on invertebrates in some ecosystems, as well as major herbivores in others (Wilson and Hölldobler 1990; Wilson and Hölldobler 2005). Recently revised molecular phylogenies of the ants have helped determine the sequence of events that have allowed ants to diversify and become ecologically dominant (Brady et al. 2006; Moreau et al. 2006). The rise of angiosperms and herbaceous insects coincide with the diversification of ants. The rise of angiosperms may have led to the diversification of ants because angiosperms produce more diverse litter that could provide a variety of suitable habitats for ants (Moreau et al. 2006) and a greater abundance of herbaceous insects may have led to the rise of ants by providing a direct food source (Moreau et al. 2006).

My study focuses on a species in the subfamily Formicinae, a species-rich, more recently diverged monophyletic group (Brady et al. 2006). The Formicinae evolved either 77-83MYA (Brady et al. 2006) or 92-101MYA (Moreau et al. 2006). Within this diverse subfamily I studied nestmate recognition in the genus *Formica*.

While this is a large genus, some generalizations can be made about *Formica* species. Ants in this genus are typically ground nesters, and the genus is more speciose in the temperate zones than in the tropics (Hölldobler and Wilson 1990). There are currently 45 described species of *Formica* in Colorado, where nests are commonly found under rocks, logs, near tree roots, and sometimes in bare soil (Gregg 1963, Bolton et al. 2007). *Formica* are divided up into several
species groups (*fusca*, *rufa*, *exsecta*, *sanguinea*, *pallidefulva* and *neogagates*) (Gregg 1963). Most species in this genus are scavengers, collecting dead arthropods, but they are also commonly tend aphids and extrafloral nectaries. Members of the Formicinae, including the genus *Formica*, lack a sting; for defense they produce formic acid which is exuded from a pore on the underside of the abdomen when the ant is disturbed (Hölldobler and Wilson 1990). This lack of a sting means that members of *Formica* are not typically aggressive predators.

Members of the genus *Formica* can be both hosts to social parasites and be parasitized by other species of ants (Chernenko et al. 2011; Czechowski 1994; Lenoir et al. 2001). In Colorado, *F. argentea* colonies are often parasitized by the ant *Polyergus breviceps* (Bono et al. 2007), an ant in the genus most closely related to *Formica* (Moreau et al. 2006). In ants, successful parasitism is contingent on invasion of the host colony, as well as the ability to remain in the host colony without being excluded or killed. The ability to both invade and remain in a nest is dependent on cuticular chemistry (Lenoir et al. 2001). Newly eclosed workers often times have only a small amount of cuticular hydrocarbons on their exoskeletons, which allows them to be virtually undetected in a nest; as time passes they acquire the colony odor (Errand et al. 1992; Lenoir et al. 1997; Soroker et al. 1995). Invaders may also use chemical mimicry to enter and remain in a nest (Dettner and Liepert 1994; Howard 1993; Stowe 1988). Regardless of the mechanisms social parasites use to be successful in parasitizing a colony, cuticular chemistry plays a vital role and deserves investigation in groups, such as *F. argentea*, which can be susceptible to social parasitism.

In order to adequately examine nestmate recognition and cuticular hydrocarbon profiles, it is vital to study an organism that is easy to manipulate and that exhibits strong nestmate recognition. *Formica argentea* (*F. fusca* group) is an ideal model organism for this investigation.
*Formica argentea* exhibits strong nestmate recognition (Bennett 1988). Colonies are typically small, around 100-150 workers, which makes it easy to collect entire colonies and rear them in the lab for experiments. Additionally, the hydrocarbon profile of *F. argentea* is complex enough to experimentally manipulate yet simple enough to examine the effects of individual compounds on ant behavior (see chapter 3). Little is known about *F. argentea* as only few published studies employ this species (Bennett 1988; Bennett 1989a; Bennett 1889b, Bono et al. 2006; Bono et al. 2007; Ouellette 2010; Snyder 1992; Snyder 1993).

**Formica cuticular hydrocarbons and nestmate recognition**

Recent studies have begun to provide information about cuticular hydrocarbon diversity and nestmate recognition in the genus *Formica*. In *Formica* it appears that cuticular hydrocarbon profiles have evolved via elevated production of (Z)-9-alkenes or via the increased production of methyl-alkanes (Martin et al. 2008a). The cuticle of *F. exsecta* contains six n-alkanes and six alkenes ranging from C$_{21}$-C$_{31}$ (Martin et al. 2008b). On the other hand the cuticular hydrocarbon of *F. japonica* (*F. serviformica* group), is far more complex consisting of n-alkanes, alkenes, methyl-alkanes and alkadienes ranging from C$_{25}$-C$_{48}$ (Akino 2006). The profile of *F. fusca* contains three n-alkanes and fourteen methyl-alkanes ranging from C$_{23}$-C$_{27}$. The cuticular hydrocarbons found on species of this genus are typically similar enough among colony members and different enough between colonies to statistically distinguish workers by colony using principal components or cluster analysis (Akino 2006; Martin and Drijfhout 2009a). In all cases the compounds found on ants within a species do not vary in identity; the variation is in the relative proportion of the compounds.

Although there has been much progress in identifying the cuticular hydrocarbon profiles in *Formica* ants, little is known about which compounds in the cuticular profile are important in
nestmate recognition. Hierarchical cluster analysis has shown that the (Z)-9-alkene signature in *F. exsecta* is enough to cluster workers by nest (Martin et al. 2008b) and behavioral data has directly linked (Z)-9-alkenes to nestmate recognition behavior (Martin et al. 2008b). Unlike in *F. exsecta* where alkenes alone were enough to evoke a nestmate recognition response, in *F. japonica* a mixture of both n-alkanes and (Z)-9-alkenes were necessary for individual workers to discriminate a nestmate from a non-nestmate (Akino et al. 2004). In *F. fusca* (*F. fusca* group) including only the C_{25} dimethyl-alkanes in a hierarchical cluster analysis was enough to accurately cluster individual workers by nest (Martin et al. 2008c). Although there is statistical evidence indicating the importance of methyl-alkanes in nestmate recognition in *Formica* ants the link between chemical variation and behavior has not been made in this genus. Using *Formica argentea* as a model species, the aim of my dissertation is to investigate nestmate recognition and cuticular hydrocarbon profiles in great detail and to test the link between cuticular hydrocarbons and nestmate recognition.

Overview of Dissertation Chapters

In the second chapter of my dissertation I provide a comprehensive summary of eusocial evolution and recognition system in social insects. I present an overview of current theory, research and experimental approaches being applied in the field of nestmate recognition. This overview provides framework for how my work on social recognition fits within the current field of study.

The third chapter of my dissertation contains identifications of the compounds present on the cuticle of *F. argentea* and aims to identify a colony-specific cuticular hydrocarbon signature. I found that *F. argentea* have 28 cuticular hydrocarbons present on their exoskeleton ranging in carbon chain length from C_{25}-C_{38}. Their cuticular hydrocarbon profile contains six n-alkanes,
four alkenes, and eighteen methyl-alkanes. A hierarchical cluster analysis revealed that using only the methyl-alkanes with a C\textsubscript{29} carbon backbone in the analysis was enough to cluster individual workers by colony.

In chapter four I look at different structural classes of cuticular hydrocarbons to see which ones evoke a nestmate recognition response in \textit{F. argentea}. I also examine different concentrations of the compounds that elicit an aggressive response to see if there is a perception threshold to changes in cuticular hydrocarbon profiles. Increasing the concentration of methyl-alkanes present on the hydrocarbon profile of one ant from a pair of nestmates increased aggression. Increasing the concentration of alkenes also increased aggression. However, altering the concentration of n-alkanes on the hydrocarbon profile of one ant from a pair of nestmates did not affect nestmate recognition behavior. Pairs of nestmate ants did not react to altered differences between their hydrocarbon profiles until a lower threshold for differences between the ants was met.

In the fifth chapter of my dissertation I test the question of whether increased differences between the hydrocarbon profiles of pairs of ants statistically predict aggression among ants. I also look at the differences in particular compounds between a pair of ants to see if those differences can predict overall aggressive behavior and the more specific highly aggressive behaviors of dragging and spraying. I found that differences in the cuticular hydrocarbon profiles do influence aggression. In support of my finding from chapters three and four, I found that difference in methyl-alkanes were the best at predicting overall aggression and spraying behavior while dragging behavior was mostly predicted by a difference in an alkene.

The sixth chapter of my dissertation focuses on nest-wide changes in the cuticular hydrocarbon profile of \textit{F. argentea} as a function of time. I found that, when kept in a uniform
environment, the total amount of cuticular hydrocarbons present on *F. argenta* workers, regardless of structural class, increased through a sixteen-week period. Interestingly the results varied when examining the relative proportion of the structural classes of compounds rather than the total amounts. I found that the relative proportion the C_{29} backbone methyl-alkanes did not vary with time while the proportions of n-alkanes, alkenes and methyl-alkanes did vary with time. These results suggest that although the hydrocarbon profile is changing with time, structural classes of hydrocarbons behave differently, with respect to relative proportion.

In the final chapter I tie together all of my dissertation research into the framework of social recognition and summarize the conclusions presented in the preceding chapters.
Chapter 2:

Eusocial Evolution and the Recognition Systems in Social Insects

Michelle O. Krasnec

Department of Ecology and Evolutionary Biology

The University of Colorado, Boulder

Boulder, Colorado, USA 80309-0334

Slightly Modified From:

Abstract

Eusocial species, animals which live in colonies with a reproductive division of labor, typically have closed societies, in which colony members are allowed entry and non-members, including animals of the same species, are excluded. This implies an ability to discriminate colony members (“self”) from non-members (“non-self”). I draw analogies between this type of discrimination and MHC-mediated cellular recognition in vertebrates. Recognition of membership in eusocial colonies is typically mediated by differences in the surface chemistry between members and non-members, and I review studies which support this hypothesis. In rare instances, visual signals mediate recognition. I highlight the need for better understanding of which surface compounds actually mediate recognition, and for further work on how differences between colony members and non-members are perceived.
Introduction

In the eusocial animals recognition of group membership is an essential component of evolutionary success (Wilson 1974). In this chapter I introduce the defining characteristics of eusocial species and their colonies. I then develop an analogy between recognition of group membership and other types of “self” versus “non-self” recognition. I develop some key theoretical issues, including phenotype matching, neutral substitution in the evolution of signal diversity, and response thresholds. Recognition of group membership has been well studied in ants, honeybees, wasps and to a certain extent in termites; I review examples in each of these types of eusocial insects.

A eusocial species is one in which colonies are formed by family groups (Choe and Crespi 1997). Some of the young in the colony have permanently diminished reproductive capacities and devote their lives caring for their sibs, defending the colony, collecting food for the colony, and constructing the nest in which the colony lives. This reproductive division of labor results in a reproductive caste, the queen (and sometimes a king) and a non-reproductive worker caste within the colony (Choe and Crespi 1997). The workers, in some instances, are further subdivided into specialized groups that perform specific tasks, like colony defense. Many of the commonly mentioned examples of eusocial species are members of the insect order Hymenoptera (Hölldobler and Wilson 1990; Hunt 2007; Michener 1974; Wilson 1974). These include honeybees (Apis), bumblebees (Bombus), ants (family Formicidae), all of which are eusocial, and social wasps, such as paper wasps (Polistes), and yellowjackets and hornets (family Vespidae, genera Dolichovespula, Vespula and Vespa). Termites, the insect order Isoptera, are also all eusocial. In addition to these species, which are all frequently encountered in the temperate zones, the stingless bees (genera Trigona and Melipona, and their relatives) and
numerous species of eusocial wasps in the vespid tribe Epiponini are found in the tropical zones. Eusocial insect colonies are remarkable for their coordination of labor among colony members and, in some cases, for their very aggressive and effective mechanisms of colony defense.

In recent years, eusociality has been discovered in a variety of other types of animals (Costa 2006). Perhaps most notable is the naked mole rat (family Bathyergidae, genus *Heterocephalus*). These small mammals, which occur in the southern part of Africa, have societies that are remarkably analogous to those of eusocial Hymenoptera and Isoptera (Clarke and Faulkes 2001; Faulkes et al. 1991; Sherman et al. 1991). Colonies of naked mole rats live in complex tunnel systems and typically have 50-100 individuals. Eusocial shrimp (genus *Synalpheus*, in the Decapoda) live within marine sponges (Duffy et al. 2002). Thrips (family Thysanoptera) and Aphids (families Hornaphididae and Pemphigidae), are both plant-feeding insects which contain species that nest in galls in the plant tissue and produce a defensive caste, soldiers, that defend the gall at the expense of individual reproductive capacity (Aoki 1980; Aoki 1987; Kranz et al. 2002).

A defining characteristic of eusocial colonies is their closed membership (Fletcher and Michener 1987; Starks 2004; Vander Meer et al. 1997). Like a multicellular organism, membership in the colony is “self”, and non-members, even if they are of the same species, are treated as “non-self”. Separation of “self” from “non-self” allows eusocial colonies to prevent invasion by parasites and predators (Vander Meer et al. 1997). This is a nearly perfect analogy to the function of the immune system in multicellular organisms, and it is worth noting that in many vertebrates odors correlated with variation in the major histocompatibility loci (MHC) facilitate social recognition processes (Penn 2002).
Colony closure can center at entrances to the nest or may extend to territorial boundaries that are distant from the nest. In the ecotypes of the western honeybee, *Apis mellifera*, found in Europe and most of North America, guard bees at the nest entrance examine incoming insects and exclude bees from other colonies as well as other species (Moore et al. 1987). The major cost of admitting non-nestmates is the risk of having honey-stores robbed, and weak colonies, which cannot effectively defend themselves, may be decimated by intraspecific robbing (Breed and Buchwald 2008). Western honeybee colonies are sometimes clustered in nature because acceptable nesting habitats—hollow trees or small caves—occur in close proximity to each other. Intraspecific nest defense in the western honeybee occurs only at the nest entrance. In other species aggressive interactions may also occur at flowers (Michener 1974). Other ecotypes of *Apis mellifera* may defend a much larger perimeter against potential vertebrate predators, but this extended defended area does not result in territorial aggression against other bees (Breed et al. 2004).

In contrast, harvester ants (genus *Pogonomyrmex*) aggressively defend not just their nest, but also an extended area around the nest in which they forage (Hölldobler 1976). This exclusion limits competition for food and results in colonies being evenly distributed across the habitat. Similarly, many species of the tropical stingless bees are aggressive at flowers in their colony’s foraging range; this also results in an even distribution of colonies within the environment. The evolutionary trade-offs that result, in some cases, in defense of the nest only and in other cases in defense of a feeding territory are not well understood. In both types of defensive systems, discrimination of colony members (“self”) from non-colony members (“non-self”) is a critical behavioral element.
There are exceptions to colonial closure among eusocial organisms and these exceptional cases merit some discussion here. In some instances, colonies of a eusocial species are isolated from other colonies of the same species, or have no significant problems with social parasites or problems with robbing, and probably as a consequence, show little expression of aggressive behavior to non-nestmates. *Apis cerana*, the eastern honeybee, fits this pattern (Breed et al. 2007). Some ant species are adapted for colonization of disturbed habitats and rapid colony expansion so that a single large colony occupies a large habitat patch. These species termed unicolonial—all ants in a population belong to the same large colony (Bourke and Heinze 1994). Unicolonial ants are often polygynous (colonies have many queens) and polydomous (a single colony occupies many nests); in these species exclusion of non-nestmates is often not expressed. *Formica podzolica*, an ant common in subalpine habitats in North America, is a good example of this social lifestyle (De Heer and Herbers 2004). Some invasive ant species, such as the Argentine ant, *Linepithema humile*, adopt a similar strategy of unicoloniality (Holway and Suarez 1999).

In sum, eusocial colonies can conveniently be viewed as multiorganism assemblages that are analogous to multicellular animals. While this superorganism analogy has limitations, it provides an excellent frame of reference for thinking about the evolution of closed societies and the importance of recognizing “self” and “non-self” in social interactions. In the next section I extend this argument to a discussion of how recognition phenotypes are constructed and perceived.

**Recognition theory and phenotype matching**

The closure of eusocial colonies relies on two mechanisms. First, there must be phenotypic features that differentiate among colonies (Breed and Buchwald 2008; Vander Meer
et al. 1997). Second, animals within a colony must be able to use this phenotypic information to discriminate members from non-members, and must be able to act in ways that exclude non-members from the colony (Breed et al. 2004).

