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Chemical and ecological analyses of host specificity in the facultative kidnapper ant, *Formica aserva* (Hymenoptera, Formicidae)

By

Kelsey Jean Scheckel

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Committee in charge:

Professor Neil D. Tsutsui, Chair
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Professor Damian O. Elias

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ABSTRACT

Chemical and ecological analyses of host specificity in the facultative kidnapper ant, *Formica aserva* (Hymenoptera, Formicidae)

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Kidnapper ants are specialized social parasites that exploit entire societies for resources such as brood care, foraging, and defense. These parasites raid nests of other ant species, steal the developing young, and rear them as a workforce in the kidnapper ant colony. Kidnapper ants in the genus *Formica* are facultative social parasites that depend exclusively on other *Formica* host species for initiating new colonies, but become less reliant on those same hosts over time. Facultative species have retained the ability to forage, nurse brood, and construct nests, unlike the obligate parasites that must continually replenish their population of captive hosts throughout the colony life cycle. This intermediate level of dependence on hosts suggests that facultative kidnapper ants represent a crucial step in the evolution of obligate kidnapping behavior in ants. In this dissertation, I explore the evolution of host specificity in the North American facultative kidnapper ant, *Formica aserva*, and how the chemical and ecological mechanisms involved in acquiring and maintaining a host population can help inform the evolutionary origins of this bizarre parasitic lifestyle.

In my first chapter, I investigate the ecological groundwork of host choice in facultative kidnapper ants, as well as their effect on host community composition within the areas immediately surrounding *F. aserva* colonies. To do so, I measured the abundance of *Formica* species (hosts and non-hosts) collected at our field site in northern California, in areas were *F. aserva* colonies were present and in areas where they were not. I found that there was no effect on *Formica* species diversity based on the presence or absence of a parasite colony. Within ecological plots containing parasite colonies, there was a general preference for two of the four potential host species available to *F. aserva*, and parasite colonies using *F. accreta* as hosts were located in areas where *F. accreta* was the most abundant host species in the community. These findings suggest that facultative kidnapper ants do not have an effect on the overall community composition of other *Formica* species. However, within areas that contain parasite colonies, some host species are more abundant than others and *F. aserva* may be selecting hosts based on their ecological availability.

In my second chapter, I examine the chemical ecology of mixed-species colonies containing *F. aserva* workers and their captive hosts and compare them to free-living colonies of host and non-host *Formica* species to assess the chemical similarity between parasite workers and their preferred hosts. Overall, *F. aserva* cuticular hydrocarbon profiles were distinct from all

the other *Formica* species (free-living hosts and non-hosts) at our field site, and host species were just as chemically different as non-host species from *F. aserva* workers. Chemical comparisons of captive and free-living host species revealed stark differences in the overall cuticular hydrocarbon composition between these groups, and captive species were more chemically similar to their heterospecific nestmates than they were to their free-living conspecifics. Based on these results, captive hosts appear to be assimilated into a parasite-centric recognition odor profile. I conclude that *F. aserva* has not evolved to mimic host recognition odors, like obligate kidnapper ants do, but instead, retains its specific recognition cues which are then shared with cohabitating host workers.

In the third chapter of my dissertation, I study the chemical deception techniques used by facultative kidnapper ants for maintaining mixed-species colonies. We separated *F. aserva* workers from their *Formica* hosts by creating experimental nests containing pure species groups, then examined the cuticular hydrocarbon profiles of parasite and host workers before and after separation. I report a significant difference in the hydrocarbon profiles of captive hosts before and after removal from physical contact with their parasitic nestmates. In contrast, the chemical profiles of *F. aserva* workers underwent only small changes after separation from host species. These chemical changes also corresponded with behavioral changes, such as greater displays of aggression between heterospecific nestmates when reintroduced after a long period of physical separation. The minor changes we observed in the chemical profiles of parasites after removal from hosts relative to the greater changes we observed within the host species suggest that facultative kidnapper ants are using a parasite-centric strategy for manipulating captive host workers. It appears that the parasites homogenize the mixed-colony odor with their dominant odors, effectively dampening the species- and colony-specific cues of their hosts through the physical transfer of hydrocarbons.

The results of my dissertation provide an understanding of the mechanisms involved in the host choice and chemical deception strategies of facultative kidnapper ants. Studies like this, that investigate the chemical ecology and life history of facultative parasites, will facilitate a better understanding of the evolution of social parasitism overall, as well as provide general insights into the coevolution of hosts and parasites.

DEDICATION

To my wild child, Rome, who has been the source of so much joy and motivation toward the end of this process. You have taught me what real endurance feels like and I am so lucky to be able to see the world all over again through your eyes. May you always revel in life's tiniest creatures and greet each day with questions, questions, questions.

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This research would not have been possible had it not been for the hard work and support of the amazing graduate students and post-docs in the Tsutsui Lab; Elizabeth Cash, Maria Tonione, Brian Whyte, Nina Pak, Rebecca Sandidge, Jan Buellesbach, Joshua Gibson, and Kaustubh Gokhale. A very special thanks to Elizabeth Cash, Ashley Adams, Brian Whyte, and Jan Buellesbach for providing assistance for the chemical analyses of this research. Further, I would like to acknowledge the tireless efforts and guidance of Elizabeth Cash and Rebecca Sandidge during the arduous field seasons collecting ecological and chemical data for my project. Additionally, I would like to thank Maria Tonione and Nina Pak for their guidance on the molecular portion of this research. The undergraduate researchers that I had the pleasure of coaching in the lab and in the field were outstanding in their efforts of these pursuits. Of these students, I owe a great deal of appreciation to Kien Nguyen who spent countless hours prepping museum collections and who helped optimize the molecular techniques of this project. There was never a shortage of laughs with this crew and I will always remember our wild trips into the field and long conversations at the end of the work day.

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Chapter 1

Ecological mechanisms of host choice in the facultative kidnapper ant, *Formica aserva*

1.0 Introduction

Symbiotic relationships in nature are fundamental to the success of all living organisms. Of these, parasitism is considered to be the most widespread life history on the planet (Thompson, 2009), defined by species interactions involving the exploitation of one organism's vital resources for the benefit of the other. These antagonistic interactions between species can result in long-term reciprocal evolution of parasitic adaptation and host defense, often referred to as an 'arms race' (Dawkins & Krebs, 1979). Thus, resource specialization by antagonistic actors is necessary in order to "keep pace in the evolutionary race" as was described by Van Valen in the Red Queen hypothesis (Van Valen, 1977). Within this context it may be beneficial for an organism to evolve specialized adaptations for a narrow range of host species as this may decrease the amount of competition during resource acquisition and can improve a parasite's ability to overcome counteradaptations by their hosts (Futuyma & Moreno, 1988). Under different circumstances, a generalist lifestyle may be favorable, as it provides access to a broader range of resources (Jaenike, 1990). Although there is empirical evidence suggesting that species can oscillate between a specialist and generalist lifestyle, it is also possible that some generalist examples of parasite-host associations are only a temporary condition in the evolutionary progression towards becoming more specialized (Habermannová et al., 2013).

Of the parasitic lifestyles, most of the described examples are endo- and ectoparasites that pose pathological threats to their hosts (ex: protozoans, arthropods, etc.) (Bush et al., 2001) but parasitic behavior can also be expressed within social environments in which parasites exploit the social resources of their hosts (i.e. parental care, food, shelter, defense) (Buschinger, 1986). Social parasitism is confined to a small number of taxonomic groups. In vertebrates, social parasitism is most evident in avian brood parasites (Soler, 2018), including the well-studied cuckoos, which constitute some of the most impressive vertebrate examples of coevolutionary arms races involving parasite adaptations and host defenses (Rothstein, 1990). Within Hymenoptera (ants, bees, wasps, and sawflies), social parasitism is widespread and highly diverse, having evolved independently on many occasions (Wilson, 1971; Buschinger, 1986; Hölldobler & Wilson , 1990; Huang & Dornhaus, 2008). In the ants (family Formicidae), social parasitism has evolved at least 60 times across six ant subfamilies (Rabeling, 2020) and there are representative species of every known category of social parasitism. The three major categories of social parasitism include temporary, dulotic (aka "slavery" or "kidnapping"), and permanent (inquilinism) parasitism, which represent different degrees of host dependence and specialization (Hölldobler & Wilson, 1990; Buschinger, 2009). Temporary social parasites are generally less specific in their adaptations to host species and are defined by their colony founding behavior, which involves the invasion of incipient host colonies by newly mated parasite queens who either kill or expel the resident queen. The existing host workers then rear the parasitic queen's offspring until the resident workers die off and are wholly replaced by the parasite workers (Wheeler, 1904). In the most extreme examples of social parasitism, permanent social parasites (i.e. inquilines) have closely evolved with their host species, sometimes in sympatry, and are tolerant of resident host queens who produce the workforce of the colony, while the inquilines typically only produce sexual offspring (Hölldobler & Wilson, 1990; Bourke & Franks, 1991; Rabeling et al. 2014).

Dulotic social parasites (i.e. slave-making ants, kidnapper ants) have only been described within the ants. This form of social parasitism begins with the dependent colony founding by

parasite queens via temporary parasitism (as described above), followed by the production of a parasite worker population that performs well-organized raids on neighboring host colonies to replenish host workers (D'Ettorre & Heinze, 2001). These host workers will perform colony tasks to varying degrees, depending on the level of dependence evolved by the dulotic species (Savolainen & Deslippe, 1996). Across the ant subfamilies, dulotic species are relatively rare, with all representatives confined to the Formicinae and Myrmicinae subfamilies. Within the Formicinae, dulotic species exhibit various degrees of host specialization. Parasitic species in the genus *Polyergus* are obligate dulotic parasites that are wholly dependent on a population of hosts for all aspects of colony life (i.e. brood care, foraging, defense) and have lost the ability to perform nearly all of these tasks themselves (Mori & Le Moli, 1988). In contrast, dulotic species in the genus *Formica* are facultative, and have maintained all of the morphological and behavioral characteristics for independent living themselves (Mori et al., 2001). Facultative parasites are dependent on their hosts during the incipient (colony founding) stage of their life cycle, and that dependence decreases through time as the abundance and efficiency of their worker population increases, eventually overtaking the contribution made by captive hosts in the colony. The evolution of host choice within the different types of social parasitism can be a predictor of the degree to which a parasite is reliant on its host species, especially for facultative parasites, which likely represent an intermediate stage in the evolution of more specialized examples of social parasitism (Hölldobler & Wilson, 1990).

Understanding the evolution of these different types of social parasitism requires insight into the evolutionary and ecological mechanisms involved in host choice, which may also affect how well-adapted and, therefore, dependent a parasite is on it host. According to Emery's Rule, social parasites commonly parasitize closely related species, and thus, the evolution of host choice can be tightly linked to the phylogenetic relatedness of a parasite to its host species (Emery, 1909). In parallel with this genetic and historical component of host selection, parasites may choose their hosts based on their behavior, morphology, and/or chemical ecology. In fact, closely related species tend to have similar lifestyles, traits, and niches making the close relatives of parasitic species easier targets for exploitation (Nash & Boomsma, 2008).

Conversely, social parasites may choose their hosts based on factors like host availability or abundance. This may be especially true for kidnapper ants which, of the categories of social parasitism, were found to parasitize more distantly related species than temporary and inquiline forms (Huang & Dornhaus, 2008). Thus, dulotic parasites may be better able to use ecological features like host species composition (e.g. abundance, nest size, nest density) when selecting a host, since they may not be as tightly confined to a narrow range of highly related species. The ecological fitting hypothesis states that mutual adaptations of species may be a result of coincidental compatibility with regard to key characteristics (Janzen, 1985). In this light, new parasite and host associations can emerge based on opportunity and access to potential hosts, the compatibility of those potential hosts with the parasite, and the ability to live together via adaptations that reduce conflict between the interacting species (Araujo et al., 2015; Parrish et al., 2008; Combes, 2001).

Ecological availability of host species is crucial for the success of social parasite colonies, especially kidnapper species which require continuous access to host colonies during the raiding season as they are replenishing their host population. In addition, kidnapper ants require close proximity to colonies of their host species for colony founding (similar to that of temporary social parasites) (Mori & Le Moli, 1988). The colony founding behavior of both obligate and facultative kidnapper species follows the same general approach. Newly

inseminated queens return to their natal nests and accompany parasite workers on raids of neighboring host colonies. The young queens enter and usurp the invaded host nest by either killing the resident queen and acquiring the adult host workers and brood (Mori et al., 1994) or expelling the resident queen and the adult workers and acquiring only the existing brood (Schumann & Buschinger, 1994). This latter approach is most representative of facultative kidnapper ants, which, despite their flexibility in the specifics related to colony usurpation, still show an obligate dependence on host species for starting new colonies (Herbers & Foitzik, 2002). The presence and density of host colonies, therefore, can greatly impact the success of kidnapper ants both with regards to maintaining a stable host population and for ensuring reproductive success.

In this study we investigate the composition of *Formica* species communities as related to host preference in facultative kidnapper ants. Since facultative social parasites potentially represent an incipient stage of parasitic evolution, the ecological mechanisms involved in host choice for these species may be useful in providing a framework for the evolution of the kidnapper syndrome as a whole. The *Formica sanguinea* group consists of 12 kidnapper ant species, which are characterized as facultative parasites based on their lack of external morphological specializations and their ability to live without captive hosts (Holldobler & Wilson, 1990). *Formica aserva* is a widespread Nearctic species within the *sanguinea* group, with colonies recorded across the US and Canada, extending as far north as Alaska (AntWeb, 2022). Within the Sierra Nevada range of Northern California, *F. aserva* uses host species in the *Serviformica* group (*F. argentea, F. subaenescens, F. accreta,* and *F. microphthalma*) and each parasite colony is believed to use only one host species.

Here, we test two hypotheses regarding the host specificity of this facultative social parasite, 1) *F. aserva* colonies parasitize the most abundant *Formica* species in its local territory and 2) the presence of *F. aserva* colonies is associated with locally reduced *Formica* species diversity.

2.0 Methods

2.1 Field Site

We mapped 13 *Formica aserva* colonies in June 2017 for our current study assessing the *Formica* community diversity within the montane habitats of the Tahoe National Forest near Truckee, California (elevation: 6,380 ft). Sagehen Creek Field Station is part of the University of California Natural Reserve System along with the Pacific Southwest Research Station. Much of our research occurred within the 9,000 acres of the Sagehen Creek Experimental Forest (Sagehen UCNRS, 2022) where the facultative kidnapper ant, *F. aserva,* lives in mixed-species nests with captive hosts belonging to the *Serviformica* clade.

Locating and identifying colonies

Formica aserva colonies were identified in the field based on their microhabitat, worker morphology and ethology, and presence of heterospecific nestmates. *Formica aserva* colonies reside in fallen logs with entrances and brood chambers existing underneath the bark and within porous cavities. Colonies containing *F. aserva* workers always consisted of at least one other cohabitating species. The *Formica* hosts of *F. aserva* in high elevation populations are phenotypically distinct from parasite workers making it easy to ascertain the presence of more than one species occupying a nest.

F. aserva workers were first identified based on their bi-coloration and large, red heads and later confirmed using the morphological characteristics defined in the *Field Guide to the Ants of New England* (Ellison et al., 2012). We prepped the *Formica* samples for museum collections as systematic references in order to accurately identify species groups based on the morphological characteristics within the "Key to Nearctic *Formica fusca* group workers" which is based on the taxonomic revisions made by Francoeur (1997) (AntWiki, 2021). All *Formica* species identifications were later confirmed by mitochondrial sequencing following the methods presented below (sections 2.3 & 2.4).