Phenotypic variation among colonies could occur in any conceivable signaling modality. Chemical cues (Vander Meer et al. 1997), visual characteristics (Tibbetts 2002), or audible signals seem, from a human point of view, to be the most plausible, but we should not lose sight of the fact that animals can use unexpected and therefore surprising means of communication. Having made this point, the overwhelming preponderance of evidence from insects suggests that chemical cues, perceived either as volatiles or by contact chemoreception, are the recognition phenotype for the vast majority of eusocial insects (Vander Meer et al. 1997). Most eusocial insects use hydrocarbons from the cuticle in phenotypic matching for nestmate recognition. In a few eusocial wasps, white or yellow markings in the cuticle, called maculations, vary among individuals and are used as visual recognition phenotypes (Tibbetts 2002).

For small colonies of eusocial animals, individual distinctiveness of the phenotypes of colony members is possible, and colony members may recognize each as discrete individuals (Breed and Bennett 1987; Tibbetts 2002). For larger colonies the sheer number of animals and the likelihood that any pair of colony members will meet infrequently during their life argues against individually distinct phenotypes (Breed and Buchwald 2008). In these species the most efficient way to accomplish recognition of colony membership is for the members to all carry the same phenotype. This could come by merging of individual phenotypes due to workers rubbing together within the nest, to the function of a gland that establishes a common (or gestalt) odor, to shared nesting materials, or to the secretion of a unique labeling mixture by the queen. All of the
mechanisms have been demonstrated, and no single rule dictates how the shared phenotype is established in eusocial colonies (Carlin 1989; Crosland 1989a; Crosland 1989b).

Animals that need to make discriminations, such as entrance guards, can then learn the phenotype of their colony and use that information in excluding non-nestmates, even if they are contacting individuals for the first time. This mechanism is termed phenotype matching and is likely the most generalizable rule in social recognition in eusocial animals (Breed and Buchwald 2008). Through phenotype matching, colony members gain a template that identifies “self” and then compare that template with the cue profile of animals they encounter. Phenotype matching is also used in social discriminations in mammals, and is particularly well studied in rodents.

Returning to the chemical cues used for discriminations, in nearly all cases hydrocarbons secreted to the outer cuticle of the insect form the basis for the recognition phenotype (Boulay 2000). These hydrocarbons probably first evolved as cuticular waterproofing and were later co-opted for social recognition. Cuticular hydrocarbons are known to serve as social signals in some other types of insects, such as the sex pheromone of houseflies, which is (Z)-9-tricosene (Wicker-Thomas 2007). The following sections present our knowledge of recognition chemistry in eusocial animals in more detail.

**Neutral substitution and phenotypic variation**

The MHC loci code for hypervariable phenotypes, which give vertebrates the flexibility to respond to novel parasites and pathogens. This phenotypic variability provides a perfect backdrop for recognition of kin or individuals, as it provides the basis for individually unique external phenotypes. Breed and Buchwald (2008) argue that when the functional requirements for a phenotype in one context are met, then “neutral substitution” of aspects of that phenotype can enhance the use of those characteristics in social recognition. For example, the morphology
of the human face is constrained by the need for a functioning jaw, open airways through the nares, and appropriately aligned eyes. Facial phenotypes, though, are hypervariable within these constraints, facilitating recognition. Noses that are wide, narrow, long, pug, or flat all serve equally well in breathing, so substitution among these shapes is neutral with respect to function but provides variability that can help to distinguish individuals.

In insects, cuticular hydrocarbons probably evolved as waterproofing and later in evolution were co-opted as social signals. Effective waterproofing requires hydrophobic compounds that will not crystallize at ambient temperatures. Many hydrocarbons of various carbon chain lengths can serve this waterproofing function. In addition, of these hydrocarbons, alkanes, alkenes, and methyl-alkanes, which are found on insect cuticles, are all effective. This means that cuticular hydrocarbon phenotype can vary substantially—facilitating social recognition—without impairing the insect’s water balance. In honeybees, fatty acids strengthen comb wax. A number of fatty acids serve equally well to enhance the mechanical properties of the wax, but can be neutrally substituted to generate variable recognition phenotypes (Buchwald et al. 2009).

Threshold models for expression of discriminations

In 1989 Reeve, investigated recognition with a unique approach. He wanted to learn what factors affect nestmate recognition in a way to maximize an organism’s inclusive fitness. Reeve investigated what constitutes an optimal acceptance threshold for the guards of a nest. This model assumes that nestmates and non-nestmates have overlapping recognition cues, which make it likely that recognition errors will occur. Guards that are too strict with their acceptance threshold may inadvertently reject true nestmates while those that are too lenient may incorrectly allow non-nestmates into the nest. With this thinking, Reeve introduced the idea that the optimal
acceptance threshold would be one that varies with the cost of accepting a non-nestmate, benefits of accepting nestmates and the frequency in which nestmates and non-nestmates are encountered.

In 2000, Downs and Ratnieks applied Reeve’s theoretical model to honeybees in the field. They found that the acceptance threshold is dependent on ecological conditions and may shift. For example, as nectar conditions improved honeybee guards were less selective about whom to allow into the nest because there was not a great cost associated with allowing an intruder into the nest. Ecological changes influence the behaviors of guards at both an individual and colony level. By rapidly increasing the number of non-nestmate intruders encountered by a guard Couvillon et al. (2008) showed that changes in the acceptance threshold of both individual guards and the colony could occur within 15 minutes. Within individual guards, mean acceptance of nestmates and non-nestmates declined. At the colony level the mean number of guards at the entrance increased (Couvillon et al. 2008).

The most common context in which the response threshold model may apply is seasonal variation in defensiveness. Defensiveness should be highest under conditions of intense competition, or when food stores within the colony are relatively large, and should be lower when competition is less fierce. In an ant, Plagiolepis pygmea, Thurina and Aron (2008) found that aggressiveness among colonies varied seasonally, peaking in the spring, when intercolonial competition for food may be at its highest. However, Kudo and Zucchi (2008) found that in a eusocial wasp, Polybia paulista, expression of nestmate recognition remained constant through the year, even though seasonally shifting competition had caused the authors to predict that they would find shifting thresholds. This is an area in which further studies will define the phenotypic flexibility of animals in the expression of social discriminations.
Ant chemistry

Most ants behave cooperatively with nestmates and exclude alien conspecifics from their nests. Typically this occurs when individuals from the same nest share a recognition cue to form a common nest odor. The collective profile of the cue is considered a template which can be learned by colony members as the shared odor of the nest. The learned template is then compared to the detected hydrocarbon profile in social discriminations. If the detected hydrocarbons are different from the nest template, then visiting insects will be considered intruders and be prevented from entering the nest (Vander Meer and Morel 1998). The use of a learned template is sometimes referred to as phenotypic matching, which I discussed above. During phenotypic matching individuals are identified as familiar and unfamiliar by having formed a template of kin (or nestmates) by learning the phenotypes from familiar individuals (or nestmates) (Lacy and Sherman 1983; Schausberger 2007).

Studies in ants have shown that the postpharyngeal gland (PPG) is important in forming nestmate recognition cues (Boulay et al. 2000; Hefetz et al. 1996; Soroker et al. 1994). After extracting hydrocarbons from the PPG of ants, Sorokoer et al. (1995) found that hydrocarbon composition in the PPG is species specific and these hydrocarbons are similar to those found on the cuticle (Soroker et al. 1995). These finding are important because the maintenance of the colony odor requires the continuous production of recognition cues that are provided by the PPG (Soroker et al. 1995; Soroker et al. 1998). The PPG is involved in the active exchange of cuticular hydrocarbons via allogrooming or trophallaxis (Soroker at al. 1994; Soroker et al. 1995).

Cuticular hydrocarbons are thought to play an important role in nestmate recognition. Cuticular hydrocarbons consist of n-alkanes, n-alkenes, and methy-lalkanes and can range in
carbon chain length from about 21 to greater than 40 carbons (Nelson and Blomquist 1995). Hydrocarbons tend to be highly species specific and intraspecific compounds typically vary in relative proportions that can be colony-specific. Because of the low volatility of cuticular hydrocarbons, acquisition of the common nest odor typically comes from the exchange of cuticular hydrocarbons via allogrooming or trophallaxis (Soroker et al 1994). Recent studies, however, have found that mixed species of ants living in close proximity, but not able to touch, can become familiarized with the neighboring species hydrocarbon signal (Errard 2008). This suggests that in addition to tactile olfactory cues volatile cues may also affect nestmate recognition template formation.

Studies on ants have examined the effects of the three classes of cuticular hydrocarbons on behavior and, although still controversial, some patterns have emerged. The profiles shown by gas chromatography may not be identical to those perceived by the insect (Dani et al. 2005; Martin et al. 2008b). Dani et al. (2005) found that in honeybees, changes in the alkene pattern and not the n-alkane pattern affects nestmate recognition. Martin et al. (2008b) determined that nestmate recognition signals in the ant *Formica exsecta* come from (Z)-9 alkene signatures even though there are other compounds present on the cuticle. Further investigation into this phenomenon by Martin and Drijfhout (2009b) suggest that the n-alkane component of the hydrocarbon profile is independent of the nestmate signal and is strongly influenced by worker task. These finding suggest that in some species of *Formica* ant the (Z)-9 alkene signature is sternly influenced by genetic factors while the n-alkane signature is influenced by environmental factors. This finding differs from previous cuticular hydrocarbon studies that typically combine all the hydrocarbons on the cuticle and perform multivariate statistics rather than by separating compound classes. Although there is evidence that alkenes are the most important hydrocarbon
class to nestmate recognition, in the ant *Formica japonica* both differences in the alkene and n-alkane signatures are necessary to elicit and aggressive response (Akino et al. 2004). Green and Gordon (2007) also found that in *Linepithema humile* behavioral nestmate recognition responses only occurs when there are mixtures of hydrocarbon structural classes. In other words, the response may be due to the structural complexity of the signal.

Although it seems that some eusocial insects use specific hydrocarbon structural classes for recognition and others use a mixture of classes, insects that rely on chemicals for social recognition must have a unique enough quantity of chemical cues to make the cues informative. Because nestmate recognition cues are colony-specific there must be more cue phenotypes in the population than there are nests. Breed and Buchwald (2008) predict that phenotypic cue profile diversity will mainly depend on the number of compounds in the cue profile and to a lesser extent depend on fine olfactory distinctions between profiles. They argue that evaluators with a template profiles composed of 13-16 compounds can discriminate high and low concentrations of compounds. Additionally, evaluators with fewer compounds in their template profile, 8-10, can discriminate compound concentrations at a finer scale. Although cue diversity is an important factor attributed to nestmate recognition, within cue diversity we must investigate acceptance thresholds with a focus on slight differences among cues.

Recognition chemistry has been investigated in many genera of ants. In the next two sections we focus on two particularly well-studied ant systems, *Formica*, which includes the wood ants, and the invasive Argentine ant, *Linepithema humile*.

**Formica ants**

*Formica* is a large, widely distributed ant genus, some members of which have been intensively studied. *Formica* ants are important members of nearly all temperate terrestrial
communities. One of the intriguing aspects of *Formica*, as a genus, is variation among species in colony structure. Some species, such as *Formica argentea* (Bennett 1989a; Bennett 1989b), have colonies with a single queen; these colonies occupy a single, discrete, nest and have relatively limited foraging territories around the nest. Many ecologically important species of *Formica*, such as *Formica podzolica* (Bennett 1989a; Bennett 1989b; DenHeer and Herbers 2004), *Formica exsecta* (Martin et al. 2008b) and *Formica aquilonia* (Sorvari and Hakkarainen 2005) are unicolonial, as described above, with multiple queens and nests within a supercolony and ecological dominance of a large area by a single extended colony.

Although eusocial insects are generally aggressive towards non-nestmates, there is still variation among how aggressive a particular species, nest, or individual should be to maximize the benefits of recognition while reducing the costs of fighting. Many studies investigate intraspecific aggressiveness. However, deconstructing interspecific aggression can lead to interesting behavioral findings that may be difficult to tease apart. Oftentimes variation in aggression is due to the context in which social insects, or animals in general, encounter another individual. Tanner and Adler (2009) investigated different factors that affect levels of aggressiveness in several species of *Formica* ants. They found that compared to ants in neutral territory, ants within their own territory tended to be more competitive towards non-nestmates showing the importance of the context competitor familiarity to levels of aggressiveness (Tanner and Adler 2009). Additionally, as resource value increases so does aggressiveness (Tanner and Adler 2009). Behavior can be affected by the behavior of their competitors; this is shown in intraspecific interactions, but Tanner and Adler (2009) have also clearly found this in an interspecific context. Many factors can affect aggressive behavior and in some species it is more context dependent (Tanner 2008; Tanner and Adler 2009).
When social animals encounter competitors they use group size to evaluate how aggressive they will be toward whom they encounter. In social insects, studies have shown that group size can affect whether an individual will enter a competition, with individuals from a larger group being more willing to enter a competition (Tanner 2008). Little is known about how social insects collect information about group size. This is important because these decisions about entering an interaction must be quick, thus insects must communicate this information in an efficient manner. Tanner (2008) found that direct contact with nestmate cuticular hydrocarbons can elicit aggressive behavior towards competitors suggesting that this is the cue some ants use to assess group number. Interestingly, it took about 25 minutes of nestmate hydrocarbon exposure to elicit an aggressive response to competition suggesting it takes a period of assimilation for ants to process group size. However, once this information was assessed the ants continued to be aggressive for 25 minutes after exposure (Tanner 2008). This suggests that the ants remember the information about group size for at least this long.

Argentine Ants

Invasive species are a concern to ecologists due to their potential to disturb native habitats. In social insects, the Argentine ant, *Linepithema humile*, is a case of great interest. The altered social structure of *L. humile* in its introduced range (for example, in California) contributes to its success as an invasive species (Holway and Suarez 1999). In its introduced range Argentine ants are unicolonial forming large supercolonies that lack territorial boundaries (Holway et al. 2002). Although nests are separated by physical space, individuals that are part of these supercolonies are tolerated when moving between nests (Holway et al. 2002). In its native range *L. humile* mainly form smaller distinct colonies that show aggression towards ants from other colonies (Suarez et al. 1999; Tsutsui et al. 2000). In a more recent study Pederson et al.
(2006) found that in its native range *L. humile* can also be unicolonial. However, unicolonial colonies in the native range are several orders of magnitude smaller than those in the introduced range (Pedersen et al. 2006). By examining differences between the Argentine ants in its native and introduced range scientists are trying to ascertain factors that have caused its altered social structure.

Several hypotheses have been proposed about how Argentine ants switched from a multicolonial social structure to a unicolonial one. Tsutusi et al. (2000) attribute multicoloniality to reduced genetic diversity. The “genetic cleaning” hypothesis proposes that unicoloniality in Argentine ants arose by selection against less common recognition alleles (Giraud 2002). Another hypothesis suggests that selection against individuals from genetically diverse groups has contributed to unicoloniality in the introduced population (Tsutsui et al. 2003). These hypotheses are based on the idea that ants in the native range are multicolonial while those in the introduced range are unicolonial. However, Pederson et al. (2006) found that unicoloniality exists in the native range of *L. humile* as well. Although unicoloniality exists in both native and introduced ranges, the levels of chemical and genetic diversity are much lower in the introduced versus the native range, suggesting that, although they may be unicolonial, these colonies are in fact different (Brandt et al. 2009). Despite the large amount of work that has been done on these questions, the matter of why these colonies are different is far from resolved.

In the Argentine ant cuticular hydrocarbons can cause intraspecific aggression (Torres et al. 2007). Vasquez et al. (2009) found that unrelated *L. humile* colonies that share similar cuticular hydrocarbons will readily fuse. This suggests that plasticity in cuticular hydrocarbon profiles maintain the fusion of unrelated *L. humile*. (Vasquez et al. 2009) Tsutsui et al. (2000) suggests that recognition cues in *L. humile* are heritable due to the genetic similarity between
individuals. Conversely, studies have shown that cuticular hydrocarbons derived from prey can affect the recognition system in Argentine ants (Liang and Silverman 2000; Liang et al. 2001). However, the affect of these environmental cues varies among introduced populations based on genetic diversity where recognition cues are more genetically based in populations with greater genetic diversity while environmentally based cues are more important populations with reduced genetic diversity (Buczkowski and Silverman 2006).