2.2 Pitfall and Visual Sampling

To assess the *Formica* species community composition at our field site as it relates to the presence or absence of parasite colonies, we first had to delineate parasite colony boundaries as well as locate sites that were free of parasite colonies and foragers that would later act as control plots for our pitfall arrays. After locating a mixed-species nest containing *F. aserva* and *Serviformica* hosts, we visually searched for other *F. aserva* workers and possible parasite nests within a 20 meter radius from the focal colony. The focal parasite colonies were deemed independent from neighboring parasite colonies based on two criteria: 1) physical distance and 2) aggressive response to neighboring conspecifics. All of the parasite colonies at our field site were separated by a large distance and the behavioral assays we performed on *F. aserva* workers from distant colonies revealed high levels of aggression (aggressive behavior defined in Tsutsui et al. (2000)) when workers were paired together in collection tubes. Similarly, we deemed sites to be "parasite-free" if they were at least 60 meters from a focal parasite colony and did not contain *F. aserva* nests or foragers.

We set up ecological plots measuring 40m x 40m around each of the 13 parasite colonies and the centroids of the 13 "parasite-free" sites in order to establish the perimeters for our pitfall arrays. The pitfall array consisted of eight traps per plot, with two traps placed in each of the four cardinal directions from the focal colony (or control center point): one at ten meters from the focal point and another at 20 meters in each direction. Each pitfall trap consisted of a 50mL polypropylene centrifuge tube with a conical base (Fisher Scientific, Hampton, NH, USA) filled with 25 mL of solution (stock solution: 2 parts distilled water, one part 70% ethanol, and 20 drops of soap detergent) and marked with a collection code. Traps were set in the ground so that the lip of the tube was flush with the soil and marker flags were placed next to each trap. The pitfall traps were collected 48 hours after placement and the contents of each trap were sorted within 12 hours of collection and samples were placed in 95% ethanol with their corresponding trap collection code.

After the pitfall traps had been collected, we conducted visual searches for *Formica* foragers at each plot following a "switchback" pattern where two researchers would start at either end of the plot and walk back and forth between the plot boundaries until they met in the middle. Each plot half was searched for 30 minutes, equating to a total of one hour of visual search time per plot. Foragers were collected with forceps or aspirators and placed in 2mL screw top vials (Sigma-Aldrich, St. Louis, MO) filled with 95% ethanol.

2.3 DNA extraction, PCR, and Sequence Alignment

We relied, primarily, on the molecular identification of *Formica* species collected in our pitfall traps and visual searches due to the inconspicuous nature of the morphological characters that define the *F. fusca* group. We extracted total genomic DNA from the hindlegs of each

sample using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following a modified version of the manufacture's protocol for ant DNA (By: M. Branstetter; edited by: M. Tonione). We modified the initial step in this protocol for breaking up tissue as follows: we placed one hindleg of each sample into a 2 ml PowerBead tube (Qiagen, Valencia, CA) followed by the lysing reagents and a 4mm stainless steel grinding ball then placed the tubes in a PowerLyzer24 Bead-Based Homogenizer (Mo-Bio Inc., San Diego, CA) and set the program to beat for 30 seconds at 500rpm for three cycles with 15 second dwells between each cycle. We followed this modified step with the remaining steps of the protocol (referenced above). Samples were stored in molecular grade, 100% ethanol (Fisher Scientific, Waltham, MA) prior to sequencing.

We used a forward primer, LCO1490 5' GGTCAACAAACATAAAGATATTGG 3', and reverse primer, HCO2198 5' TAAACTTCAGGGTGACCAAAAAATCA 3', to amplify a ~650bp fragment of the *cytochrome oxidase I* (CO1) mitochondrial gene by performing Polymerase Chain Reactions (PCR) in a total volume of 30µl containing 11.75µl of Master Mix (5X Reaction buffer, 1.2mM of $MgCl_2$, 300µM of dNTP, 0.5 µM of each primer, and 0.04 units of GoTaq Flexi DNA Polymerase (Promega, San Luis Obispo, CA)), 2µl of DNA, and 16.25µl of Ultrapure H_2O. The PCR conditions for the CO1 gene are as follows: 95°C for 4 min, 38 cycles of 95°C for 30 s, 45°C for 45 s, and 72°C for 45 s; and a final extension at 72°C for 5 min. We verified gene amplification on 1% agarose gels and purified the PCR products with ExoSAP-IT (Applied Biosystems, Waltham, MA) in a 6µl reaction volume containing 1µl of reagent and 5µl of post-PCR product following the manufacturer's temperature protocol. Purified samples were sent to the UC Berkeley Sequencing Facility and Sanger sequencing was performed from both directions. We edited and aligned our sequences using Geneious Pro v.9.0. (Biomatters Ltd, Aukland, New Zealand). We assembled our sequences and those from Torres et al. (2018) into contigs by mapping all sequences to one of our *Formica* sequences as a reference, producing a ~680bp alignment.

2.4 Phylogenetic Analyses

We used IQ-TREE to build a maximum likelihood tree inferred from our 680bp alignment using *Formica* sequences collected in our study and by Torres et al. (2018). We used a *Camponotus atrox* sequence derived from GenBank (CO1: KT159775) and a *Polyergus breviceps* sequence from Torres et al. (2018) as outgroups to root our phylogeny. We determined the best nucleotide substitution model by calculating Akaike information criterion (AIC) and Bayesian information criterion (BIC) in IQ-TREE (Nguyen et al., 2015; Hoang et al., 2018) and ran our analysis using the Jukes-Cantor (JC) model with default parameters including an Ultrafast bootstrap analysis with 1000 alignments and an approximate likelihood ratio test (aLRT) with 1000 replicates (Anisimova & Gascuel, 2006). The resulting maximum likelihood tree was used to identify the *Formica* species we collected during our ecological surveys based on their positional relationship within the clades produced by the CO1 sequences of the *Formica* species from Torres et al. (2018).

2.5 Data Analysis

To assess different components of biodiversity within and between the plot types (parasite versus control) we first prepared a rank abundance curve (Whittaker Plot) in R version 1.4.1106 as a way to visualize the evenness of the species we recorded at our sites. We further assessed the species richness and evenness of the parasite and control plots by calculating a

Shannon-Weiner Diversity Index using the formula $\sum[(p_i) \times ln(p_i)]$, where p_i is the proportion of each species, and converted these index values to expected number of species for the two plots using the exponential function (EXP) in excel. We used these expected number of species values to compare the percent difference between the two plot types. We also measured species diversity by calculating the Simpson's Diversity Index, using R packages *vegan* and *Hotelling*, for the total species count data from each plot type. The boxplots including the relative abundance for each species based on plot type were created in R using *dplyr* and *ggplot2*. In order to test whether or not species abundance was significantly different between plot types and within the different parasite plots, we ran multiple Shapiro-Wilk tests for normality on different pairwise comparisons of our data followed by Welch's two-sample *t*-tests for unequal variance for the groups of data that fit these assumptions.

3.0 Results

3.1 DNA barcoding and phylogenetics

The *cytochrome oxidase* (CO1) sequence data from our samples showed distinct clustering with the *Formica* species groups reflected in Torres et al. (2018) based on our maximum likelihood consensus tree (Figure 1). Of the monophyletic clades that were produced, most of them showed high confidence values, consisting of both our sequence data and the reference sequences. Our CO1 sequences from *F. argentea* hosts did not form a monophyletic group with the reference sequences of that same species. We used GenBank BLAST searches of our sequences along with morphological assessments of representative samples from this species to confirm the identification of our samples since we could not confidently do so with the reference sequences for that species. We included key characteristics like dense pubescence on the first four gastric tergites and short, erect hairs on the pronotum, gastric dorsum, and first tergite (Francoeur,1973; AntWiki, 2021), in making our morphological identifications. Mitochondrial polymorphisms in *F. argentea* may be the result of the discrepancies we encountered when using *F. argentea* reference sequences with our own. Despite this, we observed strong support for the monophyletic group consisting of our *F. argentea* samples.

It is important to note that the topology of our CO1 tree does not represent the phylogenetic reconstructions of the *Formica* genus made by Borowiec et al. (2021). In order to produce a more accurate topology showing the true relationships between the different clades, more sampling of the mitochondrial genome would be necessary. For the purpose of this study, however, the maximum likelihood consensus tree that we produced was sufficient for identifying the *Formica* species that we collected from our ecological surveys.

3.2 Formica *species abundance in parasite and control plots*

The rank abundance curve (Whittaker Plot) comprising the diversity of *Formica* species collected from parasite and control plots at our field site conveys an initial, steep sloping curve from the highest-ranking species to the second ranked species but the line that follows is relatively flat among the following species. This curve can be interpreted in two ways: 1) the presence of the highest ranking species is generally skewing the evenness of the species present at the different plot types and, 2) the flat line representative of the following species suggests that these remaining species have an even abundance within and between plot types (Figure 2; these proportions can also be visualized in Figure 3). For both plot types, the highest-ranking species, *F. sibylla*, was far more abundant than any of the host species based on the samples we collected

from pitfall and visual surveys. Additionally, parasite and control plots did not differ greatly in the rank abundance order of the *Formica* species collected from the combined sites (Tables 1 & 2) with the highly abundant non-host species, *F. sibylla*, ranking as the most abundant, followed by *F. accreta* and *F. microphthalma.* Although the rankings of the two least common species (*F. subaenescens* and *F. argentea*) were reversed for the control versus parasite plots, the magnitude of the difference in numbers of workers was small, and likely represents sampling variation.

The mean number of *Serviformica* individuals collected from all parasite plots (mean ± sd = 13.50 ± 10.32) and all control plots (mean ± sd= 11.55 ± 8.39) were not found to be significantly different ($t(21) = -0.51$, $p = 0.32$). Similarly, the pairwise comparisons of the relative abundance of each species based on plot type (parasite vs control) showed no significant difference in the mean number of individuals for any of the species we recorded (Figure 4). Some of the species were highly variable with regards to the number of individuals recorded at individual plots. For example, *F. sibylla* exhibited the highest variation in abundance observed among the species with some plots yielding as many as 30 individuals while other plots recorded as few as one individual.

3.3 Host preference and host abundance within parasite plots

Of the 13 parasite colonies we located, we were only able to confidently identify the captive host species for 11 colonies using mitochondrial and morphological data. Two *F. aserva* colonies containing unknown hosts from the *Serviformica* group were left out of the following assessments of host species abundance as it relates to parasite host preference. Of the *F. aserva* captive hosts that we were able to identify, *F. accreta* and *F. microphthalma* hosts were the most common captives: six *F. aserva* colonies had *F. accreta* as hosts and three were using *F. microphthalma* as hosts. *Formica argentea* and *F. subaenescens* were each found in only one *F. aserva* colony, which prohibited us from performing significance testing on host preference as it relates to host abundance at these plots. Our pairwise comparisons of host species abundance and host preference (i.e. captive host species living with *F. aserva*) revealed contrasting results for plots containing *F. aserva* colonies that were using *F. accreta* as hosts and those that were using *F. microphthalma* as hosts. Plots with parasite colonies using *F. accreta* as hosts had a significantly higher abundance of *F. accreta* workers collected in pitfall traps and by visual surveys (mean ± sd= 3.60 ± 2.41) than parasite plots where *F. aserva* was using a different host species (mean ± sd= 0.60 ± 0.55) ($t(4) = 2.72$, $p = 0.03$) (Figure 5a). Conversely, though not significant, parasite plots consisting of *F. microphthalma* captives had a very low abundance of this host species overall (mean ± sd= 0.34 ± 0.58) when compared with plots that contained *F. aserva* colonies with other host species as captives (mean ± sd= 1.86 ± 2.55) ($t(7) = -1.5$, $p = 0.09$) (Figure 5b).

3.4 Formica *species diversity in the presence and absence of* F. aserva

Our assessment of *Formica* diversity at plots that contained *F. aserva* colonies and those that did not revealed qualitative similarities with regards to species richness (S=5) and evenness (both plot types were highly skewed in the number of *F. sibylla* samples recorded but relatively even in the abundance of the other species). Similarly, the diversity indices calculated for these two plots did not reveal substantial differences in their values and inferences. The results of our Simpson's Diversity Index for parasite plots ($D = 0.645$) and control plots ($D = 0.567$) suggest that both plot types have intermediate species diversity (D values can range from 1 (high diversity) to 0 (low diversity); Table 4). Shannon-Weiner's Diversity Index revealed the same

similarity between values for parasite and control plots but the calculated diversity indices reported a lower inference of species diversity at each plot than the Simpson's diversity indices suggested. The diversity indices (*H'=ln(D)*) reported for parasite plots (*H'*=1.269) and control plots (*H'*=1.116) suggest that species diversity is similar in both of the plot types (Table 4; calculations provided in Table 5). We calculated species evenness using the relative abundance with which each species is represented in a plot (recorded as percentages with zero signifying no evenness and one signifying complete evenness). Parasite plots were about 79% even with regards to the relative abundance of each species recorded and control plots were about 70% even. Since species evenness and species diversity have an inverse relationship in which high evenness is representative of low species diversity and vice versa, then we can consider the above values (*H'*) representative of low diversity as they relate to the evenness of species reported by each plot type (%). In order to better compare the amount of diversity between the plot types, we converted these diversity indices to "true diversities", which are the effective number of species. These conversions showed a slightly higher effective number of species recorded at parasite plots (ENS = 3.56 species) than control plots (ENS = 3.06 species) suggesting that parasite plots are about 14% more diverse than control plots though both of the plot types reported low species diversity overall.

4.0 Discussion

4.1 Ecological composition of Formica *species*

We were able to identify seven monophyletic clades of *Formica* species from our pitfall trap and visual search collections using mitochondrial DNA sequence data. The seven clades consisted of the facultative kidnapper, *F. aserva*, the four host species (*F. accreta, F. subaenescens*, *F. microphthalma*, and *F. argentea*), one *F. rufa* group species (wood ants), and one *F. fusca* group species that is not a known host for kidnapper ants. Our ecological survey of the abundance of these *Formica* species with regards to the presence (parasite plots) or absence (control plots) of *F. aserva* colonies revealed that the non-host, *F. sibylla*, was the most overwhelmingly abundant *Formica* species in both the control and parasite plots. The host species at our field sites occurred on the different plot types at similar abundances relative to one another and at far lower numbers than the non-host species, *F. sibylla*. The frequent occurrence of *F. sibylla* workers in our pitfall traps and visual search collections paired with the relatively low numbers of host species collected skewed our species count data, which resulted in low species evenness among the different plot types.

There are *a priori* reasons to predict either higher or lower abundance of host species on a plot with an active *F. aserva* colony. On one hand, if *F. aserva* colonies are more likely to colonize and occupy sites where their host species is most abundant, we would expect to see higher numbers of the corresponding host species in our pitfall traps and surveys. In general, characteristics related to host demography, including nest density, are believed to be correlated with the ecological pressures imposed by social parasites (Foitzik et al., 2004) but this framework of host-parasite interactions is dynamic and likely oscillates as a result of coevolutionary adaptations between species (Wade, 2007).

Alternatively, if there is high parasite pressure on neighboring host colonies, the presence of a *F. aserva* colony may reduce the local abundance of their host, which would translate into lower numbers of the respective host in our pitfall traps and surveys. In fact, such an effect of parasite pressure on host density was revealed in studies of the kidnapper ant *Protomognathus*

americanus and their *Temnothorax longispinosus* hosts. In this system, the presence of kidnapper colonies reduced host density at particular sites where there was an abundance of ecologically favorable habitat for hosts to relocate as a result of this pressure (Foitzik et al., 2009). Although we did not directly measure the nest density of host species within our plots, it is possible that nest density, as a measure of biodiversity within and between plot types, might reveal similar findings to Foitzik et al. (2009) if our initial measures of host species abundance is an indicator for host nest density.

4.2 Host species abundance is correlated with host preference at some plots but not others

Within our field site, we found an uneven representation of the host species used by *F. aserva* colonies. *Formica accreta* was the most commonly used host species, followed by *F. microphthalma*. These two host species were also more common in our pitfall trap and visual search collections suggesting that they may be more abundant than the other two hosts, *F. subaenescens* and *F. argentea*. We did not, however, find any significant differences in species abundance between the *Formica* species at our site, which may suggest that more intensive sampling of host species may be necessary to produce a more accurate representation of *Formica* species abundance within and between plot types.

Although species did not generally differ from one another with regards to their relative abundance between plot types, we did detect differences in abundance within two of the host species relative to the type of captive species that parasite colonies were using within the parasite plots. In our comparisons of parasite plots containing *F. accreta* as captives and those that did not, we found that *F. accreta* was more abundant within the plots that contained a parasite colony with *F. accreta* captives. The opposite was true in cases where *F. microphthalma* was the host: *F. microphthalma* was generally less abundant on parasite plots where *F. aserva* was using *F. microphthalma* as a host compared to parasite plots with different captive host species, although this was not significant at the 0.05 level. The relatively lower sampling we obtained for *F. microphthalma* compared with *F. accreta* hosts may explain these differences.

Our finding that *F. aserva* colonies that choose *F. accreta* as hosts occur in areas where those same host species are more abundant may demonstrate that *F. aserva* colonies are basing their host choice on the availability of the potential hosts around them. Although host specificity of facultative parasites is not well understood (Wilson, 1971) the general models of host specificity that include the availability of host species within a parasite's range, and the tendency for social parasites to switch hosts accordingly based on this availability (Habermannová et al., 2013), suggests that the availability of *F. accreta* hosts at our field site may be driving host preference for this species for the majority of *F. aserva* colonies within the population we surveyed. The resource distribution hypothesis (Marques et al., 2000) suggests that widespread species are able to sustain a higher diversity of parasites than other species with smaller geographic ranges. Thus, the geographic range size of potential hosts is often correlated with parasite prevalence. For example, this has been demonstrated in the host-parasite dynamics of cuckoo bumblebee social parasites (subgenus *Psithyrus*), in which the species richness of parasites was positively correlated with the geographical range area of host bumblebees and, consequently, bumblebee hosts with a larger range tended to encounter more parasitism by cuckoo bumblebees (Suhonen et al., 2016; Antonovics & Edwards, 2011). Since the range size of host species are rarely stable and often, themselves, affected by parasite prevalence, social parasites may change their host preference in order to match the current ecological availability of host species (Thompson, 2005).

If host numbers decline, parasite species may start targeting a wider range of hosts or may switch their preference to a completely different host (Suhonen et al., 2015). Host switching may be more common in facultative social parasites like *F. aserva*, which are not wholly restricted to a particular host species and, therefore, may adopt a more opportunistic approach to choosing a host. Even populations of obligate parasites, like species of *Polyergus*, can target several host species in a local geographic range (Torres et al., 2018), although the ability for individual colonies to switch hosts in response to environmental changes, such as the ecological availability of those hosts, may be more limited than their facultative neighbors. Since the local abundance of species is correlated with the geographic range size of species (Soler et al., 1999), the *F. accreta* hosts at our site may be a more favorable host for *F. aserva* due to their relatively high local abundance within parasite plots and across the geographic range at our site (as suggested by the number of *F. aserva* colonies using the host across this range).

It is important to note that *F. aserva* colonies exist in sympatry with the colonies of the obligate kidnapper ant, *Polyergus mexicanus*, at our field site. Additionally, *F. aserva* and *P. mexicanus* colonies overlap in their host species preferences; both species target hosts in the *Serviformica* group. Host preference within *F. aserva* colonies may, therefore, be affected by the availability of host species as a result of the imposed pressure on host colonies by *P. mexicanus*, and vice versa, in areas where their raiding territories overlap. In other regions of sympatrically occurring kidnapper ants, the spatial distribution of raids on host colonies showed that the obligate kidnapper ant, *Polyergus breviceps*, and facultative kidnapper ants, *F. puberula* and *F. gynocrates*, avoid raiding areas that are already occupied by other kidnapper colonies (Bono et al., 2006). We cannot confirm that the same is true at our site and further studies on interspecific interactions within sympatric populations of obligate and facultative kidnapper ants would be useful in determining the ecological mechanisms contributing to host choice and the selective pressures that may be driving evolutionary changes in response to competition between these species.

4.3 Formica *species diversity is similar between parasite and control plots*

The biodiversity assessments we conducted within local territories occupied by kidnapper ant colonies and those that were "parasite-free" suggest that the presence of facultative parasitic colonies does not have a noticeable effect on the community composition of *Formica* species. The two plot types we surveyed had negligible differences in their biodiversity indices, which represent measures of species abundance and evenness, so we reject our second hypothesis that *F. aserva* colonies reduce the *Formica* species diversity within their local territory and fail to reject our null hypothesis that *Formica* species diversity is the same between parasite and control plots.

Although it is well known that parasite prevalence can affect community composition and productivity (Foitzik et al., 2001; Foitzik & Herbers, 2001), the degree to which a parasitic species depends on its hosts for survival may affect the level of change a community experiences as a result of parasite pressure. For obligate kidnapper ants like, *Harpagoxenus sublaevis* and their hosts, *Leptothorax sp.*, the presence of parasite colonies has been correlated with some evidence of decreased host production (i.e. worker abundance, number of queens, number of colonies) (Scharf et al., 2011). Similarly, repeated kidnapping raids by *Protomognathus americanus* on their *Temnothorax sp.* hosts, in areas where parasite pressure is high, have shown a destructive effect on host nests resulting in the local decrease of host nest density (Foitzik et al., 2009). In these examples, obligate kidnapper ants are shown to greatly affect the local

composition of their host species when the parasite populations are artificially increased, but the pressure that facultative parasites impose on their neighboring hosts may be weaker.

Facultative *F. aserva* social parasites are unique in that they display the same range of preference for host species as the sympatric, obligate *Polyergus*, but the degree to which they depend on those same host species changes over time and, thus, the pressure they exert on the surrounding ant community may vary throughout the parasitic colony's lifecycle. As a result of this, host species diversity may not be as affected by the presence of *F. aserva* colonies as by obligate kidnapper ants which require higher proportions of captive host workers for survival. Our personal observations suggest that all of the *F. aserva* colonies from our study site had high parasite to host worker ratios within their nests which suggests that these colonies may be more established and, therefore, less reliant on a host population. This may explain the lack of evidence we obtained for the differences in species diversity between parasite plots and parasite-free plots. As it relates to ant community diversity, little is known about the effect of kidnapper ant colonies on the composition and diversity of the ant communities that surround them and more investigation on this topic, for both obligate and facultative species, would be beneficial in understanding the ecological pressures imposed by this range of social parasites.

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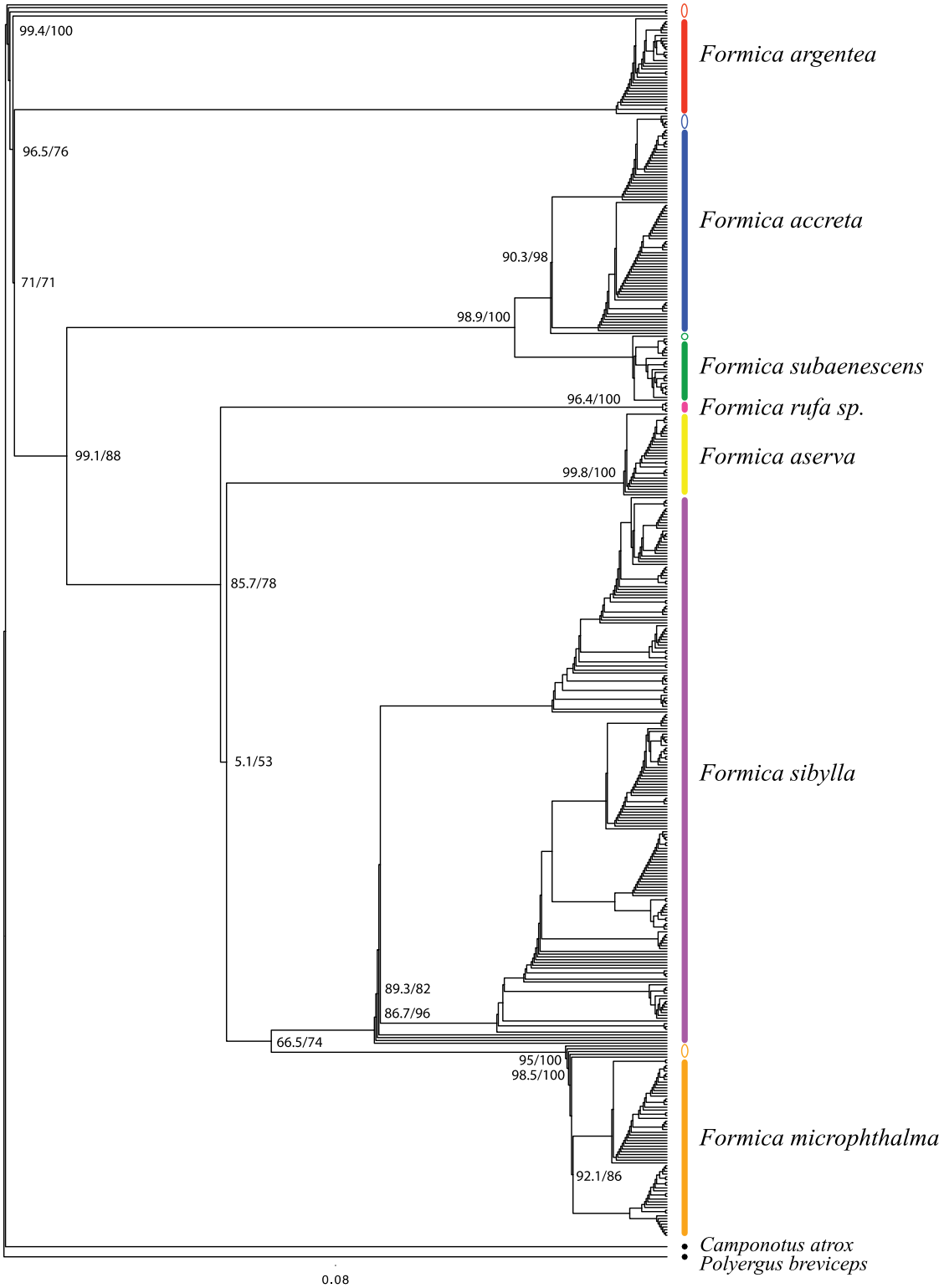

Figure 1. Maximum likelihood consensus tree based on a 680bp alignment of sequence fragments from the CO1 mitochondrial gene. Filled bars represent *Formica* samples from this study and unfilled bars represent the reference sequences we obtained from Torres et al. (2018).

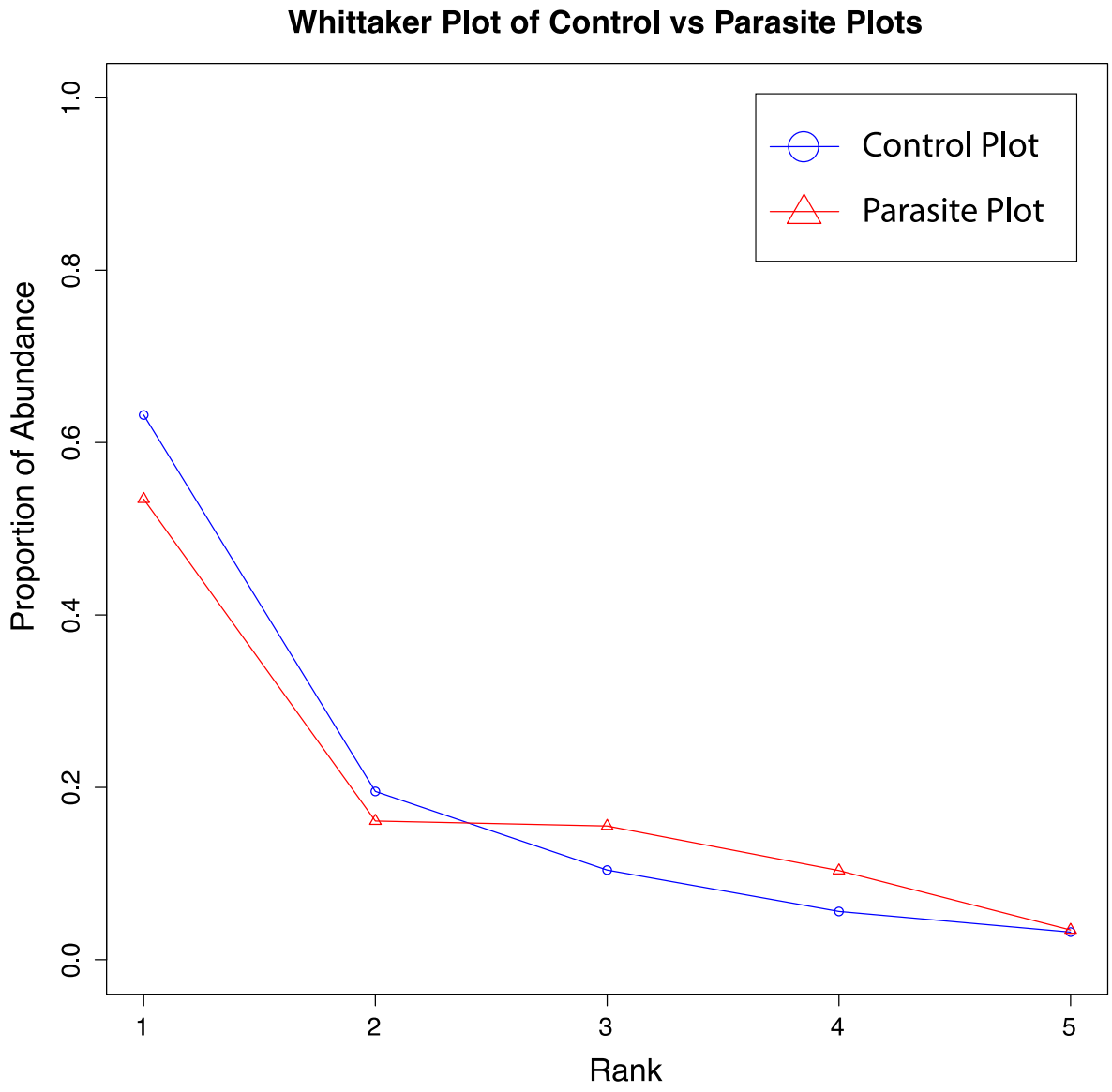

Figure 2. Rank abundance curve ("Whittaker Plot") for *Formica* species collected in pitfall traps and during visual searches from plots that contained a parasite colony and those that were "parasite-free".

Table 1. Species' abundance ranks and species richness (number of species) recorded at control plots where colonies of *F. aserva* were absent.