**The western honeybee, *Apis mellifera***

In addition to cuticular hydrocarbons, in honeybees, comb wax is important to nestmate recognition (Breed 1998a). Cuticular fatty acids found in bees are not only key compounds in nestmate recognition but also have a structural role in beeswax (Breed and Buchwald 2008). Like cuticular hydrocarbons in ants and wasps, all individuals in a honeybee colony have fatty acids but they differ in relative proportion (Buchwald et al. 2009). The primary components of bees wax are variable proportions of alkanes, wax esters and free fatty acids (Tulloch 1980). All of the fatty acids found in the comb wax, except steric acid, provide a cue for nestmate recognition (Breed 1998a). These fatty acids include saturated: palmitic acid and tetracosanoic acid and unsaturated: palmitoleic acid, oleic acid, linoleic acid and linolenic acid (Breed 1998a). In addition to varying in chemical composition comb wax varies in mechanical properties depending on the ecology of a particular species of bee (Buchwald et al. 2006; Buchwald et al. 2008). The inherently interesting connection between the mechanical and behavioral importance of fatty acids in honeybee ecology lead to interesting questions about how natural selection has acted upon these compounds.

Within a species of bee there must be enough variation in wax composition to ensure the phenotypic diversity of recognition cues. However, because of the importance of maintaining the
mechanical integrity of combwax to bee ecology, the differences in wax composition must not compromise the mechanical properties of combwax. Buchwald et al. (2009) found that phenotypic variation in the relative proportion of fatty acid composition of combwax has little impact on the mechanical properties. More specifically, variation in the majority of the unsaturated fatty acids did not affect the mechanical properties of comb wax (Buchwald et al. 2009). Interestingly, the relative proportions of these unsaturated fatty acids between nests varies, suggesting that changes in unsaturated fatty acids lead to phenotypic cue diversity without compromising nest mechanical properties (Buchwald et al. 2009). These findings suggest that in other social insects, who use cuticular hydrocarbons as recognition cues, a similar type of selection has occurred. Although it has never been tested, there is likely enough variation in the composition of hydrocarbon class within the profile to provide recognition cue diversity but this variation most likely does not affect the integrity of the waterproofing qualities of the exoskeleton.

**Social Wasps**

Like ants, several species of eusocial wasps exhibit colony-specific cuticular hydrocarbon profiles comprised of n-alkanes, methylalkanes, and alkenes (Butts et al. 1995; Dani 2006; Dapporto et al. 2006; Espelie et al. 1994). Of these hydrocarbons, in some species, methylalkanes and alkenes seem to be most critical in inducing an aggressive response typically seen when a non-nestmate tries to enter a nest (Dani et al. 2001). While in most species it is unclear which particular compounds elicit a nestmate recognition response, it is clear that combinations of these hydrocarbons are responsible for nestmate recognition (Dani et al. 2001; Gamboa et al. 1996). In addition to cuticular hydrocarbons, some species of eusocial wasp use nest paper hydrocarbons for recognition (Butts and Espelie 1995; Singer and Espelie 1992).
In paper wasps olfactory cues may not be the only factor important to communication, the variety of facial markings in *Polistes* lead investigators to examine the use of visual cues in *Polistes*. Tibbetts (2002) found that in at least one species of wasp, *Polistes fuscatus*, individuals can recognize a nestmate from a non-nestmate using facial patterns. This evidence indicates further investigation into such a phenomenon in other wasp species may lead to similar findings. Tibbetts (2004) found that eight *Polistes ssp.* had variable enough facial markings that cue diversity from the facial marking of these species could provide enough phenotypic diversity for individual recognition. Further investigation into these species’ recognition system could lead to similar findings.

**Termites**

The matter of how termites recognize a nestmate from a non-nestmate is still unresolved. Termites, although they have a different genetic structure than the Hymenoptera, also exhibit colony-specific hydrocarbon profiles (Howard 1993; Jmhasly 1998). This suggests that cuticular hydrocarbons may be mainly responsible for nestmate recognition in termites. Studies that examine the link between cuticular hydrocarbons and aggression have had mixed results with some showing increased aggression to cuticular hydrocarbons (Kaib et al. 2002) while others were not able to make this link (Su and Haverty 1991). These opposed findings have led investigators to test different avenues for recognition besides cuticular hydrocarbons.

Exogenous environmental factors may play a role in termite recognition. Researchers have linked diet to increased interspecific aggression (Florance et al. 2004). However, individuals from neighboring nests may have similar diets, which may not provide large cue diversity for recognition. There is some evidence that intestinal bacteria play an important role in nestmate recognition (Matsuura 2001). Matsuura (2001) found colony-specific microbial
communities in termite guts and that termites that had absorbed unfamiliar bacterial odor were recognized as non-nestmates. Matsuura (2001) suggests that volatile cues from fecal bacteria may be responsible for nestmate recognition cues. Further investigation is required in order to elucidate nestmate recognition in termites.

**Experimental Approaches to Recognition Studies**

Studies examining cuticular hydrocarbons need to rely on a method of detecting and identifying hydrocarbons. Typically researchers extract hydrocarbons from the cuticle of the insect using a non-polar solvent such as pentane or hexane. Extractions are then separated and identified using Gas Chromatography- Mass Spectrometry (GC-MS). Although this method is commonly used in these types of investigations, researchers must be careful to employ the proper temperature and column conditions as improper examination could lead to the underestimation of hydrocarbons present on the cuticle (Akino 2006). Once these compounds have been analyzed using GC – MS, researchers typically uses multivariate statistics to look for colony-specific patterns in hydrocarbon profiles (Martin and Drijfhout 2009a). Although this approach seems to suggest that hydrocarbons are colony-specific, they rarely link behavioral evidence with chemical evidence leaving the link between hydrocarbons and nestmate recognition highly circumstantial (Breed 1998a; Martin and Drijfhout 2009a).

To determine nestmate recognition, researchers typically perform aggression behavioral bioassays where they observe the behavior of interacting pairs or groups of individuals. In these bioassays those individuals perceived as non-nestmates typically act aggressively towards one another (Roulston et al. 2003). Although most researchers use aggression bioassays for these studies, they tend to be highly varied in many aspects including, but not limited to, duration, number of individuals, detail of observations and ways data are collected (Roulston et al. 2003).
Bioassays are critical to nestmate recognition but, because results can vary, researchers must be sure to choose the appropriate assay for the question they are trying to ask (Roulston et al. 2005). Behavioral bioassays begin to make the link between chemistry and behavior but more information is still needed to fully understand this phenomenon.

There is evidence that cuticular hydrocarbons elicit behavioral responses in social insects but the mechanism by which they perceive these odors is still poorly understood (Howard 1993). The use of electro-antennography (EAG) is a common technique used to study the perception of volatile compounds in insects. However, because cuticular hydrocarbons are not very volatile at room temperature, this technique is rarely used to investigate social insects. Some investigators have been able to successfully link antennal responses with the presence of hydrocarbons in ants (D’Ettorre 2004; Ozaki et al. 2005) and termites (Batista-Pereira et al. 2004). Future studies using this technique are needed to provide further insight into the mechanism by which odors are perceived.

**Conclusions**

Eusocial insects provide excellent models for studying discriminations of self versus non-self. Analogies are easily drawn between MHC mediated self-recognition in vertebrates and social recognition in insects. In both systems, hypervariable phenotypes provide the necessary information for self- and social recognition. In the vast majority of species, recognition in eusocial insects relies on cuticular hydrocarbons; neutral substitution among hydrocarbons can yield immense phenotypic variation for social signals.

The recent suggestion by Richard et al. (2008) that immune response, cuticular hydrocarbons, and social recognition are linked in honeybees is intriguing and merits further study. If, indeed, immune function and social recognition are linked in eusocial insects, this
would build an even stronger analogy with MHC mediated recognition systems. Comparative studies of the chemistry of social recognition will give further insight into the evolution of how social identity is signaled and perceived. This knowledge will also test the neutral substitution hypothesis, and indicate whether neutral substitution should be accepted as the primary force in generating the variable phenotypes needed in social recognition.
Chapter 3:

Colony-Specific Cuticular Hydrocarbon Profiles in *Formica argentea* Ants

Michelle O. Krasnec

Department of Ecology and Evolutionary Biology

The University of Colorado, Boulder

Boulder, Colorado, USA 80309-0334
Abstract

The cuticular hydrocarbons of the ant *Formica argentea* were identified using gas chromatography-mass spectrometry. The cuticle of *F. argentea* consists of n-alkanes, alkenes, and methyl-branched alkanes. I found that *F. argentea* workers present a colony-specific hydrocarbon profile based on their C_{29} methyl-branched alkane signature. Using this signature alone I could group worker ants by nest, suggesting the hypothesis that the C_{29} methyl-branched alkanes may be important in nestmate recognition for this species. The results support the idea that variation in positional isomers of cuticular hydrocarbons of the same carbon chain length may provide enough information for nestmate recognition. This study reinforces the idea that investigators should not treat cuticular hydrocarbon profiles as a whole but should look for colony-specific signatures embedded in parts of the profile which may provide more biologically relevant information.
Introduction

Social recognition, the ability to identify social group members, is a key mechanism underlying cooperation. In the eusocial insects, recognition of group membership is an essential component of evolutionary success (Wilson 1974). Recognition of group membership allows for eusocial insects to live in closed societies, which contributes to the ecological success of these organisms. Closed societies allow for social insects to avoid exploitation of their resources by intruders and helps increase the success of their nests. Social recognition relies on phenotypic variation among animals within populations; this variation provides cues for social discriminations. Despite the importance of the variation of cues in social discrimination, much remains to be learned about how cues vary between colonies and if there are colony-specific chemical signatures of these cues present within a species.

In many species of eusocial insects, cuticular hydrocarbons typically play an important role in nestmate recognition (Breed 1998a; Dani et al. 2001; Howard and Blomquist 2005; Ruther et al. 2002; Thomas et al. 1999; Wagner et al. 2000). These hydrocarbons consist of a mixture of low volatility linear alkanes, alkenes, and methyl-branched alkanes (Breed 1998a; Breed 1998b; Dahbi et al. 1996; Provost et al. 1993). Although all ants can internally synthesize cuticular hydrocarbons (Blomquist and Dillwith 1985), there is great variation between species in which compounds are present on the cuticle. Within a given species, individuals possess the same compounds on their cuticles but vary in the relative proportions of these compounds (Espelie et al. 1994; Gamboa 2004). Generally, this variation is correlated within their colony, so that cuticular profiles of ants vary more between colonies than within colonies (Martin et al. 2008b; Martin et al. 2009c). Examinations of the entire hydrocarbon profiles of workers provide evidence supporting colony-specific hydrocarbon profiles (Butts et al. 1993; Carlin and

The majority of studies rely on multivariate analyses of the entire profile; this may not be an appropriate method to distinguish colony-specific signatures and may not provide any biologically relevant information (Martin et al. 2008b; Martin and Drijfhout 2009a). Martin and Drijfhout (2009a) determined that multivariate analyses may not be appropriate for studies on cuticular hydrocarbons because all the compounds in the profile are not independent and minor compounds in the profile may have a disproportionate effect on the analysis. Additionally analyzing the entire hydrocarbon profile of workers may mask colony-specific signals, and hide those that are biologically relevant. Although this method successfully groups workers by colony, perhaps workers do not perceive all of the compounds that vary in concentration.

Eusocial insects typically have three structural classes of hydrocarbons on their exoskeletons; n-alkane, alkenes, and methyl-branched alkanes. While it is generally difficult to decipher which specific compounds within the hydrocarbon profile elicit a nestmate recognition response, it is clear that in some species of eusocial insect, a combination of individual structural classes of these hydrocarbons may be responsible for nestmate recognition (Dahbi et al. 1996; Dani et al. 2001; Greene and Gordon 2007; Provost et al. 1993). Examinations of parts, rather than all, of the hydrocarbon profile is necessary to decipher which compounds or structural classes are important to nestmate recognition. With this knowledge regarding nestmate recognition, investigators are looking into potential groups of compounds that may elicit a recognition response. A phylogenetic analysis by Martin and Drijfhout (2009c) found the diversity of dimethyl-alkanes on the cuticle to be highly species-specific, suggesting that further investigations should focus on these compounds as potential nestmate recognition signals.
More recent studies begin to reveal different mechanisms that may be responsible for forming a colony-specific recognition signal. When examining two species of *Formica* ant Martin et al. (2008c) proposed two different methods by which colony-specific information can be encoded in the cuticular hydrocarbon profile. Method one, as found in *F. exsecta*, relies on altering the proportion of a single type of hydrocarbon, in this case a series of (Z)-9-alkenes. Method two, as found in *F. fusca*, relies on variation in different positional isomers of a specific carbon chain length’s dimethyl-alkanes, in this case isomers of C$_{25}$-dimethyl-alkanes.

I identified the compounds present on the cuticle of *F. argentea* workers and determined if they can discriminate a nestmate from a non-nestmate. I then analyzed these compounds to determine if they vary in ways that could facilitate nestmate recognition. Finally, I examined two potential methods proposed by Martin et al. (2008c) in which colony-specific signatures may be encoded within the hydrocarbon profile to determine if either of these could apply to the ant *F. argentea*.

**Methods**

**Chemical Extraction**

I collected five colonies in the summer of 2009 in Jefferson County Colorado and froze them at -20°C. I randomly selected ten workers from each nest for chemical analysis. Prior to extraction I took worker ants out of the freezer and allowed them to defrost for 20 min. I placed each ant in 0.5ml of hexane for 10 min. Extracts were concentrated to dryness under a stream of nitrogen and re-suspended in 50 µl of distilled hexane. Re-suspended extracts were analyzed using gas chromatography- mass spectrometry (GC-MS).

**Chemical Analysis**
I injected 2 µl of extracts into an Agilent 6890N GC interfaced with an Agilent 5975 mass selective detector. Mass spectra were recorded at 70 eV in electron impact ionization mode. The GC was fitted with DB-5MS 30m x 0.25mm column (J & W Scientific, Folsom CA, USA). The temperature program began at 120°C then increased at the rate of 8°C/min. until a temperature of 300°C had been reached. This temperature was held for 10 min. prior to ending a run. Helium was the carrier gas. The injector temperature was 250°C, the ion source temperature was 230°C, and the quadrupole temperature was 150°C. 500 ng of nonadecane was used as an internal standard.

Peak Identification

Components were identified by their mass spectra using diagnostic fragmentation patterns and compared to standard alkane spectra (Sigma-Aldrich: Alkane Standard Solution C₂₁-C₄₀) and retention times. I injected hydrocarbon standards at regular intervals during sample analysis (Sigma-Aldrich: Alkane Standard Solution C₂₁-C₄₀). These standards were used to determine carbon chain length and calculate Kovat Indices. I identified methyl-branch position by diagnostic fragment ions, Kovat Indices and by the method of Carlson et al. (1998). I used DMDS derivatization to determine double bond position on the alkenes (Carlson et al. 1989).

Aggression Tests

To determine if *F. argentea* ants can discriminate a nestmate from a non-nestmate I performed behavioral bioassays with five different, field-collected, queenright colonies that were maintained in the lab. Ants were selected randomly for a total of 25 non-nestmate and 25 nestmate pairings. Behavioral interactions between pairs were observed in a Petri dish for 10 minutes and behaviors were scored by the following index: 1 = antennation, 2 = threat: open mandibles while facing each other and touching, 3 = attack: includes biting, 4 = intense attack:
includes dragging and spraying. Video recordings of the bioassays were taken to ensure unbiased scoring. I recorded the duration of each behavior and calculated the overall aggression index (AI) using the methods of D’Ettorre et al. (2000). I used the formula:

\[ AI = \frac{\sum_{i=1}^{n} AI_i \cdot t_i}{T} \]

Where \( AI_i \) is the aggression index, \( t_i \) is the duration of each act, and \( T \) is the sum of the time that the ants were in contact.