		Control Plots	
Rank	Species	Count	Proportion
1	*Formica sibylla*	79	0.618
2	*Formica accreta*	25	0.196
3	*Formica microphthalma*	13	0.102
4	*Formica subaenescens*	7	0.055
5	*Formica argentea*	4	0.032

Table 2. Species' abundance ranks and species richness (number of species) recorded at parasite plots where colonies of *F. aserva* were present.

		Parasite Plots	
Rank	Species	Count	Proportion
1	*Formica sibylla*	93	0.541
2	*Formica accreta*	28	0.163
3	*Formica microphthalma*	27	0.157
4	*Formica argentea*	18	0.105
5	*Formica subaenescens*	6	0.035

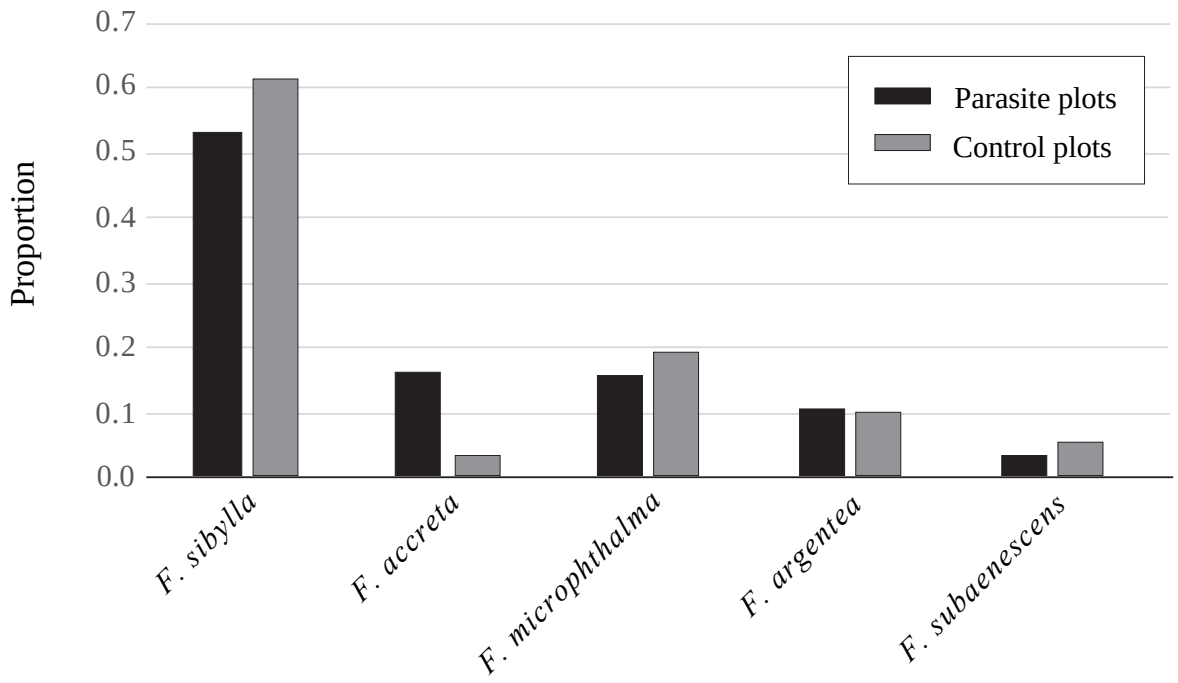

Figure 3. Proportions of individual species based on their relative abundance within parasite plots and control plots.

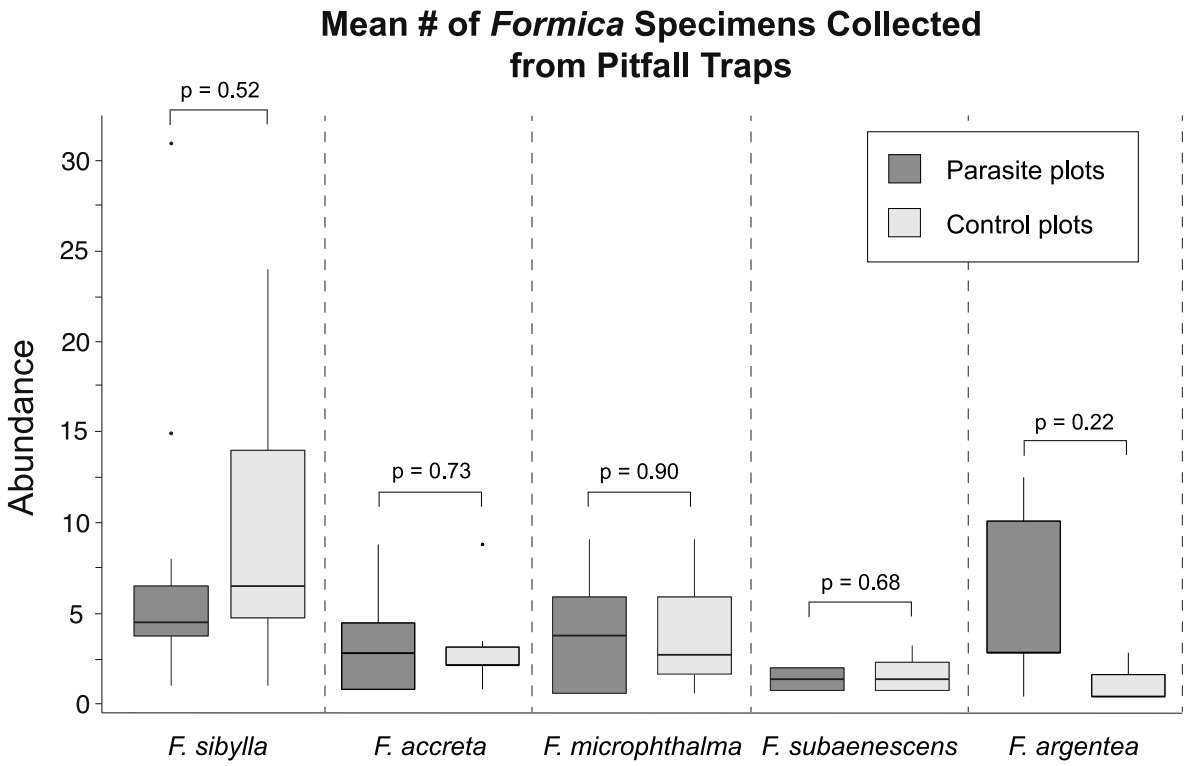

Figure 4. Multiple boxplots showing the mean number of individuals of each species collected at parasite plots and control plots. There was no significant difference between the mean number of individuals of a given species collected from parasite plots or control plots based on our t-tests.

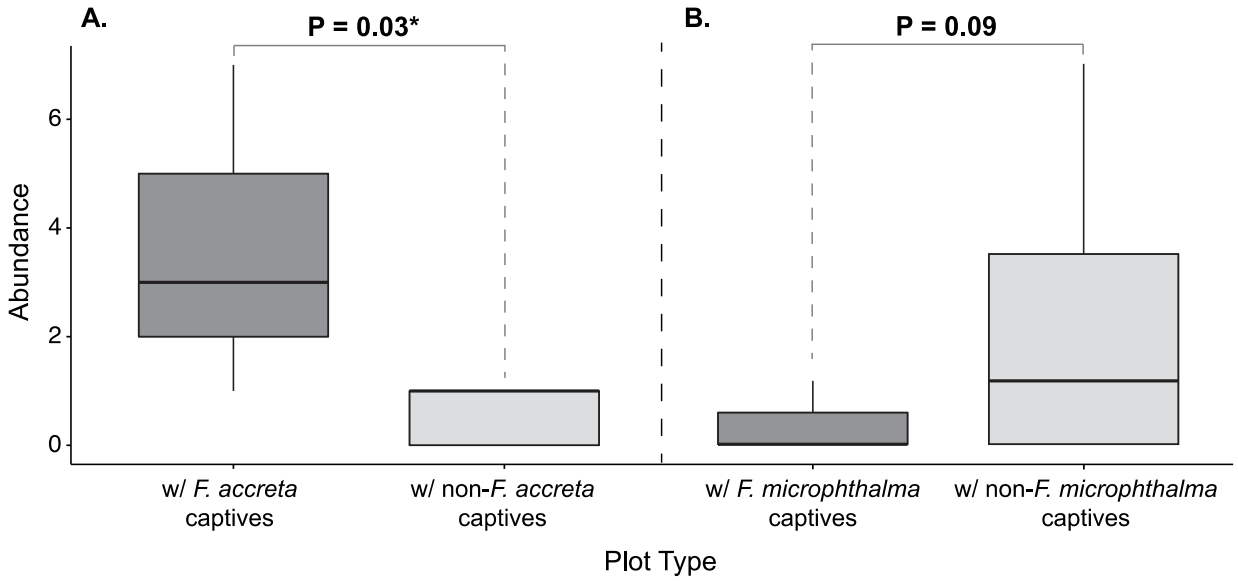

Figure 5. Boxplots showing the mean abundance of *F. accreta* workers collected from parasite plots containing *F. accreta* captives (dark grey) and parasite plots with captives of another species (non-*F. accreta* captives; light grey) (A), and the mean abundance of *F. microphthalma* hosts collected from parasite plots containing *F. microphthalma* captives (dark grey) and those that did not (light grey) (B).

Table 4. Measures of biodiversity: Shannon-Weiner and Simpson's Diversity indices for parasite and control plots along with the evenness and richness of species for both plots and the expected number of species for each plot type based on the values produced by Shannon-Weiner Diversity Index.

Plot Type	Shannon-Weiner Index (*H'*)	Expected # of species (EXP(*H'*))	Simpson's Index (*D*)	Evenness (%) (*H'*/*ln*(*S*))	Species Richness (*S*)
Parasite	1.269	3.56	0.645	79.0	5
Control	1.116	3.06	0.567	70.0	5

Table 5. Calculation of species diversity using Shannon-Weiner Index for parasite (A) and control plots (B).

A. Parasite Plots

SPECIES	ABUNDANCE	PROPORTION (p_i)	ln(p_i)	p_i ln (p_i)
F. sibylla	93	0.535	-0.627	-0.335
F. accreta	28	0.161	-1.827	-0.294
F. microphthalma	27	0.156	-1.864	-0.29
F. argentea	18	0.104	-2.269	-0.235
F. subaenescens	6	0.035	-3.368	-0.117
Total	**172**	**1.00**		**-1.269**

B. Control Plots

SPECIES	ABUNDANCE	PROPORTION (p_i)	ln(p_i)	p_i ln (p_i)
F. sibylla	79	0.632	-0.459	-0.291
F. accreta	25	0.196	-1.634	-0.319
F. microphthalma	13	0.104	-2.264	-0.236
F. argentea	4	0.032	-3.443	-0.111
F. subaenescens	7	0.056	-2.883	-0.162
Total	**128**	**1.00**		**-1.116**

Chapter 2

Chemical ecology of host specificity and the differential sharing of recognition cues by the facultative kidnapper ant, *Formica aserva*, with its *Formica* hosts

1.0 Introduction

The formation of social groups represents a major transition in evolution when individual entities began cooperating with others to avoid the constraints of living life alone (Maynard Smith & Szathmáry, 1995). Eusocial insects are prime examples of group living and are defined by their cooperative behavior and kin-based altruism. In eusocial Hymenoptera, sterile workers perform nearly all societal tasks including brood care, nest maintenance, foraging, and defense (Hölldobler & Wilson, 1990). In doing so, workers contribute to the survival and reproduction of their relatives and therefore, gain indirect fitness through their reproductive kin. Eusocial insect colonies are bounded by recognition systems that, for social insects, are predominantly composed of chemical cues (Smith & Breed, 1995). Antennal detection of non-volatile surface compounds has long been accepted as the mechanism by which social insects experience their social world (Fielde, 1901). Through a process known as olfactory discrimination, individuals can detect the chemical cues of others via antennal probing and compare these cues with their own internal neural templates that define their species- and colony-specific identity (Ozaki & Hefetz, 2014). Individuals who possess chemical cues that do not match this template are typically rejected via acts of aggression and, thus, the actor succeeds in maintaining the integrity of their altruistic society (Lahav et al., 1999).

Cuticular hydrocarbons (CHCs) are waxy lipids that cover the exoskeleton of insects and initially evolved to prevent desiccation (Blomquist & Bagnères, 2010). In many arthropods, CHCs have also acquired critical functions in communication and, in social insects, serve as recognition pheromones ("cues" or "labels") (Leonhardt et al., 2016). The CHCs expressed by insects are species-specific mixtures with variations in the relative proportions of compounds that arise from genetic differences or small environmental changes that occur intra-specifically between different colonies and populations (Lorenzi, 2003; Martin et al., 2008). Although CHCs have a strong genetic basis, once produced, they are easily transferable. The post pharyngeal gland in the head of ants is a large reservoir for colony and species-specific hydrocarbons, and colonymates actively distribute the contents of these glands during social interactions (i.e. allogrooming and trophallaxis) (Hefetz et al., 1992; Soroker et al., 1994; Meskali et al., 1995). For this reason, colony profiles can also be flexible and dynamic, and can vary across an individual's lifetime (Tsutsui, 2004).

The gestalt model for nestmate recognition proposes that individuals share their recognition cues with other colonymates to form a uniform blend of odors that is then accepted as the colony's signature (Crozier & Dix, 1979). Thus, social interactions within colonies are central to defining the recognition systems of eusocial insects. However, although these recognition systems are typically very precise and accurate, many other species have evolved mechanisms to evade them and infiltrate social insect colonies. Despite the strict recognition systems that evolved to protect the wealth of resources within insect societies, social insects are still vulnerable to parasite exploitation (Schmid-Hempel, 1998; Hölldobler& Wilson, 1990). The ants (family Formicidae) include species that display a variety of types of social parasitism. These forms include nutrient-dependent guest ants, temporary parasites that utilize hosts during colony founding, kidnapper ants that raid brood from heterospecific colonies and, the most extreme example, workerless inquilines that only produce reproductive offspring and depend on host colonies for a workforce (Buschinger, 1986).

Kidnapper ants in the genus *Formica* are facultative social parasites that are dependent on a different host species for colony founding but rely loosely on that same host at later stages of

the colony's lifecycle (Wilson, 1971). These parasites, unlike obligate kidnappers, maintain the morphological and behavioral traits of non-parasitic species, allowing them to also live independently from their hosts (Buschinger, 1986). For example, workers of these facultative social parasites are equipped with serrated mandibles that are useful for foraging and processing food, and they contribute to nest construction and actively care for brood and reproductives in the nest (Mori & Le Moli, 1988). These morphologies and behaviors contrast with obligate kidnapper ants, like those in the genus *Polyergus,* whose absolute dependence on their host has resulted in strong selection for traits that are helpful during kidnapping raids and later acquisition of a captive host worker population. Such traits include enlarged Dufour's glands and piercing mandibles, as well the loss of other behaviors necessary for foraging and nest maintenance (Buschinger, 1986; Regnier & Wilson, 1971). Like obligate kidnappers, facultative kidnapper ants lack the ability to establish colonies on their own and so begin their colonies as temporary parasites (d'Ettorre & Heinze, 2001). Many previous studies have shown the first stages of colony founding in facultative kidnapper ants, through the infiltration and take-over of an existing host colony by a newly mated parasite queen. However, the process by which facultative social parasites establish their colonies after this step remains unclear. Thus, an examination of the mechanisms involved in host choice and post-invasion cohabitation is a necessary step in understanding the life history of these rare social parasites.

Social parasitism does not come without a cost; invading queens and workers face aggressive and often deadly host defenses (Foitzik et al., 2001). Choosing the right host is therefore a crucial step that social parasites must do in order to secure their chances of survival. Some potential host species will be better targets for parasitism depending on their life history, chemical ecology, and biological similarity to the parasite (Huang & Dornhaus, 2008). The greater ease of parasitizing more similar species is believed to explain the pattern of Emery's Rule (Le Masne, 1956): social parasites tend to parasitize close relatives (Emery, 1909; Wheeler, 1901). Many examples of host specific relationships in socially parasitic Hymenoptera support this observation in the strict sense, as parasites often select sister taxa as hosts. However, a loose interpretation of Emery's Rule, proposing that phenotypic similarity rather than phylogenetic relatedness drives host selection, can be broadly applied to those more congeneric host-parasite relationships and can even extend to include other characters for defining relatedness like the chemical ecology of species (Ward, 1996; Bourke & Franks, 1991; Rabeling et al., 2014). The similarity between parasitic and host recognition cues has offered useful insights into the origins of host choice and the coevolutionary arms race that often occurs as a result of these relationships (Brandt et al., 2005). Since recognition cues are a vital part of detecting invaders in a nest, there is no question that social parasites that seek to evade those cues would specialize on species that bear a similar chemical profile to their own, improving the chances of invasion and the further maintenance of a mixed-species colony.

In this study, we investigate the chemical relationship between the facultative kidnapper ant, *F. aserva,* and several of its host species in the Sierra Nevada mountain range. In this region, *F. aserva* parasitizes members of the *Serviformica* subgenus, including *F. argentea, F. accreta, F. microphthalma,* and *F. subaenescens*. In order for kidnapper ants like *F. aserva* to successfully invade host colonies, parasites must evolve behavioral and chemical strategies for evading host defense and coercing interspecies cooperation. Thus, we expect that *F. aserva* will share components of its chemical profile with those of its chosen host species. These similarities are not expected to be present between *F. aserva* and the other, non-host, *Formica* species that occur in sympatry with parasite colonies. Additionally, *Formica* cue diversity is expected to be

higher in the species that are the target of parasitism. The focus of this diversity should be within the methyl-branched alkanes present in the species based on the results of Martin et al. (2007) and Krasnec & Breed (2013) who found that nestmate recognition in *Formica* species is dependent upon the presence, abundance, and position of double bonds on alkenes and methyl-branches on alkanes, and that these are the compound classes that should experience the strongest selective pressure in the presence of parasitism.

Here, we test two hypotheses regarding the chemical ecology of the social parasite, *F. aserva*, and its various different host species: 1) *Formica aserva* is more chemically similar to its host *Formica* species than to other, sympatric, non-host *Formica* species and 2) recognition cue diversity is higher in *Formica* species that are the target of parasitism.

2.0 Methods

2.1 Field collection

The *Formica* species used in this study were selected based on their abundance within the geographic range we surveyed in our previous chapter as well as their life history traits. We included species from the *Serviformica* sub-genus, all of which are known host species of kidnapper ants, both obligate and facultative. In addition to these host species, we collected data from workers in the *Formica rufa* species group, also known as mound-building wood ants, and workers in the *Formica fusca* species group, who are not known hosts of *F. aserva*. These species were recorded in all of our previous study plots (Chapter 1; Figure 1) and are therefore good representatives of the *Formica* species diversity within this montane habitat.

Formica aserva and workers from the aforementioned groups were hand collected from existing, GPS-marked colonies at Sagehen Creek Field Station in the Tahoe National Forest near Truckee, CA. Parasite nests were located within a grid-like plot that was marked in the summer of 2017 and measured 40 square meters around the central, parasite colony. Nine of the 13 marked parasite colonies were recovered from the summer 2017 field season and the remaining four parasite nests were no longer active. At the nine *F. aserva* colonies, 10 parasite workers and 10 host workers were hand collected at the nest entrance and within nest cavities. Specimens were kept alive in 50mL skirted centrifuge tubes that had been retrofitted with ventilated caps (Corning, Tweksbury, Massachusetts, USA). Parasites and hosts were always collected in separate vials to prevent hydrocarbon transfer between individuals. They were then freeze-killed over dry ice within 4-6 hours of collection for later chemical analysis.

We searched for free-living *Formica* nests within each of these plots and collected 10 *Formica* workers from each free-living nest we discovered, following the same procedure as above. Nests were presumed to be "free-living" and thus not parasitized by *F. aserva* if 1) they were non-host species like *F. rufa sp.*, which we identified based on their nest structure and distinct morphological features, or 2) they were found in a queenright nest with reproductives of their own species. We based this search criteria on what we know from the literature; 1) *F. aserva* is not known to parasitize *F. rufa* species and, 2) the colony founding behavior observed in facultative parasites like *F. aserva* results in foundress parasitic queens killing or expelling resident host queens upon usurpation of the nest (Topoff et al., 1990).

2.2 Chemical sampling and analysis

<u>Chemical extraction</u>

Freeze-killed specimens were removed from dry ice within 24 hours and placed in 2mL screw-top GC vials (Agilent Technologies, Santa Clara, California, USA) that were filled with 200uL of chromatography grade hexanes (HPLC grade, Fisher Scientific, Fair Lawn, New Jersey, USA). The specimens were swirled in the solvent by hand in one minute intervals for a total of 10 minutes. Ants were then removed from the solvent using hexane washed forceps and placed in 95% molecular grade ethanol for future genetic analysis. CHC extracts were kept on dry ice until arriving back to the laboratory where they were stored in a -20° C freezer.

Five of the 10 workers from each collection event were selected for further chemical analyses. The selected sample extracts were filtered through a silica gel column (200 mg silica in a glass pipette with a glass wool plug) and eluted with 1000 µL of hexane into another 2 µL GC vial which was then evaporated under a flow of nitrogen gas (Praxair, Inc., Danbury, Connecticut, USA). The dried extracts were resuspended in 100 µL of hexane and carefully swirled along the walls of the vial to collect all remaining extracts then subsequently transferred into a 250 µL GC insert (Agilent Technologies, Santa Clara, California, USA) placed inside the original GC vial. This step was repeated with another 100 µL hexane for a total of 200 µL of hexane-suspended extract within the GC insert. Suspended extracts were evaporated for a second time under a flow of nitrogen gas and then resuspended in 20 µL of hexane with 7.5ng/ µL of n-dodecane (EMD Millipore Corp., Billerica, Massachusetts, USA) as an internal standard.

GC-MS analysis of cuticular hydrocarbons

Prepared CHC extracts were analyzed using an Agilent 7890A Gas Chromatograph (GC) paired with a 5975C Mass Spectrometer (MS) operating in electron impact ionization mode. Five µL of each sample was injected with an autosampler (Agilent 7683 series) in splitless mode with an inlet temperature of 325°C. Chemical compounds were separated using a capillary column (DB-5MS, 30 m x 0.32 mm x 0.25 µm, Agilent J&W GC columns, Santa Clara, California, USA) using a temperature program optimized for *Formica* CHCs: 50°C for 5 min, 40°C/min to 200°C and then 5°C/min to 320°C for 10 min. The MS was programmed to scan from 40-600 amu.

CHC identification

A standard series of n-alkanes (C_{21}-C_{40}) was run in the GC-MS under the same temperature conditions as our samples before large sample batches and after GC-MS machine maintenance. These n-alkane series were used to detect fluctuations in retention times so that retention indices could be calculated and later used in library preparation for automated peak identification. The n-dodecane standard was also run about one for every 10 samples to monitor any fluctuations in chemical detection as well as provide a measure for standardizing peak identification.

Peak area integration and calculation was initially performed using the data analysis software "Enhanced Chemstation", G1701EA Version E.02.02 (Agilent Technologies, Santa Clara, California, USA) which was set to automatically integrate peaks with an area reject of 0, an initial peak width of 0.017, and an initial threshold of 13. Shoulder detection was turned off. All automatically detected peaks were visually assessed in a separate data analysis software "OpenChrom", Community Edition 1.3.0 (Dalton), which was used to manually adjust integration parameters to better fit our CHC data. We prepared a custom-built library of CHCs for our specific *Formica* using the adjusted area calculation under each peak, retention times, retention indices (calculated from our n-alkane series calibration based on when and under what

GC-MS conditions the specific sample was run), diagnostic ions, and mass spectra. Our custom CHC library file was used to automate compound identification with the OpenChrom software. Out of the 265 individual *Formica* samples we extracted and ran in the GC-MS, 131 were used in our study. The final samples for this study were chosen based on the quality of their GC-MS output and the confidence of our species identifications based on both morphology and mitochondrial sequencing (Chapter 1; Figure 1). Representative profiles from each group and species were manually spot-checked for accuracy and peak identifications were corrected across samples. Due to the poor resolution, co-elution of particular methyl alkanes, and ambiguous diagnostic ion pairing, co-eluting CHCs were recorded as a mixture of compounds. The majority of co-eluting compounds were consistent within and between species so we do not suspect that recording these mixtures as distinct peaks had a significant effect on our overall results.

2.3 Statistical analysis

We used R packages *vegan* and *ggplot2* to run a Nonmetric Multi-dimensional Scaling (NMDS) analysis of the 202 cuticular hydrocarbons identified across our samples. To do so, we built a matrix containing all samples, their categorical grouping, and all recorded CHCs, and then calculated the relative proportions of each compound for individual samples. This proportional matrix was plotted using NMDS to determine if the species and categories in our data set were distinguishable from one another based on these relative proportions of CHCs. We ran NMDS analyses for two data sets, one that included all samples from our study (parasites, captive hosts, free-living hosts, non-hosts) and the other, which included only free-living hosts and captive hosts. Similarly, we used this proportional matrix of total CHCs for all samples to run a heatmap analysis in base R using the *heatmap* function. We performed significance testing to determine the degree of dissimilarity between and within the categories of samples used in each of our NMDS plots using an analysis of similarity test (ANOSIM) with the R package *vegan* and function *anosim*. ANOSIM uses a rank dissimilarity matrix to test whether there is a significant difference between two or more of our categorical groups based on whether the dissimilarity is greater between the groups than within the groups. SIMPER analyses were conducted with the R package *vegan* from subsets of our proportional CHC data. These similarity percentages are based on the pairwise comparisons of sample groups by finding the average contribution of each species to the overall Bray-Curtis dissimilarities. Additionally, one-way analysis of variance (ANOVA) and *post-hoc* tests were performed in Microsoft Excel, version 16.23.

3.0 Results

3.1 Chemical composition of Formica *workers*

We identified a total of 202 hydrocarbon peaks across the eight *Formica* species in our study (Table 1; Figure 1). Some ambiguity in peak identification occurred due to poor quality output from the GC-MS after C_{32} so we focused our identifications on compounds in the range of C_{21}-C_{32}. Peaks within this range had relatively unambiguous mass spectra and CHCs in this range were fairly evenly represented across the *Formica* species examined. Some exceptions exist in the *F. argentea* and *F. sibylla* samples, which possessed fewer compounds overall when compared with the other *Formica*. *Formica sibylla* profiles consistently had fewer CHCs compared to the other *Formica* (mean ± sd = 28.0 ± 6.05) but the majority of their hydrocarbon profile fell within our focal range, therefore, the simpler profile we observed for *F. sibylla* is an accurate representation of this species and not the result of an artificially simplified profile based

on the range of hydrocarbons we selected for this study. The opposite is true for our *F. argentea* samples which we originally recorded as having a large proportion of longer chain length compounds within their CHC profile, many of which did not fall within our C_{21}-C_{32} identification range, resulting in the artificial appearance of a simpler overall CHC profile for our study's comparisons (mean ± sd = 21.75 ± 3.69). We accept this limitation in our study and the overlapping range we chose fits best with the total CHC overlap observed in all of our representative *Formica* species.

Within the mixed colonies that we collected (*F. aserva* + a *Formica* host), parasites and hosts shared about 55% of their CHCs (Figure 2). Of all the recorded peaks, 18% were unique to free-living hosts (*F. microphthalma*, *F. accreta*, and *F. argentea*) and 70% of these unique peaks were di-and trimethyl alkanes. *Formica sibylla*, one of the *Formica* species that is not parasitized by *F. aserva*, had one of the simplest chemical profiles in terms of total peaks recorded when compared with parasite profiles ($t=4.03$, $p<0.0001$). Free-living colonies of one of the host species, *F. argentea*, possessed even fewer CHCs when compared with parasites ($t=6.20$, $p=0.0008$) and the other free-living *Formica* (with the caveat noted above regarding the abundance of larger molecules that fell outside the range studied here).

The results of our one-way ANOVA comparing the effect of "life history" on cue diversity in *Formica* revealed a significant difference in recognition cue diversity between at least two life history groups (parasites, captives, free) in our study ($F_{(3,112)} = 27.53$, $p<0.0001$). *Post hoc* t-tests and Tukey's HSD test for multiple comparisons revealed that dimethyl alkane diversity (including isomers) was significantly different ($t=-3.83$, $p=0.0001$) between *F. aserva* parasites (mean ± sd = 3.54 ± 2.22) and their captive hosts (mean ± sd = 6.43 ± 2.41), with these methyl-branched compounds, as well as linear alkenes, acting as influential compounds in differentiating captive hosts from their parasitic nestmates (Tables 2, 3, & 4). The captive hosts had significantly lower dimethyl alkane diversity ($t=-4.85$, $p<0.0001$) than free-living colonies of the same host species (mean ± sd = 10.375 ± 2.41) suggesting that there is a reduction in the number of dimethyl alkanes within a host's CHC profile after they are captured and reared in the *F. aserva* colony. Similarly, parasite colonies had even fewer dimethyl alkanes within their profiles ($t=-10.91$, $p<0.0001$) compared to free-living hosts. Parasites showed no significant difference ($t=-0.85$, $p=0.399$) from non-host species like *F. rufa sp.* (mean ± sd = 4.2 ± 3.63) in terms of the number of dimethyl alkanes, suggesting that the *Formica* species in this study that are not subjected to parasitism have less dimethyl alkane diversity than the *Formica* species that are actively under parasitic pressure.

3.2 Differences in CHCs between captive hosts and their free-living conspecifics

The NMDS plot of *Formica* host species from two categories, captive and free-living, showed distinctive species and categorical grouping. Within each host species, free-living hosts clustered together and their captive counterparts clustered together, and these two categories of each species were identifiable as separate, but similar, groups. That is, although the free-living and captive forms of each species were identifiably different from each other, they were generally more similar to each other than they were to any form of different species.

Generally, captive hosts from the four representative host species cluster towards the middle of the plot (where samples from the *F. aserva* parasite were located; Figure 5), whereas free-living hosts were oriented farther away from the center (Figure 3). The results of our ANOSIM for the pairwise comparisons of captive and free workers for each host species showed that captive hosts were significantly different from their free-living conspecific counterparts (*F.*

accreta (R=0.967, p=0.001); *F. microphthalma* (R=1, p=0.006); *F. argentea* (R=0.438, p=0.019))(Figure 4). The high R values for *F. accreta* and *F. microphthalma* indicate that there is dissimilarity between the groups while the lower R value we observed for *F. argentea* suggests a more even distribution in high and low ranks between these groups. This finding fits with the less distinguishable separation of free and captive *F. argentea* in our NMDS. Greater sampling of free-living *F. argentea* colonies would provide more clarity, but they were challenging to find and collect at our study site due to inconspicuous nest entrances, difficulty locating queens, erratic and cryptic worker behavior, and uncertainty in the potential occupancy of *Polyergus* kidnapper ants in a host nest. The *F. accreta* species that we collected are a good example of the clear chemical distinction that exists when the data are supported by a more robust sample size.