Data Analysis

Quantitation of hydrocarbon components was based on integration of total ion chromatograms. Peak areas were standardized before statistical analysis using the method of Aitchison (1986). For our analyses I only included compounds that comprised at least 1% of the profile in at least one of the colonies that I examined. To investigate the two possible types of colony-specific cues suggested by Martin et al. (2008c) I examined the mean correlation values between the amounts of both homologous alkenes and n-alkanes at the colony and species level. I also examined the mean correlation values between different positional isomers of methyl and dimethyl-alkanes of the same carbon chain length both within and between colonies. Compounds that showed a high correlation within the colony but a low correlation between the colonies are good to examine for a colony-specific signature (Martin et al. 2008c). I then examined compounds that fit these criteria using a Ward’s hierarchical cluster analysis and assessed the uncertainty of clusters via multiscale bootstrap resampling (R 2.13.2, pvclust) to see if individual ants cluster by colony based on potential colony-specific signature (Martin et al. 2008c).

Results

Cuticular Hydrocarbon Identification
I identified 28 cuticular hydrocarbons (or combinations of co-eluting compounds) in the profile of *F.argentea* workers. Compounds ranged from 32-38 in their carbon chain length. I found n-alkanes, alkenes, monomethyl-branched alkanes and dimethyl-branched alkanes in the hydrocarbon profile (Table 1).

Correlations

To determine if colony-specific signatures are encoded within a hydrocarbon profile, and more specifically to determine if there were any of the colony-specific signatures proposes by Martin et al. (2008c) I examined the mean correlation values between the standardized amounts compounds within a colony and between a colony. When I investigated homologous series of alkanes and alkenes, as suggested by Martin et al. (2008c), there were no differences in the correlations of compounds between colonies and within colonies suggesting that alkenes and alkanes are not good candidates for providing a colony-specific signature because there is not great variation between colonies. I also examined the mean correlation values between standardized amounts of methyl and dimethyl-branched alkane isomers of the same carbon chain length to determine if there were any groups of methyl and dimethyl-alkanes with the same carbon chain length that was highly correlated within a colony but not between a colony. I looked at correlations among the C\textsubscript{27}, C\textsubscript{29}, and C\textsubscript{31} methyl and dimethyl-alkanes and of those only methyl and dimethyl branched C\textsubscript{29} alkanes were more highly correlated within a colony than they were between colonies (Table 2). No other set of compounds showed a similar trend, suggesting that methyl and dimethyl branched C\textsubscript{29} alkanes provide a colony-specific signature (Table 2).

Cluster Analysis
In a Ward’s hierarchical cluster analysis using only the standardized amounts C\textsubscript{29} methyl and dimethyl-alkanes, only 3 workers clustered outside of their colony (Figure 1). This analysis suggests that simply using the C\textsubscript{29} methyl and dimethyl-branched alkanes signature alone provides for discrimination between nestmates and non-nestmates. There were no other groups of compounds that did well clustering individuals by colony.

**Aggression Bioassays**

Based on aggression score, non-nestmates were significantly more aggressive toward one another than toward nestmates pairing ($T_{1, 48} = 5.78, P < 0.0001$) indicating that *F. argentea* workers can discriminate a nestmate from a non-nestmate (Figure 2). The mean aggression index when nestmates encountered each other was $0.14 \pm 0.06$ and the mean aggression index when non-nestmates encountered each other was $0.74 \pm 0.08$.

**Discussion**

Methyl and dimethyl-alkanes with a C\textsubscript{29} backbone provide a colony-specific signature that has the potential to function in nestmate recognition for *Formica argentea*. Rather than looking at differences among the entire cuticular hydrocarbon profile of individuals between colonies, investigators should look for colony-specific differences in parts of the profile, pinpointing a colony-specific signature encoding for nestmate recognition. This approach may lead to finding more biologically relevant colony-specific nestmate recognition signatures, as many studies have found that only particular parts, or structural classes of hydrocarbons, elicit a nestmate recognition response (Astruc et al. 2001; Martin et al. 2008b). For example, the cuticle of the ant *F. exsecta* contains both n-alkanes and Z-9-alkenes however only Z-9-alkenes elicited worker aggression (Martin et al. 2008b). Methyl-branched alkanes may also be important in the nestmate recognition of other species, although the behavioral link has yet to be made (Dahbi et
al. 1996; Provost et al. 1994). This study partially supports Martin and Drijfhout's (2009c) extensive comparative study on published cuticular hydrocarbons to look for potential compounds that, due to species-specific diversity, may be good candidates to provide nestmate recognition signals. They determined that dimethyl-alkanes should be investigated further as they may be likely candidates for providing a nest-specific signature (Martin and Drijfhout 2009c). I found that both methyl and dimethyl-alkanes are important in statistically distinguishing a nestmate from a non-nestmate in *F. argentea*.

In addition to finding a colony-specific hydrocarbon signature, I found that *F. argentea* workers can distinguish a nestmate from a non-nestmate. The cuticle of *F. argentea* contains approximately 28 different cuticular hydrocarbons. Similar to cuticular hydrocarbons found on other insects the hydrocarbons on *F. argentea* workers range in carbon chain length from 25-38, with profile containing n-alkanes, alkenes and methyl-branched alkanes. Monomethyl and dimethyl-alkanes predominated the hydrocarbon profile and only four of the 28 most prominent hydrocarbons present were alkenes. These data support the finding of Martin et al. (2008c) who examined the hydrocarbon profiles of 13 different species of *Formica* and found that they are either dominated by 9-alkenes or by dimethyl-alkanes, although *F. argentea* contains both monomethyl and dimethyl-alkanes.

Cuticular hydrocarbon profiles typically contain a series of homologous hydrocarbons (Martin et al. 2008c; Martin and Drijfhout 2009c; Van Wilgenburg et al. 2010). A series of methyl-branched alkanes are homologous when the compounds have a methyl branch on the same position but the compound differs in carbon chain length. Similarly, a series of alkenes are homologous when they contain a double bond on the same position but differ in carbon chain length. Recent studies have shown that many of the homologous series of cuticular hydrocarbons
can be highly correlated (Martin and Drijfhout 2009b; Van Wilgenburg et al. 2010). Some of these correlated homologous compounds, although differing in structure, do not differ in biological relevance or meaning (Van Wilgenburg et al. 2010). In this study I examined the correlations of homologous series of cuticular hydrocarbons between and within colonies and found many compounds highly correlated both within and between colonies.

Deciphering patterns within cuticular hydrocarbon profiles has advanced investigators into looking for nestmate specific signatures that may be used as cues for nestmate recognition. Martin et al. (2008c) found that in the case of species recognition, rather than investigating the entire cuticular hydrocarbon profile, there are species specific signatures in different species of *Formica*, that include specific parts and patterns in the hydrocarbon profile. Extending this idea to discrimination among conspecifics, Martin et al. (2008c) examined two different methods in which colony-specific signatures can be encoded in the cuticular hydrocarbon profile. One method can be by colonies differing in the proportion of a single type of homologous hydrocarbon, as in *F. exsecta*, in which colony-specific information is encoded within a single homologous series of Z-9-alkenes (Martin et al. 2008b; Martin et al. 2008c). In the other proposed method, some species may rely on colony-specific information through variation in positional isomers of the same carbon chain length as seen in *F. fusca* where variation in the C_{25}-dimethylalkanes was enough to provide a colony-specific signature (Martin et al. 2008c). My study confirms that, like *F. fusca*, *F. argentea* have differing methyl and dimethyl-alkane isomers of the same carbon chain length that are colony-specific. While *F. argentea* relies on both dimethyl and monomethylalkanes for a colony-specific signature, *F. fusca* relies on only dimethylalkanes.
A widely accepted hypothesis as to how individuals can recognize a nestmate from a non-nestmate is based on Crozier and Dix (1979) idea that there is a “gestalt” colony odor. This colony odor is maintained between individuals within the nest via trophallaxis, creating a uniform colony odor that, even if dynamic, allows individuals in a nest to always smell the same because of this exchange of fluids (Soroker et al. 1994). The colony odor, hydrocarbons in particular, can have both a genetic component and an environmental component (Heinze et al. 1996; Vander Meer and Morel 1998; Liang and Silverman 2000). The influence of colony odor by both environmental and genetic factors may make a big difference in the “gestalt” odor. Compounds that are influenced by environmental factors may change drastically throughout the season, therefore changing the colony odor for those specific compounds. Compounds that are genetically influenced may not change as much though time. The distinction between how different compounds are acquired is indicative of the importance of discerning specific patterns in cuticular hydrocarbon profiles, especially since studies have shown that genetic “gestalt” is sufficient for nestmate recognition (Van Zweden 2010). Once compounds responsible for colony-specific recognition cues are identified, these compounds can be examined to see if they are genetically of environmentally influenced and provide new insight into how the colony odor is formed.

This study begins to sort out cuticular hydrocarbon profiles and supports other studies that have found colony-specific hydrocarbon signatures, rather than treating the hydrocarbon profile as a whole. The next step for this study, and others like it, is to isolate these specific compounds and determine if they elicit a behavioral response. This has been difficult for many investigators because most of these compounds are not commercially available and will need to be synthesized for future behavioral experiments. Understanding nestmate recognition cues as a
whole can be a difficult task but beginning to find colony-specific signatures gives researchers an idea about what compounds to pinpoint for behavioral responses. Additionally, deciphering which compounds are genetically influenced and which ones are environmentally influenced can provide more information about how the information regarding recognition cues are actually used and formed within a nest.
Tables and Figures:

Figure 1: Ward’s hierarchical cluster analysis to assign individual *Formica argentea* workers to colonies by using only the standardized amounts $C_{29}$ methyl and dimethyl-alkanes in hydrocarbon profile. Out of 50 ants only 3 clustered outside of their nests. Dotted boxes represent statistically significant clusters.
Figure 2: In *F. argentea* non-nestmates are significantly more aggressive to one another than nestmates ($T_{1,48} = 5.78$, $P < 0.0001$).
### Table 1: Identification of the 28 hydrocarbon compounds (or combination of coeluting compounds) found on *F. argentea* workers. Only compounds that comprised more than 1% of the hydrocarbon profile in at least one of the nests examined were included.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Retention</th>
<th>MW</th>
<th>Carbon Chain</th>
<th>Diagnostic Fragment Ions (M/Z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentacosane</td>
<td>17.174</td>
<td>352</td>
<td>25</td>
<td>43, 57, 71, 85, 99, 352</td>
</tr>
<tr>
<td>9-Hentacosene</td>
<td>18.79</td>
<td>378</td>
<td>27</td>
<td>41, 55, 69, 83, 97, 378</td>
</tr>
<tr>
<td>Hentacosene</td>
<td>19.192</td>
<td>380</td>
<td>27</td>
<td>43, 57, 71, 85, 99, 380</td>
</tr>
<tr>
<td>7-Methylheptacosane</td>
<td>19.552</td>
<td>394</td>
<td>27</td>
<td>112, 308, 379</td>
</tr>
<tr>
<td>3-Methylheptacosane</td>
<td>19.633</td>
<td>394</td>
<td>27</td>
<td>57, 365, 394</td>
</tr>
<tr>
<td>(9,13), (10,14), (1,15) Dimethylheptacosane</td>
<td>19.819</td>
<td>409</td>
<td>27</td>
<td>140/141, 154/155, 168/169, 196/197, 210/211, 218, 224/225, 239, 267, 295</td>
</tr>
<tr>
<td>(7,11) Dimethylheptacosane</td>
<td>19.918</td>
<td>408</td>
<td>27</td>
<td>112/113, 183, 252/253, 323</td>
</tr>
<tr>
<td>Octacosane</td>
<td>20.079</td>
<td>394</td>
<td>27</td>
<td>43, 57, 71, 85, 99, 394</td>
</tr>
<tr>
<td>Methyloctacosane</td>
<td>20.122</td>
<td>408</td>
<td>27</td>
<td>43, 57, 71, 393</td>
</tr>
<tr>
<td>9-Nonacosene</td>
<td>20.553</td>
<td>408</td>
<td>27</td>
<td>43, 57, 71, 85, 99, 408</td>
</tr>
<tr>
<td>Nonacosane</td>
<td>20.755</td>
<td>408</td>
<td>27</td>
<td>43, 57, 71, 85, 99, 408</td>
</tr>
<tr>
<td>15, 13, 11-Methylnonacosane</td>
<td>21.004</td>
<td>422</td>
<td>29</td>
<td>168/169, 196/197, 224/225, 252/253, 280/281, 422</td>
</tr>
<tr>
<td>9-Methylnonacosane</td>
<td>21.081</td>
<td>422</td>
<td>29</td>
<td>140/141, 308/309</td>
</tr>
<tr>
<td>7-Methylnonacosane</td>
<td>21.282</td>
<td>422</td>
<td>29</td>
<td>112/113, 336/337</td>
</tr>
<tr>
<td>3-Methylnonacosane</td>
<td>21.462</td>
<td>422</td>
<td>29</td>
<td>56/57, 392/393</td>
</tr>
<tr>
<td>Triacantane</td>
<td>21.62</td>
<td>422</td>
<td>30</td>
<td>57, 71, 85, 99, 183, 211, 253, 281, 420</td>
</tr>
<tr>
<td>3-Methyltriacantane</td>
<td>22.045</td>
<td>437</td>
<td>30</td>
<td>56/57, 379, 408</td>
</tr>
<tr>
<td>14-Methyltriacantane</td>
<td>22.423</td>
<td>437</td>
<td>30</td>
<td>210/211, 280/281, 421</td>
</tr>
<tr>
<td>Hentriacontane</td>
<td>22.578</td>
<td>437</td>
<td>31</td>
<td>43, 57, 71, 85, 99, 436</td>
</tr>
<tr>
<td>9-Hentriacontene</td>
<td>22.913</td>
<td>434</td>
<td>31</td>
<td>41, 55, 69, 83, 97, 404, 434</td>
</tr>
<tr>
<td>9, 11, and 13-Methylhentriacontane</td>
<td>23.037</td>
<td>450</td>
<td>31</td>
<td>43, 57, 71, 140/141, 168/169, 196/197, 280/281, 308/309, 435</td>
</tr>
<tr>
<td>7-Methylhentriacontane</td>
<td>23.403</td>
<td>450</td>
<td>31</td>
<td>112/113, 336/337, 364/365, 392/393</td>
</tr>
<tr>
<td>2-Methyldotriacontane</td>
<td>24.918</td>
<td>465</td>
<td>32</td>
<td>42/43, 420/421, 449</td>
</tr>
<tr>
<td>Octatridacantene</td>
<td>28.213</td>
<td>531</td>
<td>38</td>
<td>41, 55, 69, 404, 533</td>
</tr>
</tbody>
</table>
Table 2: The mean correlation values ($r^2$) between the standardized amount of C$_{29}$ methyl and dimethyl-alkanes in the cuticular hydrocarbon profile of *Formica argentea* ants. (a) Correlations within nests. (b) Correlations between nests (species wide). Greater correlations within nests compared to between nests indicates C$_{29}$ methyl and dimethyl-alkanes may provide a colony-specific signature in this species.
Chapter 4:

Recognition Cues and Perception Thresholds to Changes in Cuticular Hydrocarbon Profiles in *Formica argentea*

Michelle O. Krasnec

Department of Ecology and Evolutionary Biology

The University of Colorado, Boulder

Boulder, Colorado, USA 80309-0334
Abstract

The cuticular hydrocarbon profile of *Formica argentea* worker ants consists of a mixture of n-alkanes, alkenes, and methyl-alkanes. Recent studies show that many species of ants act aggressively when they encounter another individual who differs from itself in some structural classes of hydrocarbons but not others. The goal of this study was to determine which structural classes of hydrocarbons (n-alkanes, alkenes, and methyl-alkanes) elicit nestmate recognition responses in *F. argentea*. I found that increasing the proportion of methyl-alkanes and alkenes on the hydrocarbon profile *F. argentea* workers caused increased aggression when they were paired with nestmates, suggesting methyl-alkanes and alkenes are important in nestmate recognition. In contrast, increasing the n-alkane hydrocarbon profile did not increase aggression. This study also quantifies perceptual thresholds in nestmate recognition. Workers do not act aggressively towards a nestmate until differences in the hydrocarbon profile reach the perceptual threshold in both methyl-alkanes and alkenes. These results suggest that investigations of cuticular hydrocarbon profiles in nestmate recognition need to treat each hydrocarbon structural class separately, as ants react aggressively when exposed to some classes of compounds and not others.
Introduction

One key mechanism underlying cooperation among animals is social recognition, the ability of animals in a society to identify social group members and to discriminate members from non-members. Social recognition relies on phenotypic variation among animals within populations. This variation provides cues for social discriminations that allow for phenotype matching of an individual’s learned template for group members with the cues provided by the individual it encounters (Hepper and Cleland 1998; Heth et al. 1998; Lacy and Sherman 1983; Mateo 2004; Schausberger 2007; Sherman et al. 1997). Recognition cues can be visual, as those used by humans, auditory, as in birds, crickets, and frogs (Gerhardt 1978; Ligon 1991; Pollack and Hoy 1979) or olfactory, as in some mammals and insects (Breed 1998a; Halprin 1991). Recognition cues are needed to provide an effective means of communication within groups.