3.3 Chemical comparisons and species-related differences in cuticular hydrocarbons of Formica

The NMDS plot of all recorded hydrocarbons across *Formica* species revealed a clear distinction between *F. aserva* parasites, free-living host *Formica*, and non-host *Formica*. There was no distinction between parasite samples and their captive hosts, as seen by the overlapping clusters of parasite and captive host species in the center of the plot. Three of the four captive host species clustered close together with the *F. aserva* samples whereas their free-living counterparts and non-host *Formica* were oriented farther away from the captive and parasite samples at the center (Figure 5). The fourth captive host, *Formica argentea*, did not cluster as closely with *F. aserva* as did the other hosts and, instead, was positioned more closely with its free-living conspecifics. We conducted significance testing on each parasite colony to determine if parasite workers were significantly different from their captives. Our ANOSIM results of the comparisons of *F. aserva* CHCs and their corresponding captive host CHCs showed that workers in these mixed colonies, although cohabitating, still retained significant differences between their CHC profiles (Figure 6). Thus, although hosts appear more chemically similar overall to *F. aserva* when captured, they still maintain distinct chemical features that are likely species and colony-specific.

Other pairwise ANOSIM comparisons included *F. aserva* with each of the free-living *Formica* species from this study (Figure 7) which we performed to test if *F. aserva* was more different from non-hosts than the species it selects as hosts. Based on these ANOSIM results, we cannot conclude that *F. aserva* is more chemically similar to and therefore, "less different from", its host species than non-host species. The R values for each pairwise comparison showed that *F. aserva* is highly dissimilar to all of the free-living *Formica* (host and non-host) with one exception; *F. aserva* was less dissimilar from the non-host *F. rufa sp.* (R=0.348) (Figure 7d) than it was from the other *Formica*. This result parallels the close positional relationship of *F. aserva* and *F. rufa sp.* workers in the NMDS (Figure 5). Thus, *F. aserva* actually appears to be the most chemically similar to one of the species it does not target as a host, which is in stark contrast with our hypothesis that *F. aserva* would appear more chemically similar to host species than to non-host species.

The results of the heatmap comparing the relative proportions of CHCs of the *Formica* species from the different categories in our study (parasite, captive host, free-living host, and non-host) revealed three of the four captive host species (*F. microphthalma, F. accreta, F. subaenescens*) clustering closely together while appearing farther apart from their free-living species (with the exception of captive *F. subaenescens*, whose free-living counterparts were not collected) (Figure 8). Captive and free-living *F. argentea* clustered closely together, reflecting the similar result we observed in the NMDS (Figures 3 & 5) where *F. argentea* samples

resemble each other despite parasitism. *Formica aserva* and *F. rufa* also clustered closely together revealing the aforementioned similarities between these two species. Free-living *F. microphthalma, F. accreta,* and non-host, *F. sibylla,* are dispersed across the heatmap and do not cluster in any apparent pattern.

To identify the CHCs that were most similar and different among *F. aserva,* the respective host *Formica,* and free-living colonies of the same host *Formica* species, we calculated similarity percentages (SIMPER) by discriminating CHCs between groups using Bray-Curtis dissimilarities. Since each *F. aserva* colony we assayed was found using only one host species, we grouped our data into sets corresponding to the three different host species: *F. microphthalma, F. accreta,* and *F. argentea*. We did not include a set of data for the fourth host, *F. subaenescens,* because we did not collect any free-living colonies of this species. For each set, we recorded the most influential CHCs driving the differences between the pairwise comparisons within the set (Tables 2, 3, & 4). The influential compounds recorded within the pairwise comparisons for each set contained representatives of each of the varying compound classes we identified (n-alkanes, alkenes, branched alkanes) but the majority of influential compounds accounting for the pairwise differences were branched alkanes.

The overall average dissimilarity was also calculated using SIMPER and we found that in the *F. microphthalma* and *F. accreta* sets, *F. aserva* and its captive hosts were always less dissimilar to each other than they were to free-living hosts (Table 5). These results were not consistent in the *F. argentea* set, however, where parasites and captive hosts were more dissimilar (62.3%) than captive hosts were to their free-living conspecifics (57.7%), suggesting that *F. argentea* more closely resembles the CHC profile of its free-living species even when parasitized. This result matches the NMDS positioning of *F. argentea* samples from free-living and captive nests, in which captive *F. argentea* were not oriented as close to *F. aserva* as were the other host species (*F. microphthalma, F. accreta, F. subaenescens*), and were positioned closely to the free-living *F. argentea* (Figures 3 & 5).

4.0 Discussion

Kidnapper ants and their hosts are fascinating model systems for fundamental questions in evolutionary biology, behavioral ecology, and chemical ecology. By examining the cuticular hydrocarbon profiles of the kidnapper ant, *F. aserva,* and four of its sympatric host species in the same genus (*Formica*), this study advances our understanding of the chemical ecology that underlies this system. Moreover, by comparing free-living and captive individuals of these host species, we have been able to identify specific changes that are associated with being parasitized.

4.1 Captive host species conform to a parasitic gestalt

In this study we examined the CHC profiles of *Formica* species in a location where social parasitism is common and where the facultative kidnapper ant, *F. aserva,* is abundant. Through a combination of colony mapping, behavioral observation, field collection, and chemical techniques, we were able to extract and identify over 200 hydrocarbon peaks from the cuticles of 130 individual *Formica* workers. We found that captive host workers of two hosts, *F. microphthalma* and *F. accreta* had a higher proportion of shared semiochemicals with *F. aserva* colonymates than with free-living workers of their own species. This finding suggests that there is congruency between parasite and host chemical profiles as a result of interspecific cohabitation, and that this results in a close resemblance in the chemical phenotypes of captive

hosts and parasites, regardless of the particular host species used by *F. aserva*, as it relates to these two host species. Cohabitation likely results in homogeneity of the chemical gestalt as a result of the close interactions that occur between colonymates and the mechanical transfer of colony-specific recognition cues through allogrooming and trophallaxis, and previous studies indicate that these interactions may occur at an even higher rate in mixed species colonies (Vienne et al., 1995; Bagneres et al., 1991). The contribution of the *de novo* biosynthesis of CHCs of heterospecific colony mates in artificially mixed colonies has been tested by Vienne et al. (1995) but their results confirmed that CHC transfer via social interactions accounts for the shared colony signature observed. Further studies by Hefetz et al. (1992) tested the mechanisms responsible for the chemical similarity observed in mixed species colonies, the results of which showed that mechanical transfer of CHCs is mainly responsible for the mixed colony odor. The primary organ responsible for this mechanical transfer is the post pharyngeal gland which has been referred to as the "gestalt organ", acting as a reservoir of hydrocarbons to be transferred during social interactions (Hefetz et al. 1992).

As noted above, uniformity in the cuticular hydrocarbons used for colony recognition is central to maintaining accurate colony recognition behavior. However, CHCs also play a critical role in the behavioral development of social insects. The process of behavioral imprinting that occurs soon after workers emerge from pupation has been documented in many species of ants and is believed to be the basis of how newly eclosed workers establish their discriminatory behavior and recognition cue "templates" (Le Moli & Mori, 1982, 1984). The establishment of the recognition template via imprinting is exploited by social parasites as they begin their new colonies and establish a cooperative, interspecific worker population (Ozaki & Hefetz, 2014; d'Ettorre, 2013). Pioneers like Adele Fielde (1903) and Carlin and Holldobler (1983) were among the first to demonstrate the importance of early olfactory learning in ants by founding artificial mixed social insect colonies. Since that time, myriad studies have highlighted the importance of the imprinting stage in social insects and its evident role in social parasitism. Similarly, in non-ant Hymenoptera, such as polistine wasps, social parasites were believed to acquire the majority of their hosts' recognition cues through repeated interactions with the host and nest substrate (Pfennig et al., 1983) and newly emerged wasps were shown to learn recognition odors upon emergence from pupation (Gamboa, 2004). Early olfactory imprinting would best explain the behavioral cohesion we observed between *F. aserva* and their hosts given the degree of dissimilarity that still existed between the chemical profiles of parasites and their captives.

In all of these examples, colony founding behavior is believed to play a key role in the evolution of recognition cue matching or other less sophisticated examples of chemical integration with a host. In obligate social parasites, like *Polyergus breviceps*, laboratory experiments that examined colony founding by newly mated parasite queens always showed killing of the resident host queen and the subsequent take-over of the resident host workers already present in the invaded nest (Topoff et al., 1990). This method of colony founding requires a sophisticated degree of chemical deception by foundress parasite queens, and later, her brood, as their survival depends on their acceptance by the host workers in the nest. Chemical camouflage (obtaining compounds from host interactions), chemical insignificance, in which parasite queens and newly eclosed workers lack an abundance and complexity of CHCs, and chemical mimicry (active biosynthesis of host recognition cues) are all believed to facilitate colony founding in obligate social parasites (Guillem et al., 2014). In facultative parasites, however, early observations of colony founding in laboratory experiments have revealed

behaviors by the foundress queen and resident host queen and workers that may not be accelerating the evolution of host cue mimicry in these intermediary parasites. These observations include foundress queens of multiple facultative species entering host colonies and driving out resident queens and their adult workers, leaving the host brood behind (Topoff et al., 1990). Thus, newly eclosed parasite workers may not be imprinting on host-specific cues. As it relates to the evolution of chemical mimicry in social parasites, colony founding events like this, in which the initial parasitic brood is being reared by the parasitic queen rather than an already present host cast, suggest that the mechanisms necessary for cue mimicry to evolve may be absent in facultative kidnapper ants. Though these behaviors are incredibly challenging to observe in nature, early experiments like these provide examples of the divergent and diverse invasion strategies used by facultative and obligate kidnapper ants.

If newly eclosed facultative parasites are not basing their colony odor on host-specific cues, then these parasites must be using a unique method, not readily observed in other social parasites, to chemically deceive their hosts. In fact, the conformity of CHCs we observed in captive hosts of *F. aserva* was also observed in captive host species of the European facultative kidnapper ant, *F. sanguinea*. In this example, *F. sanguinea* caused a reduction in their hosts' species-specific cues by masking them with their own recognition cues (Wlodarczyk & Szczepaniak, 2017). Some paper wasp facultative parasites are also known to use similar strategies of chemical deception. For example, foundress queens of *Polistes biglumis* deposit their unique blend of methyl-branched hydrocarbons on the nest substrate of the usurped nests to increase their chances of being accepted by newly emerging captives, effectively masking the existing gestalt with their own scent (Lorenzi et al., 2011). It is likely that, in these examples and our own, the selective pressure on more complex strategies of chemical deception might be too weak to accelerate the evolution of host cue mimicry for facultative parasites (d'Ettorre, et al., 2002, Wlodarczyk & Szczepaniak, 2017).

4.2 Parasites are more chemically different from free-living host species than non-host species

In parallel with our finding that captive hosts and their free-living conspecifics bear significantly different CHCs, despite their genetic relatedness and likely kinship, we found that parasite colonies were also significantly different in their CHC composition from these same free-living species. In addition, there was no obvious distinction in the degree of dissimilarity between *F. aserva* and some non-host species. In our analysis of the differences between *F. aserva* and free-living members of its various host species, we found that *F. aserva* workers were qualitatively quite different in their CHC composition from all free-living host species, and these differences were not smaller than the differences between *F. aserva* and the non-host, *F. sibylla*. Essentially, *F. aserva* was just as chemically different from their hosts than this non-host species.

The most striking finding in this analysis was that *F. aserva* was actually the most chemically similar to the non-host species, *F. rufa sp.*. This was evident both in our NMDS and statistical analyses evaluating the degree of dissimilarities between the species. This finding is contrary to our prediction of host specific choices in facultative parasites; that *F. aserva* selects host species to which they are *most* chemically similar. Therefore, we cannot conclude that chemical congruency with a host species is the key determinant in host selectivity for this facultative kidnapping ant. In fact, host choice likely involves a combination of chemical, behavioral, biological, and ecological factors.

The raiding behavior of kidnapper ants is a spectacular observation in nature that has attracted the attention of myrmecologists for the past century and beyond. In fact, the organized

raids conducted on neighboring ant colonies to replenish host workers in the parasitic nest is the most important behavior distinguishing kidnapper ants from other forms of social parasitism. Thus, these behaviors can provide useful insights for understanding how kidnapper ants are actively choosing hosts on the ground and to what degree they are specializing on these hosts. Previous studies of another well-studied facultative kidnapper ant, *F. sanguinea*, have shown that this species selectively raids colonies of the *Serviformica* species group, despite the presence of other genera in their raiding range (Mori et al., 2001). This finding is in accordance with Emery's Rule in that *F. sanguinea* workers are selecting hosts to which they are phylogenetically closely related (Emery, 1901). Similarly, it is likely that *F. aserva* is more chemically similar to closely related *Formica* than to other genera, loosely in support of our chemical interpretation of Emery's rule, although not tested in this study. Though members of the *sanguinea* complex, including *F. aserva* studied here, appear to be selecting hosts in the *Serviformica* group, observations of raiding behavior in *F. sanguinea* report the propensity of this facultative parasite to conduct raids on multiple *Serviformica* colonies resulting in parasitic colonies with more than one host species present (Mori et al. 2001). This suggests that, in some cases, there may be a lack of true host specificity in facultative kidnapper ants (Mori et al., 1991, 2001). The predatory-like raiding behavior of facultative parasites helps explain these observations in nature since facultative kidnappers are aggressive, predatory species that have been shown raiding for prolonged periods in the season on multiple host colonies and are often found with food during raids (Mori et al. 2001). The predatory origins of kidnapping behavior (Topoff, 1990) and *F. aserva's* relatively simple natural history compared to other species on the evolutionary spectrum, may help explain the lack of chemical specificity we observed in *F. aserva*.

Despite previous observations that suggest a somewhat generalist approach to host choice by facultative kidnappers based on the presence of multiple host species in the parasitic nest, we observed host fidelity in all nine *F. aserva* colonies at our study site (each *F. aserva* colony had only one host species in the nest). The mechanisms resulting in host fidelity in kidnapper ants are believed to be the result of captive host workers rearing parasitic brood, potentially resulting in young parasite queens that have already come in contact and imprinted on host-specific compounds from interactions and nest substrate who will subsequently target those same species during future colony foundation (Jongepier & Foitzik, 2016). As a result, host fidelity is believed to be a driver of recognition cue diversity in ants as the repeated and prolonged pressure of parasites on their specific hosts results in the evolution of host defenses that result in more cue complexity. Although it is still unclear whether or not foundress queens of facultative social parasites acquire both host brood and resident host workers after invasion (that would be rearing parasitic brood from the start), the brood rearing that occurs afterward by captured hosts acquired from raids may still have an effect on host-specific cue imprinting by newly eclosed parasite workers and queens. This may be possible due to the presence of unique compounds still present on captive host workers even after parasitism, which we found in samples from our study, despite their general conformity to the parasitic profile (see Chapter 3). Since *F. aserva* colonies appear to be selecting one host species repeatedly during raids, then this particular facultative parasite may be responsible for a degree of the cue diversity we see in the hosts of this study and, thus, may be evidence for the varying degrees with which facultative parasites occur on the evolutionary spectrum of kidnapping behavior.