In many species of eusocial insects, communication is well developed. Workers in colonies use communication to make complex social decisions. Effective means of communication are vital to many aspects of the life-history of eusocial insects (Hölldobler and Wilson 1990). For example, social insects use alarm pheromones to communicate with others of an imminent danger (Hölldobler and Wilson 1990). Many species of social insects will use trail marking pheromones to lead nestmates to food or a suitable nesting location (Hölldobler and Wilson 1990). Interestingly social insects also use chemical communication for other, less established purposes. For example, task allocation within colonies can be chemically mediated in ants (Greene and Gordon 2003; Greene and Gordon 2007; Martin and Drijfhout 2009b). Chemical cues can also be used by workers to discriminate among queens and fertile and non-fertile workers (Dietemann et al. 2003). Chemical cues are also important in recognition systems, where individuals need to be aware of group membership in order to cooperate with nestmates.
Recognition systems play a crucial role in the evolution of cooperation by giving animals the ability to make discriminations among other members of a population based on kinship or group membership (Hamilton 1964). Typically in eusocial insects nestmate recognition occurs via olfactory cues present on the insect cuticle (Lahav et al. 1999; Thomas et al. 1999; Vander Meer and Morel 1998; Wagner et al. 1999). Individuals can distinguish a nestmate from a non-nestmate based on the detection of differences in concentrations of cuticular hydrocarbons between the profile of their own colony and the profile of a potential intruder (Dani et al. 2001; Ichinose and Lenoir 2010; Ruther et al. 2002; Thomas et al. 1999; Wagner et al. 2000). Little is known about which components of the hydrocarbon profile are important in nestmate recognition and even less is known about the minimum concentrations of compounds required for perception of signals used in this type of communication.

In most eusocial insects, hydrocarbons on the cuticle mediate nestmate recognition responses. The hydrocarbons consist of a mixture of relatively low-volatility linear alkanes, alkenes, and methyl-alkanes that are detected thorough antennal contact (Dahbi et al. 1996; Provost et al. 1993). Although each colony tends to have a colony-specific cuticular hydrocarbon profile (Dahbi et al. 1996; Martin et al. 2008a; Martin et al. 2008c; Martin and Drijfhout 2009b; Provost et al. 1994), it is clear that all the compounds in the cuticular hydrocarbon profile are not involved in nestmate recognition (Breed 1998a; Breed 1998b; Dani et al. 2001; Dani et al. 2005; Dahbi et al. 1996; Provost et al. 1993). Cuticular hydrocarbons that serve communication functions tend to be subsets of the larger array of compounds on the surface of insects (Dani et al. 2001; Dahbi et al. 1996; Provost et al. 1993). Recent studies focus on the role of specific structural classes of hydrocarbons in nestmate recognition. In the honeybee, *Apis mellifera*, n-alkenes and fatty acids are utilized by guard bees for nestmate discrimination (Breed 1998a;
Breed 1998b; Dani et al. 2005). In paper wasps methyl-alkanes and linear alkanes are the important compounds in causing aggression (Dani et al. 2001). In some ants, methyl-alkanes or alkenes evoke an aggressive response (Astruc et al. 2001; Martin et al. 2008b). Studies such as these, that tease apart the cuticular hydrocarbon profile into different structural classes, are more informative about which cues are responsible for eliciting a nestmate recognition response.

For nestmate recognition cues to be informative, variation in recognition cues must occur between individuals or colonies (Breed and Buchwald 2008; Cini et al. 2009; Ichinose and Lenoir 2010). Within-species variation is almost always in relative concentrations of compounds, with the same compounds present on all insects of that species (Espelie et al. 1994; Gamboa 2004). Conspecific colonies typically vary enough in relative proportion of cuticular compounds to allow for discrimination of a nestmate from a non-nestmate (Breed and Buchwald 2008; Dahbi et al. 1997; Nielsen et al. 1999; Vander Meer et al. 1989). Variation in the concentrations of cuticular hydrocarbon compounds between nests is important to nestmate recognition because it provides the cue diversity needed to allow for nestmate recognition.

While identification of nestmate recognition cues is an immense advancement in studies of cooperation in eusocial insects (Astruc et al. 2001; Breed 1998a; Breed 1998b; Dani et al. 2001; Dani et al. 2005; Martin et al. 2008b), further research needs to address quantitative perceptual thresholds of these compounds. Studies that examine specific cue compounds in nestmate recognition generally use methods in which individuals are exposed to compounds and then the behavioral response is assayed (Astruc et al. 2001; Dani et al. 2001; Martin et al. 2008b). In other words, the investigators expose an individual to one concentration of a compound and record if there is a behavioral response. Although variation in cuticular hydrocarbon profiles is important for nestmate recognition (Breed and Buchwald 2008; Dahbi et
al. 1996; Nielsen et al. 1999; Vander Meer et al. 1989), little is known about the quantitative perceptual threshold necessary to evoke an aggressive response. An acceptance threshold in nestmate recognition is defined as the maximum amount of differences between the template and the cue that a guard would allow without rejecting an individual that it encounters (Reeve 1989). Individuals that fall below that threshold would be considered nestmates while individuals that fall above that threshold would be considered non-nestmates and be excluded from the nest.

In this chapter, using the ant *Formica argentea* as my model system, I aimed to determine the specific class(es) of cuticular hydrocarbons responsible for evoking a nestmate recognition response and established a quantitative perceptual threshold of compound concentrations required to cause this behavioral change. I applied varying concentrations of compounds representing different structural classes of cuticular hydrocarbons to nestmate ants to see if altering their cuticular hydrocarbon profile, with respect to a particular hydrocarbon structural class, elicited a nestmate recognition response. I also established the minimum concentrations of compounds required for the perception of a recognition signal.

**Methods**

To determine which structural class of cuticular hydrocarbons act as cues for nestmate recognition, I applied different structural classes of hydrocarbons to *F. argentea* workers and looked for a behavioral response. For each class of hydrocarbon I applied that compound to one ant from a pair of nestmate ants. This allowed me to see how much the hydrocarbon profile of an ant had to change for it to no longer be perceived as a nestmate. I also used varying concentrations of each compound, which fell into a biologically relevant range, to determine a nestmate recognition perception threshold for this species.
For this investigation I used a subset of possible compounds, as is often done in these types of studies (Dani et al. 2005, Greene and Gordon 2007, Meskali et al. 1995), because of both the difficulty and cost of acquiring and/or synthesizing many cuticular hydrocarbons. Methyl-alkanes are not available for purchase and are difficult to synthesize, for this experiment I tested methyl-alkanes that had been acquired via separation from the entire ant cuticular hydrocarbon wash using the methods of Greene and Gordon (2007). I used the same technique to acquire alkenes but additionally purchased some synthetic alkene standards. The technique that separates the hydrocarbon classes led to the loss of the natural n-alkanes on the profile. The n-alkanes tested in this experiment were all purchased (see below for chemical information).

Chemical Treatment

For each compound and concentration tested, I randomly selected five pairs of nestmate ants, each from five different colonies, for a total of 25 trials per concentration (50 trials per concentration for the C$_{21}$-C$_{40}$ standard alkane solution). I tested various concentrations of hydrocarbons in a hexane solution, that fell within the approximate average range of natural hydrocarbons found on *F. agenta* (5.1 ± 1.5 µg/per ant): (for the: alkanes (C$_{21}$-C$_{40}$ standard alkane solution (Sigma-Aldrich 99%): 0, 2, 3, 20, 30 µg; pentacosane (Sigma-Aldrich 99%): 0, 2, 3, 20, 30 µg; heneicosane (Sigma-Aldrich 99%): 0, 2, 3, 20, 30 µg), alkenes ((Z)-9-heneicosene (Sigma-Aldrich 97%): 0, 2, 3, 20, 30 µg; (Z)-9-tricosene (Sigma-Aldrich 97%): 0, 0.05, 1, 2, 3, 20, 30 µg) and methyl-alkanes from washes (0, 0.04, 1, 2 µg). Methyl-alkanes were acquired by a separation technique modified from Greene and Gordon (2007), I altered the concentrations of methyl-alkanes used for these experiments by varying the number of ants used in the original pentane wash (Fig. 1).
During each trial I randomly selected one ant from the pair to treat with a specific concentration of compound. I prepared the compounds by placing 100ul of the desired concentration of compound in a glass vial and concentrating it to dryness under a nitrogen stream. I then placed the ant that was receiving the treatment into the treated vial and vortexed the ant for a total of one minute. A new vial was used for each ant and as a control the other ant from the pair was vortexed for one minute in a vial of 100ul hexane that was concentrated to dryness. After both ants from the pair were vortexed I waited 10 minutes before beginning behavioral bioassays. Vortexing did not appear to affect the ant’s behavior or cause significant mortality.

Behavioral Bioassay

Both the treated and untreated ants were placed in a glass Petri dish (50 mm dia. X 7 mm h) for five minutes. Behavioral interactions between pairs were observed for 5 minutes and behaviors were scored using the following index: 1 = antennation, 2 = threat: open mandibles while facing each other and touching, 3 = attack: includes biting, 4 = intense attack: includes dragging and spraying. To ensure unbiased scoring, video recordings of the bioassays were made for later analysis. I recorded the duration of each behavior and calculated the overall aggression index (AI) using the methods of D’Ettorre et al. (2000). I used the formula:

$$ AI = \frac{\sum_{i=1}^{n} AI_i \cdot t_i}{T} $$

Where $AI_i$ is the aggression index, $t_i$ is the duration of each act, and $T$ is the sum of the time that the ants were in contact. At the end of five minutes, ants were frozen and stored at -20°C for further chemical analysis. I subsequently chemically analyzed the treated ants using GC-MS to confirm that the intended compound had been properly transferred to the treated ant. The
amounts of compounds transferred to ants were within natural cuticular hydrocarbon concentrations.

Data Analysis

To determine what compounds increase aggression and to determine threshold response limits for these compounds I performed an ANOVA with Tukey HSD pairwise comparisons on each of the compounds that I tested (JMP Pro 9).

Results

Alkanes

Changing the recognition profile of *F. argentea* workers by increasing the amount of individual alkanes on their surface did not increase aggression. A commercially available alkane standard contained nearly all of the alkanes found on the surface of this species. The addition of this C$_{21}$-C$_{40}$ alkane standard did not significantly influence aggression ($F_{4,245} = 0.43$, $P= 0.78$). From this mix of alkanes, two alkanes, pentacosane and heneicosane, were chosen as representative compounds to test more closely for a behavioral response. Pentacosane did not significantly affect aggression ($F_{4,120} = 1.93$, $p = 0.11$) indicating that pentacosane did not elicit a nestmate recognition response (Fig. 2). Heneicosane also did not significantly affect aggression in *F. argentea* workers ($F_{4,120} = 1.43$, $p = 0.23$) indicating that heneicosane did not elicit a nestmate recognition response (Fig. 3).

Alkenes

Changing the recognition profile by increasing the amount of two representative alkenes on *F. argentea* workers affected aggression. The addition of (Z)-9-heneicosene significantly affected aggression in *F. argentea* nestmates ($F_{4,120} = 7.94$, $p = 0.0001$). The perceptual threshold to changes in Z-9-Heneicosene levels was after the addition of 2-3 µg of (Z)-9-
heneicosene to the hydrocarbon profile (Fig. 4). (Z)-9-tricosene also significantly affected aggression in *F. argentea* nestmates (*F*<sub>6,168</sub> = 73.97, p <0.001). The perceptual threshold to changes in the (Z)-9-tricosene hydrocarbon profile was after the addition of 1-2 µg of (Z)-9-tricosene (Fig 5). Alkene washes also affected aggression however this study focuses on a subset of synthetic alkenes because this yields to a more accurate quantification of perceptual thresholds.

**Methyl-alkanes**

Increasing the amount of methyl-alkanes on the cuticular profile of *F. argentea* workers affected aggression. The addition of methyl-alkane acquired from washes of *F. argentea* workers affected aggression in *F. argentea* nestmates (*F*<sub>3,93</sub> = 4.03, p =0.0096). The perception threshold to changes in the methyl-alkane profile was after the addition of 1-2 µg of methyl-alkanes (Fig. 6).

**Discussion**

Ants respond differentially to different structural classes of hydrocarbons. Increasing the amount of methyl-alkanes on the hydrocarbon profile of one ant from a pair or nestmates evoked an aggressive response. I also found that altering the alkene profile of *Formica argentea* elicited an aggressive response. Based on the alkane standard and two sample alkanes tested, altering the alkane profile of *F. argentea* did not increase aggression. These results show the importance of separating out the different structural classes of hydrocarbons when investigating questions about nestmate recognition.

The behavioral changes seen after the addition of methyl-alkanes to the hydrocarbon profile of *F. argentea* support my finding from Chapter 3, that methyl-alkanes with a C<sub>29</sub> carbon backbone are an important component of the colony-specific recognition signature for *F.*
argentea. Methyl-alkanes statistically separate workers among nests in several species of ant, although the direct link to behavior has only been tested in a few cases (Dahbi et al. 1996; Martin et al. 2008a; Martin et al. 2008c; Provost et al. 1994). Lucas et al. (2005) found that methyl-alkanes do play a role in the nestmate recognition of three species of Pachycondyla ants. These results are consistent with my finding that methyl-alkanes are important nestmate recognition cues in F. argentea.

The addition of alkenes also elicited an aggressive response in F. argentea. However, these results are more difficult to interpret. Although I cannot exclude the possibility that alkenes contribute to nestmate recognition in F. argentea, in chapter 3 I found that there was not enough variation in the alkene profiles of F. argentea workers to provide a colony-specific signal that could statistically differentiate workers into colonies. For a recognition cue to be informative there must be enough possibilities for cue diversity to differentiate amongst groups (Breed and Buchwald 2008). In F. argentea there are only four alkenes in the hydrocarbon profile, which may simply not be enough to provide an informative nestmate recognition cue, but when used in combination with other hydrocarbons alkenes may be important in nestmate recognition. In some species of Formica, alkenes have also increased worker aggression although in these cases there were at least five different alkenes present on the cuticle (Akino et al. 2004; Martin et al. 2008b; Martin et al. 2009c).

Another important conclusion from this study is that there is a minimum threshold for ants to perceive changes to the cuticular hydrocarbon profile in F. argentea. Workers do not act aggressively towards a nestmate until differences in the hydrocarbon profile reach the perception threshold. This information supports the idea that individuals compare the cuticular hydrocarbon profile of an ant they encounter to their internal template, if there is enough of a difference
between the template and the cue then the individual will behave aggressively. Evidence of a
chemical perception threshold has been demonstrated in the paper wasp, *Polistes dominulus*, (Cini et al. 2009) and two species of ants (Ichinose and Lenoir 2010, Ozaki and Wada-Katsumata 2010). Studies that examine quantitative thresholds for perception in nestmate recognition are vital to the understanding of nestmate recognition systems as a whole. The majority of studies that look at the effects of specific compounds to nestmate recognition fail to test different concentrations of compounds to establish a perception response threshold (Dahbi et al. 1996; Dani et al. 2001; Greene and Gordon 2007; Lucas et al. 2005; Martin et al. 2008b; Martin et al. 2008c; Provost et al. 1993). The existence of a minimum perception threshold for a particular compound demonstrates how much information is needed for eusocial insects to no longer recognize an individual as a nestmate. Establishing a perceptual response threshold for nestmate recognition in ants opens the door for examinations of broader ecological questions that combine the ideas of nestmate recognition with overall colony fitness.

Reeve (1989) defines an optimal acceptance threshold in nestmate recognition as the maximum difference between the template and the cue that a guard would allow without rejecting an individual that it encounters. He (Reeve 1989) described the idea that the acceptance thresholds of a colony may shift in a constantly changing environment in order to maximize colony fitness. Reeve’s (1989) optimal acceptance threshold model describes varying acceptance thresholds depending on the cost of accepting kin and rejecting non-kin. For example, in honeybees during periods when resources were low, guards were more selective about whom they allowed into the hive, while when resources were high guards became less selective about who they allowed into the hive (Couvillon et al. 2008; Downs and Ratnieks 2000). Although Reeve (1989) is describing an optimal threshold for the phenotype matching internal template, a
similar line of reasoning can apply to perceptual thresholds. Now that investigators are beginning to establish perception acceptance thresholds in ants, further work needs to be done to establish whether ants have an optimal acceptant threshold that shifts to maximize colony fitness. Although this idea has not been directly tested, the ant, *Plagiolepis pygmea*, seasonally varies in aggressiveness (Thurin and Aron 2008) suggesting the possibility that the acceptance threshold can change with ecological conditions in ants.

Investigators need to be cautious when interpreting cuticular hydrocarbon profiles and the potential role of hydrocarbons in nestmate recognition. Typically studies on this topic rely on multivariate analyses of the entire profile; as pointed out by Martin and Drijfhout (2009a), these methods may not always be appropriate. As made clear by this study, the entire hydrocarbon profile is not necessarily responsible for eliciting a nestmate recognition response. Studies that treat the hydrocarbon profile as a whole, when making claims about nestmate recognition, may be passing over biologically relevant information. To thoroughly examine nestmate recognition, investigators should determine what compounds elicit a nestmate recognition response and determine the perception thresholds of those compounds. These results will broaden our knowledge about nestmate recognition and help us understand how the ecology of the nest affects nestmate recognition systems.
Figure 1: A schematic of the methods used to separate methyl-alkanes from the rest of the cuticular hydrocarbon profile of *F. argentea* workers. The isolated methyl-alkanes that I acquired through this procedure were added to *F. argentea* workers to alter their methyl-alkane hydrocarbon profile. The concentrations of methyl-alkanes that were added varied by changing the number of ants that were included during the first pentane wash (0, 12, 40, 60 ants). This method was adapted from Greene and Gordon (2007).
Figure 2: The mean aggressions score for pairs of nestmate ants where one ant from the pair was treated with pentacosane while the other was not. The addition of pentacosane did not significantly affect the level of aggression between nestmates.
Figure 3: The mean aggression scores for pairs of nestmate ants where one ant from the pair was treated with heneicosane while the other was not. The addition of heneicosane did not significantly affect the level of aggression between nestmates.
Figure 4: The mean aggression scores for pairs of nestmate ants where one ant from the pair was treated with \((Z)-9\)-heneicosene while the other was not. The addition of \((Z)-9\)-heneicosene significantly increases the level of aggression between nestmates. Aggression increases beginning with the addition of 2–3 µg \((Z)-9\)-heneicosene to the treated ant indicating the lower threshold response limit.
Figure 5: The mean aggression scores for pairs of nestmate ants where one ant from the pair was treated with (Z)-9-tricosene while the other was not. The addition of (Z)-9-tricosene significantly increases the level of aggression between nestmates. Aggression increases beginning with the addition of 1-2 µg (Z)-9-tricosene to the treated ant indicating the lower threshold response limit.
Figure 6: The mean aggression scores for pairs of nestmate ants where one ant from the pair was treated with methyl-alkane washes from *F. Argentea* workers while the other was not. The addition of methyl-alkanes significantly increases the level of aggression between nestmates. Aggression increases beginning with 1-2 µg of methyl-alkanes indicating the lower threshold response limit.
Chapter 5:

Differences in Cuticular Hydrocarbon Profiles and Their Effects on the Behavior of *Formica argentea*

Michelle O. Krasnec

Department of Ecology and Evolutionary Biology

The University of Colorado, Boulder

Boulder, Colorado, USA 80309-0334
Abstract

Social recognition is an important factor contributing to the ecological success of eusocial insects. Individuals must be capable of recognizing a group member from a non-group member for social recognition to be effective. Most eusocial insects use phenotypic matching, employing hydrocarbons on their cuticle as recognition cues, however, little is known about exactly how phenotypic matching is used during social recognition. Individual ants that encounter one another very rarely have perfectly “matching” phenotypes. Information is needed on how similar or how different chemical phenotypes need to be to stimulate a nestmate recognition response. In this study, I examine how differences in the cuticular hydrocarbon profile of pairs of ants translate to behavioral responses. In other words, how different do recognition phenotypes need to be to evoke a behavioral response? I found that as differences between the overall cuticular hydrocarbon profile between pairs of ants increased, overall aggression index score also increased. I also found that differences in two methyl-alkanes (7-methylheptacosane and 7-methylnonacosane) and one alkane (triacontane) were statistically important in predicting aggression scores within pairs of ants. When examining the two most aggressive behaviors performed by *F. argentea*, dragging and spraying, dragging behavior was predicted by differences in the chemical profiles of 9-heptacosene, heptacosane, 15,13,11 methylnonacosane, and hentriacontane and spraying behavior was affected by differences in several methyl-alkanes (15,13,11 methylnonacosane, 7-methylnonacosane, and 8,12 methyldotriacontane). Based on these results, it appears that differences in methyl-alkane profiles are most important in predicting aggression, supporting my results from Chapter 4 that methyl-alkanes increase aggression in *Formica argentea.*
Introduction

Social recognition relies on phenotypic variation among animals within populations; this variation provides cues for social discriminations. Eusocial insects predominantly use olfactory cues, in the form of cuticular hydrocarbons, in nestmate recognition (Lockey 1998). An individual social insect that encounters another individual uses phenotypic matching to determine whether it should or should not behave aggressively toward that individual (Crozier and Dix 1979). The “guarding” individual has an internal template of its nest odor, essentially the average cuticular hydrocarbon profile present on individuals from its nest, which it then tries to compare to the encountered individual’s cuticular hydrocarbon profile. If there is not a close enough “match” in hydrocarbon profiles, the “guarding” individual often behaves aggressively toward the individual that it has encountered (Crozier and Dix 1979). Few studies have attempted to quantify how large cuticular profile differences need to be between the two individuals to cause a behavioral response (Cini et al. 2009). Even less is known about how specific differences in the cuticular hydrocarbon profile mediate specific behavioral components of increased aggression.

Differences in cuticular hydrocarbon profiles of eusocial insect workers are generally good at statistically distinguishing workers between nests (Akino et al. 2004, Dapporto et al. 2006; Espelie et al. 1994; Martin et al. 2008c; Martin and Drijhout 2009; Singer et al. 1998). Principal components analysis or discriminant function analysis can correctly classify workers by nest, based on cuticular hydrocarbon profiles (Akino et al. 2004, Dapporto et al. 2006; Espelie et al. 1994; Lockey 1991; Maritn et al. 2008a; Martin and Drijhout 2009a; Singer et al. 1998). These studies indicate that each nest presents its own fingerprint cuticular hydrocarbon profile. Most of these studies generally do not correlate behavior with cuticular hydrocarbon differences.
However, recently some studies have begun to convincingly link differences in insect chemistry to behavior (Dani et al. 2001; Lorenzi et al. 2004; Martin et al. 2009b; Ruther et al. 2002).

Cuticular hydrocarbons that serve communication functions tend to be subsets of the larger array of compounds on the insects’ surface. In the honeybee, *Apis mellifera*, n-alkenes and fatty acids function as recognition cues (Breed 1998a; Breed 1998b). In some ants, methyl-branched alkanes and alkenes function in communication (Dahbi et al. 1996; Lucas et al. 2005; Martin et al. 2009b; Provost et al. 1993). Gamboa et al. (1996) provided behavioral evidence that mixtures of methyl-branched alkanes are responsible for social recognition in *P. fuscatus*. Dani et al. (2001) found that in *P. dominulus* monomethyl alkanes and alkenes induce behavioral responses but n-alkanes had no effect. Additionally, in Chapter 4, I found that both methyl-alkanes and alkenes increased aggression in *Formica argentea*, while n-alkanes did not.

Effective social recognition is important in social insect colonies because they often face many external pressures that could be harmful to the fitness of the nest. Parasites, heterospecific predators and conspecific predators often attempt to steal food, shelter, and other resources from well-established colonies. There is typically considerable incoming traffic at the nest entrance and effective guarding requires that eusocial insects be good at distinguishing a nestmate from a non-nestmate. However, recognition cues between nestmates and non-nestmates often vary slightly within a nest and also tend to overlap between nests making discrimination a difficult task (Getz 1981; Lacy and Sherman 1983). Individuals guarding the nest that require a perfect “match” in recognition cues may often erroneously exclude a nestmate, while individuals that loosely interpret recognition cues may put the entire colony at risk by allowing access to individuals that do not belong into the nest. Although links between cuticular hydrocarbons and
nestmate recognition behaviors are beginning to be established, little is known about how different cuticular profiles must be between individuals to elicit an aggressive response.

Cue diversity is important, and often overlooked, in social recognition. Cues need to be diverse enough to be informative, yet too much diversity within the cue may lead to inefficient social recognition. The importance of cue diversity becomes apparent in groups that exhibit individual recognition. Individual recognition is the most exact form of recognition, and occurs when one individual identifies another according to the individual distinctive characteristics (Dale et al. 2001; Tibbetts and Dale 2007). *Polistes* paper wasps maintain their intracolony dominance hierarchy by being able to identify individuals from their nests based on their facial and abdominal markings (Tibbetts 2002). In instances such as these, where individual recognition occurs, an extremely variable recognition cue can be advantageous; a more distinctive wasp may be less of a threat because its rank is easily identifiable where as a non-distinctive wasp can be seen as a threat because its rank in the colony is less understood (Dale et al. 2001; Tibbetts and Dale 2007). Although most species of eusocial insects do not exhibit individual recognition, cue diversity remains crucial in all instances of social recognition including those using individual recognition and phenotypic matching. In the cases where an individual is using phenotypic matching for nestmate recognition, investigating the amount of cue diversity within a colony and how “choosy” individuals are when using phenotypic matching for nestmate recognition, can lead to a better understanding about the mechanisms underlying nestmate recognition in these systems.

In this chapter I examined the differences in cuticular hydrocarbon profiles of pairs of nestmate *Formica argentea* ants and compared those differences with behavioral data to determine if an increased difference in cuticular hydrocarbon profile corresponds with increased
aggression. I predicted that the larger the difference in cuticular hydrocarbon profile between individuals within a pair the more aggressive they will be. I also examined which compounds are important in predicting aggressive behavior. I predicted that methyl-alkanes would be important because methyl-alkanes produce a colony-specific signal (chapter 3). When investigating specific aggressive behaviors, rather than an aggression score, to see if differences in particular compounds influence particular highly aggressive behaviors, I predicted a large difference between methyl-alkanes and alkenes in the profile will predict aggressive behaviors.

Methods

Study Organism

Five colonies of *Formica argentea* were collected in Jefferson County Colorado in the Spring of 2010. Colonies were collected at least 500 meters apart from one another to ensure the collection of separate nests. After field collection, live colonies were housed separately in the lab in plastic containers (29 x 16 cm and 10 cm high) with a plaster floor. Nests were kept at room temperature, 23 ± 2 °C, with a 12:12 h light:dark cycle. Ants were provided with water and a 10% sucrose solution ad libitum and given frozen crickets two times a week. All behavioral bioassays were performed within one month of the time that I collected the nests from the field.

Behavioral Bioassay

I classified 14 different behaviors exhibited by *F. argentea* (Table 1). Aggressive behaviors were considered: attacking, charging, dragging, biting, lunging, stalking, spraying and threatening; Non-aggressive behaviors were considered: antennation, following, avoiding, standing next to, and looking. Pairs of ants were selected haphazardly from one of the five nests and placed in a Petri dish (50 mm dia. X 7 mm h). I performed bioassays for a total of 25 nestmate pairings and 25 non-nestmate pairings. Specific behaviors between pairs were observed
for 10 minutes. The frequency of each behavior was recorded. I recorded the proportion of time the ants spent performing a specific behavior and additionally calculated an Aggressions Index Score (AI) for each pair of ants as done by D’Ettorre et al. (2000). To calculate the AI, behavioral interactions between pairs were observed for 10 minutes and behaviors were scored using the following index: 1 = antennation, 2 = threat: open mandibles while facing each other and touching, 3 = attack: includes biting, 4 = intense attack: includes dragging and spraying. I recorded the duration of each behavior and calculated the overall aggression index (AI) using the methods of D’Ettorre et al. (2000). I used the formula:

\[ AI = \frac{\sum_{i=1}^{n} AI_i \times t_i}{T} \]

Where \( AI_i \) is the aggression index, \( t_i \) is the duration of each act, and \( T \) is the sum of the time that the ants were in contact. After observations ants were frozen and stored at -20 °C for subsequent chemical analysis.

Chemical Analysis

To determine the differences in cuticular hydrocarbon profiles between pairs of ants I used Gas Chromatography- Mass Spectrometry (GC-MS) to analyze chemical extracts from individual ants. Prior to chemical extraction, ants were taken out of the freezer and allowed to defrost for 20 min. Each ant was then placed in 0.5 ml of hexane for 10 min. Extracts were concentrated to dryness under a stream of nitrogen and re-suspended in 50 µl of distilled hexane, then injected into an Agilent 6890N GC interfaced with an Agilent 5975 mass selective detector. Nonadecane was used as an internal standard (500 ng). Mass spectra were recorded at 70 eV in electron impact ionization mode. The GC was fitted with DB-5MS 30m x 0.25mm column (J & W Scientific, Folsom CA, USA). Sample runs began at 120°C then increased at the rate of 8°C/min. until a temperature of 300°C had been reached. Samples were held at this temperature
for 10 min. prior to ending a run. Injections were made in a splitless mode with a 0.2ml/m purge at 2 min. Helium was the carrier gas. The injector temperature was 250°C, the ion source temperature was 230°C, and the quadrupole temperature was 150°C.

Peak Identification

I identified n-alkanes by comparison of GC and MS data with purchased standard reference compounds (Sigma-Aldrich: Alkane Standard Solution C₂₁-C₄₀). Methyl-alkanes and alkenes were identified by combining the evidence mass fragmentation patterns, diagnostic fragment ions (compared to those in the NIST library), and retention indices in accordance with Carlson et al. (1998). Double bond positions were identified through DMDS derivatization (Carlson et al. 1989).

Data analysis

I examined the differences in hydrocarbon profile between pairs of nestmate ants. Quantitation was based on integration of total ion chromatograms to determine the relative proportion of each compound present on the hydrocarbon profile. The relative proportion of each compound was arcsine transformed for data analysis. For each pairing of ants I took the absolute value from subtraction of the transformed proportion of each compound on ant one from the transformed proportion of each compound on ant two. This gave me a score for the difference in the relative proportion of each compound in the cuticular hydrocarbon profile between each pair of ants.

I also summed up the differences in compounds between each pair of ants to get a score for how different the cuticular hydrocarbon profile was between members of the pair.

Statistical Analysis

*Relationship between aggression and chemical differences*
To determine if aggression increased as the difference in chemical profile increased, I performed a regression looking at the relationship between the sum of the differences between the pairs compared to the aggression index score that the pair received.

To determine which compounds were statistically good predictors of aggression score, I performed a stepwise multiple regression.

Specific Compounds

To determine what compounds are important in statistically predicting aggression, a stepwise discriminant analysis was performed. To investigate how differences in specific compounds correspond with differences in specific behaviors, a stepwise discriminant analysis was performed.

Results

Relationship between aggression and chemical differences

I performed a linear regression to determine if a larger difference in the cuticular hydrocarbon profile between a pair of ants affects aggressive behavior. The difference in cuticular hydrocarbon profiles between a pair of ants significantly correlates with the aggression index score between the pair although the relationship is not linear (p = 0.0004, R² = 0.25, F₁,₄₀ = 14.77).

Compounds that statistically predict aggression

To test which compounds are good predictors of aggression index score, a stepwise multiple regression analysis was performed. Levels of F to enter and levels of F to remove corresponded to probability levels of 0.05 and 0.1, respectively. Tests for multicollinearity indicated that there were low levels of multicollinearity for the compounds entered in the model. Results of the stepwise multiple regression indicate that differences in 7-methylheptacosane, 7-
methylnonacosane, and triacontane were sufficient enough to influence aggression (P < 0.001, R^2 = 0.44, F_{3,47} = 11).