4.3 Formica *hosts have higher cue diversity than non-host* Formica

Our analysis of the cuticular hydrocarbons of free-living host species and non-host *Formica* revealed that species that are the targets of parasitism possess more di- and trimethyl alkanes than those that are not. Since recognition in ants is determined by the suite of CHCs a colony possesses, our results suggest that host species may be experiencing selective pressure on the chemical complexity of their CHC profiles and, thus, could be adapting their defensive strategy for combating parasitic invasion. That is, because high colony label diversity (i.e. CHC diversity) may enable more effective colony recognition, the higher CHC diversity in host species may reflect historical evolutionary changes in response to parasitism by *F. aserva*. Although our findings did not reveal a sophisticated degree of chemical specificity of *F. aserva* on its host species, e.g. *F. aserva* was not more chemically similar to hosts than non-hosts, the field colonies we assayed still demonstrated a high level of host fidelity and, thus, *F. aserva's* presence could be contributing to the complexity we observed in its host species.

Although previous studies have focused on the totality of compounds in the hydrocarbon profile for making comparisons between colonies, newer findings suggest that colony-specific differences in specific parts of the profile may be more useful, and that dimethyl alkanes are the only compounds believed to be contributing to colony-specific recognition in *Formica* (Martin et al., 2011; Akino et al., 2004). Kidnapper ants, more than other varieties of social parasites, are likely to impose strong negative frequency dependent selection on these specific compound classes (Jongepier & Foitzik, 2016). This is due to the extended pressure and strain that kidnapper ants impose on their hosts as a result of the frequent raids on free living colonies (Jongepier & Foitzik, 2016). For this reason, social parasites play an important role in driving cue diversity in their hosts. Studies that tested the effect of the European facultative kidnapper ant, *F. sanguinea,* on the recognition cues of their hosts found that host populations under high parasitic pressure had more diverse profiles, especially with regard to dimethyl alkanes (Martin et al. 2008; Martin et al. 2011). This finding suggests that even facultative parasites at their intermediary stage of kidnapping behavior can drive the evolution of more complex discriminatory systems in their host species. With the increased cue complexity we observed in the *Formica* hosts of our study, particularly with respect to the number and variety of dimethyl alkanes on the cuticle, it is reasonable to suggest that *F. aserva* is contributing to the evolution of host defensive strategies. Although there is certainly a correlation between the cue complexity we observed and the presence of *F. aserva* in the ant communities we assayed, it is likely that other factors may also affect host cue diversity.

Polyergus breviceps is a well-studied obligate kidnapper ant that also occurs at our study site. These colonies coexist in sympatry with *F. aserva* colonies and both of these kidnapper species use the same *Formica* species as their hosts. Hosts are therefore experiencing pressure from these social parasites, which likely affects the evolution and expression of their colony recognition cues, creating stricter chemical systems to defend against colony intruders (Martin et al., 2011). Although comparative studies of obligate and facultative forms of kidnapper behavior in ants have been conducted, we are unaware of a study that directly assesses the effect of the interaction between facultative and obligate forms of parasitism in the areas where representatives of these groups overlap both geographically and with respect to host species. Future studies on kidnapper ants in areas where obligate and facultative species overlap should focus on the interactions of this combined parasitism on the overall ant community.

In conclusion, it appears that captive host species are conforming to the colony gestalt of *F. aserva* and becoming more chemically divergent from their free-living counterparts, while *F.*

aserva maintains a homogenous mixture of CHCs that appear fairly uniform across the different host species they parasitize. This is likely due to the behavioral ecology of this facultative parasite whose life history, including colony founding and host specificity, does not appear as specialized as that observed in obligate kidnapper ants. Thus, *F. aserva* has not evolved to mimic host odors but, instead, homogenizes their host's odors to appear more like their own, likely through repeated social interactions. We cannot explain why *F. aserva* was the most chemically similar to the non-host *F. rufa sp.* than it was to all the other free-living *Formica* in this study or why that chemical similarity has not resulted in parasitism of *F. rufa sp.*. However, based on our field observations, mound building *F. rufa sp.* ants form large colonies with very aggressive workers that have a large body size similar to *F. aserva* workers. These colonies are also famously polydomous and polygynous, which makes them difficult targets for the type of parasitism that *F. aserva* has evolved (Ellis & Robinson, 2014). Finally, our finding that the free-living host species in our study have higher cue diversity than non-host *Formica* is in agreement with other findings regarding the effect of parasite pressure on host cue diversity. This suggests that the particular host species in our study are evolving more complex recognition systems in response to parasitic species like *F. aserva.* However, we cannot decouple the effect of *Polyergus* parasites on the recognition cues of *Formica* species at our study site they have an overlapping range with *F. aserva*, and may therefore be raiding the same host colonies as *F. aserva*. Since our qualitative findings of the CHC composition of *F. aserva,* their hosts, and non-host *Formica* did not indicate any degree of advanced chemically deceptive strategy like chemical mimicry, then it would not be correct to assume that the cue diversity we recorded in the host species is solely due to the presence of our facultative kidnapper ant. Despite this, there is no doubt that *F. aserva* has evolved an effective strategy for developing multi-species colonies and that strategy includes dominating the colony gestalt with their own recognition cues.

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Table 1. Mean relative abundances ± standard deviation of 202 cuticular hydrocarbons from the different sample groups, with peak numbers presented in the order of their retention times. The (+) indicates the presence of co-eluting compounds, for example, peak 37 is potentially a mixture of 4,8 DiMe C_{24} *and* 4,10 DiMe C_{24}.

			Mixed colonies		Free-living host colonies			Non-host colonies	
Peak Number	Compound	Retention Time (min)	*F. aserva*	*F. sp.* Captives	*F. accreta A*	*F. accreta B*	*F. argentea*	*F. rufa sp.*	*F. sibylla*
1	C_{21}	12.52	0.012±0.006	0.008±0.005	0.015±0.008	0.001±0.001	0±0	0.006±0.006	0.003±0.002
2	9 + 11 Me C_{21}	12.86	0±0	0±0	0.003±0.002	0±0	0±0	0±0	0±0
3	5 Me C_{21}	12.99	0±0	0±0	0±0	0±0	0±0	0.001±0.001	0±0
4	3 + 5 Me C_{21}	13.19	0.008±0.005	0.01±0.007	0±0	0±0	0±0	0.001±0.001	0±0
5	3 Me C_{21}	13.25	0.005±0.003	0.005±0.003	0.006±0.007	0±0	0±0	0±0	0±0
6	3 + 4 Me C_{21}	13.48	0±0	0±0	0±0	0±0	0±0	0±0	0.002±0.001
7	4 Me C_{21}	13.53	0±0	0.003±0.002	0±0	0±0	0±0	0±0	0±0
8	C_{22} monoene	13.28	0±0	0±0	0±0	0±0	0±0	0±0	0.002±0.001
9	C_{22}	13.47	0.003±0.003	0.004±0.005	0.002±0.001	0.001±0.001	0±0	0.001±0.001	0.002±0.001
10	9 Me C_{22}	13.85	0±0	0±0	0±0	0±0	0±0	0±0	0±0
11	9 + 10 Me C_{22}	13.86	0±0	0±0	0.01±0.006	0±0	0±0	0±0	0±0
12	3 Me C_{22}	13.92	0±0	0.001±0.001	0±0	0±0	0±0	0±0	0.002±0.001
13	4 + 10 + 11 Me C_{22}	13.93	0±0	0±0	0±0	0±0	0±0	0±0	0.001±0.001
14	4 Me C_{22}	14.07	0.001±0.001	0.001±0.001	0.027±0.014	0±0	0±0	0±0	0±0
15	4,10 + 4,12 + 6,10 + 6,12 DiMe C_{22}	14.25	0±0	0±0	0±0	0±0	0±0	0±0	0±0
16	4,8 + 4,10 + 4,12 + 6,10 + 6,12 DiMe C_{22}	14.43	0±0	0±0	0±0	0±0	0±0	0±0	0±0
17	C_{23} diene	14.28	0±0	0±0	0±0	0±0	0±0	0±0	0.019±0.008
18	C_{23} monoene	14.33	0.002±0.001	0.018±0.032	0.004±0.002	0±0	0±0	0.001±0.001	0.14±0.088
19	C_{23}	14.58	0.026±0.011	0.027±0.018	0.033±0.008	0.009±0.005	0.009±0.009	0.034±0.027	0.078±0.031
20	7 Me C_{23}	14.83	0±0	0.001±0.001	0±0	0±0	0±0	0±0	0±0
21	11 Me C_{23}	14.95	0.002±0.001	0.007±0.008	0±0	0±0	0±0	0±0	0.005±0.003
22	9 + 11 Me C_{23}	15.02	0.001±0.001	0.005±0.007	0.226±0.188	0±0	0±0	0±0	0.002±0.001
23	5 Me C_{23}	15.15	0.001±0.001	0.002±0.002	0.006±0.003	0±0	0±0	0.002±0.001	0.003±0.002
24	3 + 5 Me C_{23}	15.38	0.006±0.004	0.01±0.006	0±0	0.002±0.001	0±0	0.001±0.001	0±0
25	3 Me C_{23}	15.41	0.005±0.003	0.004±0.003	0.025±0.01	0.002±0.001	0±0	0.001±0.001	0.004±0.002
26	9,13 DiMe C_{23}	15.32	0±0	0±0	0.006±0.003	0±0	0±0	0±0	0±0
27	5,13 DiMe C_{23}	15.5	0±0	0±0	0±0	0±0	0±0	0±0	0±0
28	C_{24} diene	15.35	0±0	0±0	0±0	0±0	0±0	0±0	0.005±0.003
29	C_{24} monoene	15.49	0.001±0.001	0.003±0.001	0±0	0±0	0±0	0±0	0.006±0.004
30	C_{24}	15.73	0.008±0.011	0.007±0.012	0.008±0.005	0.003±0.002	0±0	0.004±0.003	0.003±0.001
31	3 + 8 Me C_{24}	15.84	0±0	0±0	0.01±0.005	0±0	0±0	0±0	0.028±0.013
32	11 + 12 + 13 Me C_{24}	16.13	0.001±0.001	0.006±0.002	0±0	0±0	0±0	0±0	0±0

33	10 + 11 + 12 + 13 Me C_{24}	16.15	0±0	0±0	0.058±0.031	0±0	0±0	0±0	0±0
34	4 Me C_{24}	16.4	0.001±0.001	0.002±0.001	0.038±0.02	0.001±0.001	0±0	0±0	0±0
35	3 Me C_{24}	16.57	0±0	0.008±0.003	0±0	0±0	0±0	0±0	0±0
36	3 + 5 Me C_{24}	16.62	0±0	0±0	0.003±0.002	0±0	0±0	0±0	0±0
37	4,8 + 4,10 DiMe C_{24}	16.81	0±0	0±0	0.004±0.003	0±0	0±0	0±0	0±0
38	4,8 DiMe C_{24}	16.82	0±0	0±0	0±0	0±0	0±0	0±0	0±0
39	6,8 DiMe C_{24}	16.06	0±0	0±0	0±0	0±0	0±0	0±0	0.002±0.001
40	6,10 + 6,12 DiMe C_{24}	16.24	0±0	0±0	0±0	0±0	0±0	0±0	0.003±0.002
41	C_{25} diene	16.47	0.001±0.001	0.026±0.012	0±0	0±0	0±0	0±0	0.029±0.033
42	C_{25} monoene	16.68	0.01±0.006	0.041±0.077	0.044±0.024	0±0	0.007±0.008	0.007±0.005	0.387±0.107
43	C_{25}	17.02	0.187±0.076	0.084±0.037	0.082±0.033	0.029±0.017	0.029±0.027	0.21±0.034	0.06±0.022
44	9 + 11 Me C_{25}	17.37	0±0	0.003±0.001	0±0	0±0	0±0	0±0	0±0
45	11 + 13 Me C_{25}	17.39	0.003±0.002	0.01±0.012	0.152±0.082	0.01±0.002	0±0	0.002±0.002	0.005±0.005
46	5 Me C_{25}	17.57	0.005±0.002	0.007±0.005	0±0	0.002±0.001	0±0	0.002±0.001	0±0
47	9 Me C_{25}	17.57	0.007±0.002	0±0	0±0	0±0	0±0	0±0	0±0
48	13 Me C_{25}	17.69	0±0	0±0	0.037±0.02	0±0	0±0	0±0	0±0
49	3 + 5 Me C_{25}	17.72	0.011±0.01	0.017±0.018	0.028±0.026	0±0	0±0	0±0	0.008±0.004
50	4 Me C_{25}	17.79	0.002±0.001	0.003±0.002	0±0	0±0	0±0	0±0	0±0
51	3 + 4 + 8 + 13 Me C_{24}	17.83	0.001±0.001	0±0	0±0	0±0	0±0	0±0	0±0
52	3 + 4 + 8 + 13 Me C_{25}	17.83	0.001±0.001	0±0	0±0	0±0	0±0	0±0	0±0
53	3 Me C_{25}	17.91	0.031±0.02	0.045±0.03	0.016±0.009	0.017±0.004	0.001±0.001	0.002±0.001	0.013±0.01
54	11,13 DiMe C_{25}	18.01	0±0	0±0	0±0	0±0	0±0	0.002±0.001	0±0
55	9,13 + 11,13 + 13,15 DiMe C_{25}	17.74	0±0	0±0	0±0	0±0	0±0	0±0	0±0
56	9,13 + 11,13 DiMe C_{25}	17.75	0±0	0±0	0.007±0.004	0±0	0±0	0±0	0±0
57	C_{26}	18.25	0.019±0.016	0.011±0.014	0.005±0.004	0.005±0.003	0.004±0.004	0.014±0.003	0.002±0.001
58	3 + 8 Me C_{26}	18.38	0.003±0.001	0±0	0±0	0±0	0±0	0±0	0±0
59	3 + 6 Me C_{26}	18.39	0±0	0±0	0±0	0±0	0±0	0±0	0.005±0.003
60	10 Me C_{26}	18.57	0±0	0.004±0.002	0±0	0±0	0±0	0.001±0.001	0±0
61	11 + 12 + 13 + 14 Me C_{26}	18.61	0.001±0.001	0.004±0.002	0±0	0±0	0±0	0±0	0±0
62	10 + 11 Me C_{26}	18.62	0±0	0±0	0±0	0.013±0.007	0±0	0±0	0±0
63	10 + 12+ 13 + 14 Me C_{26}	18.64	0.003±0.002	0.004±0.003	0±0	0±0	0±0	0±0	0±0
64	12 + 13 + 14 Me C_{26}	18.64	0±0	0.002±0.001	0.01±0.006	0.011±0.006	0±0	0.001±0.001	0±0
65	8 + 10 + 13 + 14 Me C_{26}	18.73	0.002±0.001	0±0	0±0	0±0	0±0	0±0	0±0
66	11 Me C_{26}	18.82	0.003±0.001	0±0	0±0	0±0	0±0	0±0	0±0
67	6 Me C_{26}	18.83	0±0	0.001±0.001	0±0	0.002±0.001	0±0	0±0	0±0
68	12 Me C_{26}	18.98	0±0	0±0	0±0	0.011±0.006	0±0	0.001±0.001	0±0
69	4 Me C_{26}	19	0.005±0.002	0.004±0.003	0.005±0.003	0.007±0.004	0±0	0.001±0.001	0±0
70	4,10 + 6,10 DiMe C_{26}	19.14	0±0	0±0	0±0	0.004±0.001	0±0	0±0	0±0
71	10,12 + 10,14 DiMe C_{26}	19.27	0±0	0±0	0±0	0.005±0.003	0±0	0±0	0±0
72	4,8 DiMe C_{26}	19.34	0±0	0±0	0±0	0±0	0±0	0±0	0±0
73	6,10 DiMe C_{26}	19.36	0±0	0±0	0±0	0.012±0.007	0±0	0±0	0±0