Compounds that statistically predict specific behaviors

To test which compounds are good predictors of the occurrence of specific behaviors a stepwise multiple regression analysis was performed. Levels of F to enter and levels of F to remove corresponded to p levels of 0.05 and 0.1. Tests for multicollinearity indicated that there were low levels of multicollinearity for the compounds entered in the model. For dragging behavior, the results of the stepwise multiple regression indicated that differences in 9-heptacosene, heptacosane, 15,13,11 methylnonacosane, and hentriacontane were enough to predict dragging behavior (P = 0.0004, R^2 = 0.38, F_{4,46} = 6.44).

For spraying behavior, 15,13,11 methylnonacosane, 7-methylnonacosane, and 8,12 methylldotriacontane were enough to predict spraying behavior (P < 0.0001, R^2 = 0.43, F_{3,47} = 10.96).

Discussion

Differences in cuticular hydrocarbon profiles between pairs of ants significantly correlated with aggression. As the difference between the cuticular profiles of the individuals increased, the level of aggression between the pair increased. Several studies have shown that cuticular hydrocarbon profiles can be colony-specific, however the behavioral link between differences in cuticular hydrocarbon profiles in ants and aggressive behavior is rarely made (reviews: Hefetz 2007; D’Ettorre and Lenoir 2010). In support of these results, in chapter 4 I demonstrated that increasing the difference of methyl-alkanes and alkene hydrocarbon profiles between pairs of ants caused increased aggression in *Formica argentea*. These results provide
support indicating that differences in cuticular hydrocarbon profiles are associated with aggression, strongly supporting their possible importance in nestmate recognition.

A stepwise regression demonstrated that two methyl-alkanes (7-methylheptacosane and 7-methyl-nonacosane) and one alkane (triacontane) were important in influencing aggression scores. These results partially support my findings from chapter 3 and those of other investigators, that changes in methyl-alkanes influence aggression (Lucas et al. 2005). This study provides a good link between behavior and chemical differences, something that is lacking in other studies. Other investigators have found that methyl-alkanes are important in statistically separating workers by colonies (Dahbi et al. 1996; Martin et al. 2008a; Martin et al. 2008c; Provost et al. 1994). These are interesting results, but, due to the difficulty and extensive time requirement, the studies have not linked chemical differences to differences in behavior. Studies, such as mine, take a detailed approach that tries to link chemical differences with aggression and indicate that differences in cuticular hydrocarbon profiles can predict aggression.

When looking at specific aggressive behaviors, rather than an aggression score, I found that differences in certain compounds do influence both dragging and spraying behavior, the most aggressive behaviors exhibited by *F. argentea*. I examined both dragging behavior and spraying behavior to determine if differences in the cuticular profile of specific chemicals affect either of these behaviors. Dragging behavior was predicted by differences in the chemical profiles of 9-heptacosene, heptacosane, 15,13,11 methyl-nonacosane, and hentriacontane. Spraying behavior was affected by differences in several methyl-alkanes (15,13,11 methyl-nonacosane, 7-methyl-nonacosane, and 8,12 methyl-dotriacontane).

Differences between pairs of ants in both an alkene and a methyl-alkane were most important in statistically predicting dragging and spraying behavior, supporting my findings from
Chapter 4 that both alkenes and methyl-alkanes increase aggression in *F. argentea*. These results are supported by other studies where in some species of *Formica* alkenes have increased worker aggression (Akino et al. 2004; Martin et al. 2008b; Martin and Drijfhout 2009a) and methyl-alkanes evoke a nestmate recognition response in three species of *Pachycondyla* ants (Lucas et al. 2005). These studies pinpoint the compounds that are important in nestmate recognition but do not examine the relative differences between compounds that evoke a behavioral response.

This chapter aimed to develop our understanding of olfactory social recognition at a finer scale. Recognition cues often vary slightly within a nest and also tend to overlap between nests, making phenotypic matching in the context of nestmate recognition a difficult duty (Getz 1981; Lacy and Sherman 1983). Individuals guarding the nest do not require a perfect match in recognition cues because accepting only perfect matches would lead to many recognition errors (Couvillon et al. 2008; Downs and Ratnieks 2000; Reeve 1989). My study examined variations between the hydrocarbon profile to determine how these differences affect behavior. To fully understand the intricacies of phenotypic matching with respect to social recognition further studies are warranted. These studies should alter the proportions of specific compounds on pairs of ants to decipher precisely how these chemical differences affect specific behaviors.
Table 1: A list of behaviors that were observed during behavioral bioassays between pairs of *Formica argentea* workers.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description of Behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bite</td>
<td>Biting</td>
</tr>
<tr>
<td>Chase</td>
<td>Lunge towards ant open mandibles, contact</td>
</tr>
<tr>
<td>Drag</td>
<td>Pull antennae or other body parts</td>
</tr>
<tr>
<td>Attack</td>
<td>Contact with other ant open mandibles</td>
</tr>
<tr>
<td>Stalk</td>
<td>Lunge towards ant open mandibles, no contact</td>
</tr>
<tr>
<td>Threat</td>
<td>Antennae waving in threatening way with open mandibles</td>
</tr>
<tr>
<td>Antennation</td>
<td>Touching with antennae closed mandibles</td>
</tr>
<tr>
<td>Follow</td>
<td>Touching and following ant closed mandibles</td>
</tr>
<tr>
<td>Avoid</td>
<td>Touch and then run away closed mandibles</td>
</tr>
<tr>
<td>Look</td>
<td>Looking at close range to ant, no touching, closed mandibles</td>
</tr>
<tr>
<td>Standing Next To</td>
<td>Standing next to at close range, no touching, closed mandibles</td>
</tr>
</tbody>
</table>
Chapter 6:

Temporal Variation of Cuticular Hydrocarbons in *Formica argentea* Workers: Differences Among Hydrocarbon Structural Classes

Michelle O. Krasnec

Department of Ecology and Evolutionary Biology

The University of Colorado, Boulder

Boulder, Colorado, USA 80309-0334
Abstract

The cuticular hydrocarbon profile of ant colonies can vary temporally, however most studies that examine this variation investigate the hydrocarbon profile as a whole. This approach may not be very informative in understanding nestmate recognition, as specific structural classes of hydrocarbons may differ in chemical properties and in importance in nestmate recognition. In this study I tracked the changes in worker cuticular hydrocarbon profiles in laboratory colonies of *Formica argentea* every two weeks for sixteen weeks. I examined quantitative and proportional differences in n-alkane, alkene, methyl-alkanes, and methyl-alkanes with a C$_{29}$ backbone. In a uniform environment, the total amount of hydrocarbons increased with time for all structural classes. However, the relative proportions of the different structural hydrocarbon classes behaved differently. The relative proportion of n-alkanes in the profile increased while the relative proportions of alkenes and methyl-alkanes in the profile decreased. Interestingly, the proportion of the C$_{29}$ backbone methyl-alkanes did not vary with time. Finding no change in the C$_{29}$ methyl-alkanes suggests that although the colony cuticular hydrocarbon profile changes over time, one of the proposed colony recognition signals remains constant (Chapter 3). *F. argentea* workers maintained their ability to discriminate a nestmate from a non-nestmate even after being kept in this uniform environment and workers maintained the same level of aggression between non-nestmates until the end of the sixteen-week experiment. These results may have implications for understanding nestmate recognition in this and other species because the C$_{29}$ backbone methyl-alkanes are important to the colony-specific recognition signal.
Introduction

Animal species that rely on external phenotypes for social recognition may face difficulties if the phenotype changes ontogenetically or seasonally. The potential changes in the external recognition phenotype raises concerns as to their reliability as recognition cues. In eusocial insects, cuticular hydrocarbons, chemical cues used for nestmate recognition, have been shown to change over time (Dahbi and Lenoir 1998; Lenoir et al. 2001; Liu et al. 1998; Newey et al. 2009; Nielsen et al. 1999; Provost et al. 1993; Suarez et al. 2002; Vander Meer et al. 1989; Van Zweden 2010). In this chapter I examine time-based changes in cuticular hydrocarbon profiles and determine if colonies that are kept in a uniform environment lose their ability to recognize a nestmate from a non-nestmate in the ant *Formica argentea*.

In insects, hydrocarbons are internally synthesized by oenocytes associated with either epidermal cells or within the peripheral fat body (Bagnères and Blomquist 2010). The newly synthesized hydrocarbons are transported to the hemolymph in association with the lipophorin molecule (Bagnères and Blomquist 2010). The process of hydrocarbon transport from the oenocytes to the cuticle can be very selective, as previous studies have found differences in quantitative amounts of hydrocarbons between the hemolymph and the cuticle (Sevala et al. 2000; Schal et al. 2003). Some cuticular hydrocarbon odors directly reflect genetic variation among ants (Obin and Vander Meer 1988; Thurin and Aron 2008; Van Zweden 2009). Other cuticular hydrocarbon odors can be influenced by environmental factors, such as food consumed by the ants (Crosland 1989c; Jutsum et al. 1979; Liang and Silverman 2000; Liang et al. 2001; Van Zweden et al. 2009), or be modified by exposure to soil or other nesting materials (Bos et al. 2011). Oftentimes cuticular hydrocarbon profiles are shaped by an interaction of genetic variation and environmental factors (Liu et al. 2001; Martin and Drijfhout 2009c; Obin and
Vander Meer 1988; Stuart 1987). Multiple factors contribute to insect cuticular hydrocarbon profiles, however in social insects the relative contributions to the cuticular hydrocarbon profile of hydrocarbons that are synthesized by the insect and those that are sequestered from dietary or other environmental sources is poorly understood.

Some studies have found that, although members of each colony have a uniform odor, this odor can vary seasonally (Liu et al. 1998; Liu et al. 2001; Nielsen et al. 1999; Vander Meer 1989). These results are interesting, but in these cases changes in the cuticular hydrocarbon profile may be due to changing environmental conditions. Interestingly, when some species of ants are placed in a uniform laboratory environment their cuticular hydrocarbon profiles continue to change with time (Lahav et al. 2001; Provost et al. 1993; Van Zweden et al. 2009). Placing colonies in a uniform lab environment allows for studies that begin to distinguish whether hydrocarbons are mainly acquired genetically or from the environment (Stuart 1987; Van Zwenden et al. 2009). Studies that take these factors into account can determine how specific parts of the colony odor, particularly the ones involved in recognition systems, vary with time.

Nestmate recognition in social insects relies on cuticular hydrocarbon cues. Cuticular hydrocarbon profiles of the same species typically have the same set of compounds, but those compounds vary in relative proportion (Bagnères and Wicker Thomas 2010; Nowbahari et al. 1990). There are three major structural classes of hydrocarbons n-alkanes, alkenes and methyl-alkanes present on the insect cuticle. The three structural classes of cuticular hydrocarbons have different properties that may cause them to behave differently on the cuticle when exposed to varying environmental factors. n-Alkanes have a high melting temperature and my therefore be better at preventing water loss than alkenes or methyl-alkanes of the same carbon chain length (Gibbs 1998; Gibbs et al. 2003). While investigators generally accept that cuticular hydrocarbons
can vary temporally, the majority of studies of shifts in cuticular hydrocarbon profiles focus on the changes to the whole hydrocarbon profile rather than hydrocarbon classes embedded in the overall signature (Nielsen et al. 1999; Liu et al. 1998; Liu et al. 2001; Vander Meer et al. 1989).  The differences in chemical properties of the different structural classes of hydrocarbon provides support for the importance of examining ontogenetic and temporal changes in the particular structural classes of hydrocarbons, rather that changes to the profiles as a whole.

The aim of this chapter was to closely examine how the different structural classes of cuticular hydrocarbons change over time in *Formica argentea* colonies kept in a uniform environment. I focused on closely investigating different structural classes of compounds. My finding from chapters four and five indicate that *F. argentea* workers react differently to the different structural classes of compounds, thus separating the cuticular hydrocarbon profile into structural classes was important for this study. More specifically, I wanted to test whether one of the proposed colony nestmate recognition signatures, C29 backbone methyl-alkanes, changes over time (Chapter 3). I also examined nestmate recognition abilities at the beginning of this study and again after colonies had been kept in a uniform environment after 16 weeks to test if aggression levels changed after individual colonies encountered uniform environmental conditions.

**Methods**

To examine how the different structural classes of cuticular hydrocarbons changed over time I placed four whole colonies of field-collected individuals in a uniform laboratory environment. Bioassys were performed on field-collected colonies at week 0 to record their initial average aggression. Every two weeks for 16 weeks I analyzed the chemical profile for 18 different ants in the colony and looked for changes in both quantity and relative proportion of n-
alkanes, alkenes, methyl-alkanes, and C$_{29}$ methyl-alkanes in their hydrocarbon profile. I examined the quantity and of these compounds because variation in the quantity of hydrocarbons may differentially affect their waterproofing ability and, in turn, affect insect survival. I investigated the proportions of these compounds because other studies have hypothesize that discrimination using chemical compounds is based on ratios among those compounds, making relative proportions of compounds crucial to investigate (Breed and Buchwald 2008).

Colony collection

I collected four entire colonies in the summer of 2010 in Jefferson County Colorado and brought them back to the lab. To control for the possible effects of colony size and the presence of a queen, each colony selected for this experiment contained about 200 workers and a queen. Each nest was placed in an individual plastic shoebox container that contained a bottom layer of plaster of paris and silicone spray around the top edge to prevent the ants from escaping. Every two weeks, beginning with day 0, I haphazardly collected 18 individual ants from each nest. Immediately after collection, ants were frozen at -20°C and held until analysis. Ants were collected in this manner every two weeks for a total of 16 weeks. To ensure uniform conditions between colonies, colonies were all kept at the same temperature (23°C- 26°C), given the same light conditions (12:12 LD), given two dead crickets for food per colony once a week, and received water and a 10% sucrose solution *ad libitum*.

Chemical Extraction

Prior to extraction of the cuticular hydrocarbons, I took worker ants out of the freezer, allowed them to defrost for 20 min. and placed each ant in 0.5ml of hexane for 10 min. Extracts were concentrated to dryness under a stream of nitrogen and re-suspended in 50 µl of distilled
hexane. The re-suspended extracts were analyzed using gas chromatography-mass spectrometry (GC-MS).

Chemical Analysis

I injected 2 µl of extracts into an Agilent 6890N GC interfaced with an Agilent 5975 mass selective detector. Mass spectra were recorded at 70 eV in electron impact ionization mode. The GC was fitted with DB-5MS 30 m x 0.25 mm column (J & W Scientific, Folsom CA, USA). Samples began at 120°C then increased at the rate of 8°C/min. until a temperature of 300°C had been reached. Samples were held at this temperature for 10 min. prior to ending a run. Injections were made in a splitless mode with a 0.2 ml/min purge at 2 min. Helium was the carrier gas. The injector temperature was 250°C, the ion source temperature was 230°C, and the quadrupole temperature was 150°C. 500 ng nonadecane was used as the internal standard.

Data Analysis

I grouped compounds based on their hydrocarbon structural class; n-alkane, alkene, methyl-alkane, and methyl-alkane with a C$_{29}$ carbon backbone. These analyses only included compounds that comprised at least 1% of the profile in at least one of the colonies that was examined.

I quantified the total amount of the different hydrocarbons present on each worker in two different ways for analysis: 1) Quantitation was based on determining the adjusted relative proportion of each compound present in the hydrocarbon profile 2) Quantitation was based on integration of total ion chromatograms. Peak areas were standardized before statistical analysis using the method of Aitchison (1986). To determine if the standardized mean amount of each of the four different hydrocarbon structural classes differs through time I performed a nested ANOVA with two nominal variables (nest and week) with nest nested within week. To
determine if the mean relative proportions of the four different structural classes of compound in the hydrocarbon profile vary through time I performed a nested ANOVA with two nominal variables (nest and week) with nest nested within week. The relative proportion data were log transformed for statistical analysis (JMP Pro9).

Behavioral Bioassays

To determine if *F. argentea* ants continue to discriminate a nestmate from a non-nestmate after being placed in a uniform environment for 16 weeks I performed behavioral bioassays at week 0 and at week 16 with workers from four different field collected colonies that were housed in the lab. Ants were selected randomly for a total of 25 non-nestmate and 25 nestmate pairing. Behavioral interactions between pairs were observed in a Petri dish for 10 minutes and behaviors were scored by the following index: 1 = antennation, 2 = threat: open mandibles while facing each other and touching, 3 = attack: includes biting, 4 = intense attack: includes dragging and spraying. Video recordings of the bioassays were taken to ensure unbiased scoring. I recorded the duration of each behavior and calculated the overall aggression index (AI) using the methods of D’Ettorre et al. (2000). I used the formula:

\[
AI = \frac{\sum_{i=1}^{n} A_i t_i}{T}
\]

Where \( A_i \) is the aggression index, \( t_i \) is the duration of each act, and \( T \) is the sum of the time that the ants were in contact.

Results

Standardized Peak Area

The mean quantity of hydrocarbons present on *F. argentea* workers increased through time. For n-alkanes, there was significant variation of mean standardized peak area among nests within week \( (F_{16,2563} = 8.29, p < 0.001) \). There was also significant variation of mean
standardized peak area between the weeks, with the mean standardized peak area of n-alkanes increasing with time ($F_{7, 2563} = 37.67, p < 0.001$), Fig. 1.

For alkenes there was significant variation of mean standardized peak area among nests within week ($F_{16,1706} = 7.33, p < 0.001$). There was also significant variation of mean standardized peak area between the weeks, with the mean standardized peak area of alkenes increasing with time ($F_{7, 1706} = 24.77, p < 0.001$), Fig. 2.

In methyl-alkanes there was a significant variation of mean standardized peak area among nests within week ($F_{16,7738} = 12.11, p < 0.001$). There also was a significant variation of mean standardized peak area between the weeks, with the mean standardized peak area of methyl-alkanes increasing with time ($F_{7, 7738} = 39.29, p < 0.001$), Fig. 3.

When examining the only the methyl-alkanes with a $C_{29}$ carbon backbone there was significant variation of mean standardized peak area among nests within week ($F_{16,2565} = 25.48, p < 0.001$). There is also significant variation of mean standardized peak area between the weeks, with the mean standardized peak area of methyl-alkanes with a $C_{29}$ carbon backbone increasing with time ($F_{7, 2565} = 33.24, p < 0.001$), Fig. 4.

Relative Proportion of Hydrocarbon Components

Among the n-alkanes there was significant variation in the transformed relative proportion of mean peaks among nests within week ($F_{16,2563} = 2.76, p < 0.001$). There was also significant variation in the mean transformed relative proportion peaks between the weeks, with the mean relative proportion of n-alkane peaks increasing significantly with time ($F_{7, 2563} = 5.04, p < 0.001$), Fig. 5.

There was significant variation among the alkenes in transformed relative proportion of mean peaks among nests within week ($F_{16,1706} = 5.60, p < 0.001$) and significant variation in the
mean transformed relative proportion of peaks between the weeks, with the mean relative proportion of alkenes peaks decreasing significantly with time ($F_{7, 1706} = 2.40, p = 0.0140$), Fig. 6.

For the methyl-alkanes there was a significant difference in transformed relative proportion of mean peaks among nests within week ($F_{16, 7738} = 3.06, p < 0.001$) and significant variation in the mean transformed relative proportion of peaks between the weeks, with the mean relative proportion of methyl-alkane peaks decreasing significantly with time ($F_{7, 7738} = 4.10, p < 0.001$), Fig. 7.

Among the methyl-alkanes with a C$_{29}$ carbon backbone there was significant variation in transformed relative proportion of mean peaks among nests within week ($F_{16, 2565} = 49.01, p < 0.001$). There was no significant variation of the mean transformed relative proportion peak of methyl-alkanes with a C$_{29}$ backbone between the weeks ($F_{7, 2565} = 1.02, p = 0.42$), Fig. 8.

Aggression Bioassays

Aggression bioassays done at week 0 indicated that non-nestmate pairings were significantly more aggressive towards one another than nestmate pairing (non-nestmate mean AI score = 0.74 ± 0.06, nestmate mean AI score = 0.13 ± 0.083, $T_{1, 48} = 5.78, P < 0.0001$) indicating that initially $F. argentea$ workers can discriminate a nestmate from a non-nestmate.

At the end of the 16 week period $F. argentea$ workers were still able to discriminate a nestmate from a non-nestmate (non-nestmate mean AI score = 0.69 ± 0.015, nestmate mean AI score = 0.057 ± 0.089, $T_{1, 48} = 5.40, P < 0.0001$). Aggression score between non-nestmates did not significantly differ between week 0 and week 16 ($T_{1, 49} = 0.29, p = .7760$).

Discussion
Throughout the 16 week time period the mean relative proportion of methyl-alkane peaks with a C_{29} backbone did not differ, while the mean relative proportions of n-alkanes, alkene, and methyl-alkane peaks as a whole did differ through time. This suggests that, if the methyl-alkanes with C_{29} carbon backbone are one of the colony-specific recognition signatures, the colony-specific nestmate recognition signature remains constant within the cuticular hydrocarbon profile while the relative proportions of other parts of the profile are constantly changing. Numerous studies have shown that under natural conditions cuticular hydrocarbon profiles change over time (Lahav et al. 2001; Liu et al. 2001; Nielsen et al. 1999; Provost et al. 1993; Vander Meer et al. 1989). These studies investigate changes to the entirety of the cuticular hydrocarbon profile, when realistically only specific parts of the profile are used in nestmate recognition. By taking this approach investigators may miss how the colony-specific signal is changing. This study separates the cuticular hydrocarbon profile by structural classes to investigate changes in the profile. In a uniform environment the relative proportion of the structural classes of hydrocarbons do not behave the same.

The mean relative proportion of n-alkane peaks increased with time while the mean relative proportion of alkene and methyl-alkane peaks decreased with time. Van Zweden et al. (2009) looked at how the mean relative proportions of individual compounds changed through time in a uniform environment. They found that the relative proportion of n-alkanes and some methyl-alkanes increased over time. This study supports their finding that the relative proportion of n-alkanes increases but found that the relative proportions of methyl-alkanes as a whole decreases with time. Throughout the 16-week time period in a uniform environment the total amount of hydrocarbons present on the cuticle of *F. argentea* workers increased. Most interestingly, although the methyl-alkanes with a C_{29} backbone increased in the mean total
amount, the relative proportion of these compounds in comparison to the other compounds on the worker did not change. The constancy of the relative proportion of methyl-alkanes with a C_{29} backbone suggests that they may be an important signal in nestmate recognition.

Further experiments need to be performed to determine why there was an increase in hydrocarbon production. One potential explanation to this change could be attributed to the ant diet in uniform conditions. It is possible for the hydrocarbon of a prey item to show up on the hydrocarbon profile of a predator (Liang and Silverman 2000; Liang et al. 2001). In this study the increase in hydrocarbon production is most likely not attributed to diet because I did not find any unique compounds present on the cuticle of *F. argentea* between week 0 and week 16. Another possible explanation for the increase in hydrocarbon production is that an increase in hydrocarbons is a physiological response to the environment. This is possible considering the primary function of cuticular hydrocarbons is to prevent desiccation (Howard and Bloomquist 2005). Further studies are needed to determine the mechanisms responsible for increased cuticular hydrocarbon production.

Before and after being placed in a uniform environment for 16 weeks, *F. argentea* workers were able to discriminate a nestmate from a non-nestmate. These finding are supported in a few other species of ants that, when maintained in a uniform environment, did not lose their nestmate recognition abilities (Stuart 1987; Van Zweden et al. 2009). On a similar note, several investigators have examined the effects of changes in cuticular hydrocarbon profiles of colonies that have been field collected, split, and kept in a separate uniform environment (Dahbi and Lenoir 1998; Lahav et al. 2001; Newey et al. 2009). They found that the separation caused changes in the hydrocarbon profiles but those changes were not enough induce aggression between the separate colonies (Dahbi and Lenoir 1998; Lahav et al. 2001; Newey et al. 2009).
These studies focused on changes to the entirety of the hydrocarbon profiles and may have missed what was specifically happening to the colony-specific recognition signature.

While it is clear that in many species the hydrocarbon profile changes with time, it is unclear how to interpret those changes in an evolutionary or ecological context. Liu et al. (1998) provide two possible explanations for these changes. They first propose that the temporal change in the hydrocarbon profile provides protection to the colony from social parasites that may, in a static environment, be able to mimic the colony’s profile and in turn rob resources. Constant changes in the profile can possibly deter this type of exploitation. Secondly they propose that the temporal changes in the hydrocarbon profile are a physiological response to the changing environment. Regardless of why the hydrocarbon profile is changing, more information is needed about how the specific parts of the profile change and how cuticular hydrocarbons remain important in nestmate recognition under this changing environment.

My data show that although the different structural classes of hydrocarbons may change with time, the one of the proposed colony-specific signatures does not change. This finding suggests a new understanding of the formation of the internal template formed by guarding ants in nestmate recognition. Many studies conclude that the internal template of guarding individuals updates itself in a changing environment (Vander Meer and Morel 1998). If the colony-specific recognition signal remains constant in a changing environment, guarding individuals may not be constantly updating their internal template in regards to nestmate recognition. This study illustrates the importance of looking at parts of the cuticular hydrocarbon profile rather than making broad statements about the collective profile and establishes a new approach for looking at changes in the cuticular hydrocarbon profile.
Figure 1: Changes in the mean standardized peak area of n-alkanes on the cuticle of *F. argentea* with time when exposed to a uniform environment. The mean amount of n-alkanes present per worker increased significantly with time ($F_{7,2563} = 37.67$, $p < 0.001$).
Figure 2: Changes in the mean standardized peak area of alkenes on the cuticle of *F. argentea* with time when exposed to a uniform environment. The mean amount of alkenes present per worker increased significantly with time ($F_{7,1706} = 24.77, p < 0.001$).
Figure 3: Changes in the mean standardized peak area of methyl-alkanes on the cuticle of *F. argentea* with time when exposed to a uniform environment. The mean amount of methyl-alkenes present per worker increased significantly with time ($F_{7,7738} = 39.29$, $p < 0.001$).
Figure 4: Changes in the mean standardized peak area of methyl-alkanes with a C$_{29}$ carbon backbone on the cuticle of *F. argentea* with time when exposed to a uniform environment. The mean amount of methyl-alkanes with a C$_{29}$ carbon backbone present per worker increased significantly with time ($F_{7, 2565} = 33.24, p < 0.001$).
Figure 5: Changes in the mean transformed relative proportion of n-alkane peaks on the cuticle of *F. argentea* with time when exposed to a uniform environment. The mean relative proportion of n-alkane peaks present per worker increased significantly with time ($F_{7, 2563} = 5.04$, $p < 0.001$).
Figure 6: Changes in the mean transformed relative proportion of alkene peaks on the cuticle of *F. argentea* with time when exposed to a uniform environment. The mean relative proportion of alkene peaks present per worker decreased significantly with time ($F_{7, 1706} = 2.40, p = 0.0140$).
Figure 7: Changes in the mean transformed relative proportion of methyl-alkane peaks on the cuticle of *F. argentea* with time when exposed to a uniform environment. The mean relative proportion of methyl-alkane peaks present per worker decreased significantly with time ($F_{7, 7738} = 4.10$, $p < 0.001$).
Figure 8: Changes in the mean transformed relative proportion of peaks with a methyl-alkane with a C\textsubscript{29} carbon backbone on the cuticle of *F. argentea* with time when exposed to a uniform environment. The mean relative proportion of peaks with a methyl-alkane with a C\textsubscript{29} carbon backbone present per worker did not differ significantly with time (F\textsubscript{7, 2565} = 1.02, p = 0.42).
Chapter 7:

Synthesis and Conclusions

Michelle O. Krasnec
Department of Ecology and Evolutionary Biology
The University of Colorado, Boulder
Boulder, Colorado, USA 80309-0334
Social recognition is a very complex process relying on the expression, detection and perception of recognition cues. In most species of social insects, cuticular hydrocarbons play some sort of role as recognition cues (Breed 1998; Dani et al. 2001; Ruther et al. 2002; Thomas et al. 1999; Wagner et al. 2000). However, little is known about what parts of the profiles are used as recognition cues and how variations in surface chemistry are interpreted by individuals. Working with the ant *Formica argentea*, I examined nestmate recognition and cuticular hydrocarbon profiles and determined how surface chemistry varies between individuals and if variation in cuticular chemistry influences nestmate recognition behaviors.

Deciphering patterns within cuticular hydrocarbon profiles has pushed investigators into looking for colony-specific signatures that may be used as a cue for nestmate recognition. In the past, investigators examined the cuticular hydrocarbon profile in its entirety to determine if workers could be statistically separated by colony using cuticular hydrocarbon profiles alone (Martin and Drijfhout 2009a). While this approach works well at separating workers by colony, the use of the entire hydrocarbon profile in these analyses may mask more precise colony-specific signatures in the hydrocarbon profile (see Martin and Drijfhout 2009a).

I found that in *F. argentea*, the C$_{29}$ methyl-alkanes found in the profile alone can successfully statistically separate workers by nest, indicating that C$_{29}$ methyl-alkanes may provide a colony-specific signature. The diversity of C$_{29}$ methyl-alkanes between nests indicates that the expression of these compounds may play a role in the recognition cue of this species. Furthermore, future studies of nestmate recognition need to closely examine cuticular hydrocarbon profiles in search of colony-specific signatures, and test if these compounds evoke an aggressive response.
Making the experimental link between cuticular chemistry and behavior is crucial to studies of nestmate recognition and is often overlooked (Dahbi et al. 1996; Martin et al. 2008a; Martin et al. 2008c; Provost et al. 1994). In my dissertation I demonstrate that, although the cuticular hydrocarbon of *F. argentea* include n-alkanes, alkenes and methyl-alkanes, workers behave aggressively when exposed to individuals that differ from them in alkene and methyl-alkane profiles. Altering the n-alkane profile in *F. argentea* ants did not influence nestmate recognition behavior. My results suggest that only parts of the profile, not the cuticular hydrocarbon profile in its entirety, are used as a nestmate recognition cue in *F. argentea*. Studies such as this are important because they allow for a precise examination of nestmate recognition, directly demonstrating which compound or compounds evoke a behavioral response. My study also begins to take a quantitative approach to answering questions about nestmate recognition, a topic that is often ignored but crucial to gaining a deep understanding of social recognition.

My dissertation explains olfactory social recognition at a finer scale. I examined variation between the hydrocarbon profiles of individuals to closely examine phenotypic matching and determine how differences in the cuticular hydrocarbon profile affect behavior. I found that differences between the cuticular hydrocarbon profile of individuals led to increased aggression, and methyl-alkanes were the most important in statistically predicting aggressive behavior. These results offer an understanding about phenotypic matching in the context of social recognition. To gain a full understanding of the process of nestmate recognition, techniques, such as the ones used in my dissertation, which show how differences in the cue profiles of individuals affect specific behaviors, should be employed by more investigators.

Furthermore, I demonstrated that the relative proportions of n-alkanes, alkenes, and methyl-alkanes vary over a 16 week period. The relative proportions of n-alkanes in the profile
increased while the relative proportions of alkenes and methyl-alkanes in the profile decreased. Interestingly, the relative proportion of the C\textsubscript{29} methyl-alkanes did not change. Considering that the C\textsubscript{29} methyl-alkanes provide a colony-specific hydrocarbon signature in \textit{F. argentea} and that the relative proportion of these compounds do not vary over time could lead to interesting interpretations. These findings suggest that if the colony-specific recognition signal remains constant in a changing environment, guarding individuals may not be constantly updating their internal template in regards to nestmate recognition. This study demonstrates the importance of examining the different parts of the cuticular hydrocarbon profile. Making broad statements about the collective profile may lead to inaccurate conclusions.

Much progress has been made in the understanding of nestmate recognition, however, further investigation is required to gain a full understating of social recognition. The use of synthetic hydrocarbons can lead to precise identifications of the major recognition cues used by social insects. The formation of a library of synthetic compounds could provide the key to fully understanding the mechanisms involved in nestmate recognition and is vital to the progression of this field of study. Investigators can then ascertain which compounds act as nestmate recognition cues and examine how these cues are interpreted and how vary in an ever changing environment. Including all of these factors in future studies of nestmate recognition will greatly further our understanding of social recognition.
References


Su NY, Haverty MI (1991) Agnostic behavior among colonies of the Formosan subterranean termite, Coptotermes formosanus Shiraki (Isoptera: Rhinotermitidae), from Florida and Hawaii:


Tanner CJ (2008b) Aggressive group behavior in the ant Formica xerophila is coordinated by direct nestmate contact. Animal Behaviour 76: 1335-1341.