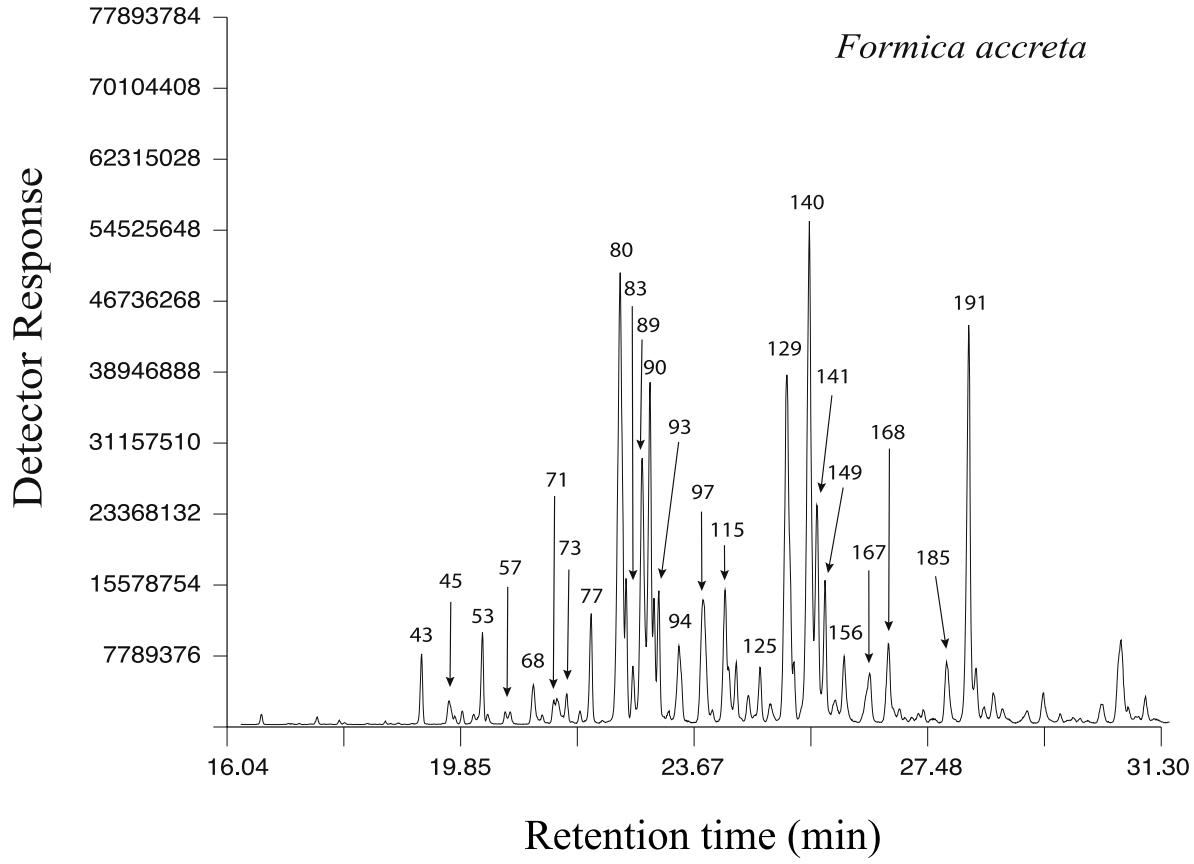
74	4,8 + 4,10 DiMe C_{26}	19.4	0±0	0±0	0±0	0.001±0.001	0±0	0±0	0±0
75	C_{27} diene	18.89	0.004±0.004	0.009±0.007	0.014±0.01	0±0	0±0	0±0	0.006±0.006
76	C_{27} monoene	19.21	0.14±0.078	0.138±0.093	0.263±0.141	0.003±0.002	0±0	0.015±0.009	0.157±0.149
77	C_{27}	19.59	0.248±0.119	0.1±0.065	0.048±0.026	0.035±0.017	0.086±0.086	0.367±0.082	0.019±0.014
78	3 + 11 + 13 Me C_{27}	19.81	0±0	0.016±0.006	0±0	0±0	0±0	0±0	0±0
79	13 Me C_{27}	19.96	0.003±0.001	0.056±0.01	0.034±0.018	0±0	0.005±0.003	0±0	0.012±0.006
80	11 + 13 Me C_{27}	19.98	0.015±0.015	0.031±0.023	0.018±0.01	0.154±0.028	0±0	0.005±0.003	0.004±0.003
81	3 + 7 Me C_{27}	20.03	0.01±0.007	0.024±0.013	0±0	0±0	0±0	0±0	0±0
82	7 Me C_{27}	20.1	0.026±0.009	0.019±0.009	0±0	0±0	0±0	0±0	0±0
83	5 Me C_{27}	20.24	0.005±0.002	0.011±0.006	0±0	0.006±0.004	0.003±0.002	0.002±0.001	0.002±0.001
84	3 + 5 Me C_{27}	20.37	0.017±0.012	0.019±0.012	0±0	0±0	0±0	0±0	0.008±0.004
85	3 Me C_{27}	20.42	0.018±0.014	0.018±0.01	0.011±0.011	0±0	0.026±0.01	0±0	0±0
86	4 Me C_{27}	20.43	0.002±0.001	0.003±0.001	0±0	0±0	0±0	0±0	0±0
87	5,9 + 5,13 DiMe C_{27}	20.13	0±0	0±0	0±0	0±0	0±0	0±0	0.001±0.001
88	9,13 + 11,13 DiMe C_{27}	20.22	0±0	0±0	0.005±0.003	0±0	0±0	0±0	0±0
89	11,15 DiMe C_{27}	20.38	0.005±0.003	0.012±0.007	0±0	0.056±0.015	0±0	0.001±0.001	0.002±0.002
90	7,11 DiMe C_{27}	20.44	0.029±0.023	0.016±0.014	0±0	0.057±0.036	0±0	0.003±0.003	0.002±0.001
91	5,11 + 5,15 DiMe C_{27}	20.64	0.004±0.001	0.007±0.002	0±0	0±0	0±0	0±0	0±0
92	5,15 + 11,15 DiMe C_{27}	20.77	0.021±0.006	0±0	0±0	0±0	0±0	0±0	0±0
93	5,15 DiMe C_{27}	20.93	0±0	0±0	0±0	0.024±0.012	0±0	0±0	0±0
94	3,11 + 3,13 DiMe C_{27}	21.18	0±0	0±0	0±0	0.025±0.013	0±0	0±0	0.011±0.003
95	7,11,15 TriMe C_{27}	20.75	0±0	0±0	0±0	0.003±0.002	0±0	0±0	0±0
96	C_{28} monoene	20.65	0.035±0.018	0.042±0.023	0.074±0.039	0±0	0±0	0±0	0±0
97	C_{28}	20.87	0.013±0.014	0.015±0.014	0.005±0.004	0.021±0.021	0.019±0.01	0.011±0.003	0.003±0.002
98	3 + 8 Me C_{28}	20.83	0±0	0±0	0±0	0.022±0.011	0±0	0±0	0±0
99	3 + 12 Me C_{28}	20.94	0.009±0.002	0±0	0±0	0±0	0±0	0±0	0±0
100	3 + 10 + 12 + 14 + 15 Me C_{28}	20.95	0.01±0.002	0±0	0±0	0±0	0±0	0±0	0±0
101	10 Me C_{28}	21	0±0	0±0	0±0	0±0	0±0	0.002±0.001	0±0
102	12 Me C_{28}	21.18	0.002±0.001	0.007±0.003	0±0	0±0	0±0	0±0	0.001±0.001
103	13 + 14 + 15 Me C_{28}	21.18	0±0	0.004±0.002	0.004±0.002	0±0	0±0	0±0	0±0
104	10 + 12 + 14 + 15 Me C_{28}	21.21	0±0	0.012±0.004	0±0	0±0	0±0	0±0	0±0
105	11 + 12 Me C_{28}	21.23	0±0	0±0	0±0	0±0	0±0	0.002±0.001	0±0
106	12 + 14 + 15 Me C_{28}	21.27	0.006±0.004	0.009±0.006	0±0	0.03±0.015	0±0	0±0	0±0
107	10 + 12 + 14 + 15 Me C_{28}	21.31	0±0	0±0	0±0	0±0	0±0	0±0	0.001±0.001
108	4 + 14 + 15 Me C_{28}	21.52	0±0	0.002±0.001	0±0	0±0	0±0	0±0	0±0
109	4 + 10 Me C_{28}	21.53	0±0	0±0	0.001±0.001	0±0	0±0	0±0	0±0
110	4 Me C_{28}	21.63	0.003±0.001	0.003±0.002	0±0	0±0	0±0	0±0	0±0
111	10 + 12 Me C_{28}	21.87	0±0	0±0	0.001±0.001	0±0	0±0	0±0	0±0
112	8,10 + 8,12 + 10,12 DiMe C_{28}	21.21	0±0	0±0	0±0	0.07±0.035	0±0	0±0	0±0
113	12,14 + 13,15 + 14,16 DiMe C_{28}	21.3	0±0	0.008±0.003	0±0	0±0	0±0	0±0	0±0
114	8,12 DiMe C_{28}	21.41	0.003±0.002	0±0	0±0	0±0	0±0	0.002±0.002	0±0

115	10,14 DiMe C_{28}	21.65	0±0	0.004±0.002	0±0	0.033±0.012	0±0	0±0	0.001±0.001
116	6,14 DiMe C_{28}	21.72	0±0	0.006±0.003	0±0	0±0	0±0	0±0	0±0
117	6,10 DiMe C_{28}	21.78	0±0	0±0	0±0	0.012±0.006	0±0	0±0	0±0
118	4,8 + 4,14 DiMe C_{28}	21.89	0±0	0±0	0±0	0.007±0.002	0±0	0±0	0±0
119	4,8 + 4,10 + 4,12 DiMe C_{28}	21.97	0±0	0±0	0±0	0.002±0.001	0±0	0±0	0±0
120	4,12 + 4,14 DiMe C_{28}	21.99	0±0	0.002±0.001	0±0	0.006±0.002	0±0	0±0	0±0
121	6,10 + 6,14 DiMe C_{28}	22.16	0±0	0±0	0±0	0.015±0.006	0±0	0±0	0±0
122	4,14 DiMe C_{28}	22.36	0±0	0±0	0±0	0.007±0.002	0±0	0±0	0±0
123	C_{29} diene	21.43	0.01±0.01	0.012±0.009	0.035±0.021	0±0	0±0	0±0	0.003±0.002
124	C_{29} monoene	21.72	0.059±0.032	0.062±0.038	0.09±0.048	0.029±0.015	0±0	0.022±0.016	0.009±0.009
125	C_{29}	22.14	0.054±0.026	0.074±0.08	0.017±0.01	0.016±0.007	0.108±0.071	0.162±0.03	0.063±0.052
126	14 + 15 Me C_{29}	22.35	0±0	0.002±0.001	0±0	0±0	0±0	0±0	0±0
127	11 + 13 Me C_{29}	22.49	0±0	0±0	0.013±0.007	0±0	0±0	0.004±0.003	0±0
128	11 + 13 + 15 Me C_{29}	22.49	0.01±0.009	0.023±0.013	0±0	0.154±0.076	0±0	0.01±0.006	0.003±0.002
129	13 + 15 Me C_{29}	22.54	0.012±0.004	0.036±0.023	0.009±0.005	0.101±0.057	0.026±0.003	0±0	0.003±0.003
130	3 + 7 Me C_{29}	22.6	0.024±0.015	0.036±0.021	0±0	0±0	0±0	0±0	0±0
131	5 Me C_{29}	22.72	0±0	0.022±0.008	0±0	0±0	0.024±0.016	0.002±0.001	0±0
132	7 Me C_{29}	22.72	0±0	0.041±0.015	0±0	0±0	0.03±0.009	0.004±0.002	0.001±0.001
133	4 Me C_{29}	22.76	0±0	0.006±0.001	0±0	0±0	0±0	0±0	0±0
134	3 + 5 Me C_{29}	22.84	0.005±0.001	0±0	0±0	0±0	0±0	0±0	0±0
135	6 Me C_{29}	22.89	0.002±0.001	0±0	0±0	0±0	0±0	0±0	0±0
136	3 Me C_{29}	22.96	0.005±0.002	0.011±0.006	0.043±0.024	0±0	0±0	0.002±0.001	0.002±0.001
137	11,13 + 13,15 DiMe C_{29}	22.78	0±0	0±0	0±0	0±0	0±0	0±0	0.001±0.001
138	9,13 + 11,13 DiMe C_{29}	22.83	0±0	0±0	0.004±0.002	0±0	0±0	0±0	0±0
139	9,13 + 9,15 DiMe C_{29}	22.23	0±0	0±0	0±0	0.011±0.006	0±0	0±0	0±0
140	11,15 DiMe C_{29}	22.89	0.017±0.01	0.033±0.02	0±0	0.12±0.031	0±0	0.003±0.002	0.005±0.003
141	7,11 DiMe C_{29}	22.9	0.006±0.005	0.016±0.01	0±0	0.041±0.015	0±0	0.002±0.002	0.001±0.001
142	7,15 DiMe C_{29}	22.91	0±0	0.036±0.017	0±0	0.022±0.008	0±0	0±0	0±0
143	7,11 + 7,13 + 7,15 DiMe C_{29}	22.92	0±0	0±0	0±0	0±0	0±0	0±0	0.001±0.001
144	9,15 DiMe C_{29}	22.93	0±0	0.026±0.005	0±0	0±0	0±0	0±0	0±0
145	7,11 + 7,13 DiMe C_{29}	23.04	0.05±0.02	0.055±0.02	0±0	0±0	0±0	0±0	0±0
146	5,9 DiMe C_{29}	23.05	0±0	0±0	0±0	0.011±0.005	0±0	0±0	0±0
147	5,15 + 13,15 DiMe C_{29}	23.1	0±0	0±0	0±0	0±0	0±0	0.008±0.005	0±0
148	5,9 + 5,11 DiMe C_{29}	23.14	0±0	0±0	0±0	0.009±0.005	0±0	0±0	0±0
149	5,15 DiMe C_{29}	23.2	0±0	0.029±0.025	0±0	0.027±0.013	0.199±0.064	0±0	0.002±0.001
150	4,14 + 4,16 DiMe C_{30}	23.35	0±0	0±0	0±0	0.022±0.011	0±0	0±0	0±0
151	3,11 + 3,13 DiMe C_{29}	23.35	0±0	0±0	0±0	0±0	0±0	0±0	0.005±0.002
152	7,15 + 13,15 DiMe C_{29}	23.36	0±0	0±0	0±0	0±0	0.065±0.018	0±0	0±0
153	6,12 DiMe C_{29}	23.53	0±0	0.002±0.001	0±0	0±0	0±0	0±0	0±0
154	9,13,17 TriMe C_{29}	23.13	0±0	0±0	0.002±0.001	0±0	0±0	0±0	0±0
155	C_{30} monoene	23.2	0.004±0.006	0.004±0.002	0±0	0±0	0±0	0±0	0±0

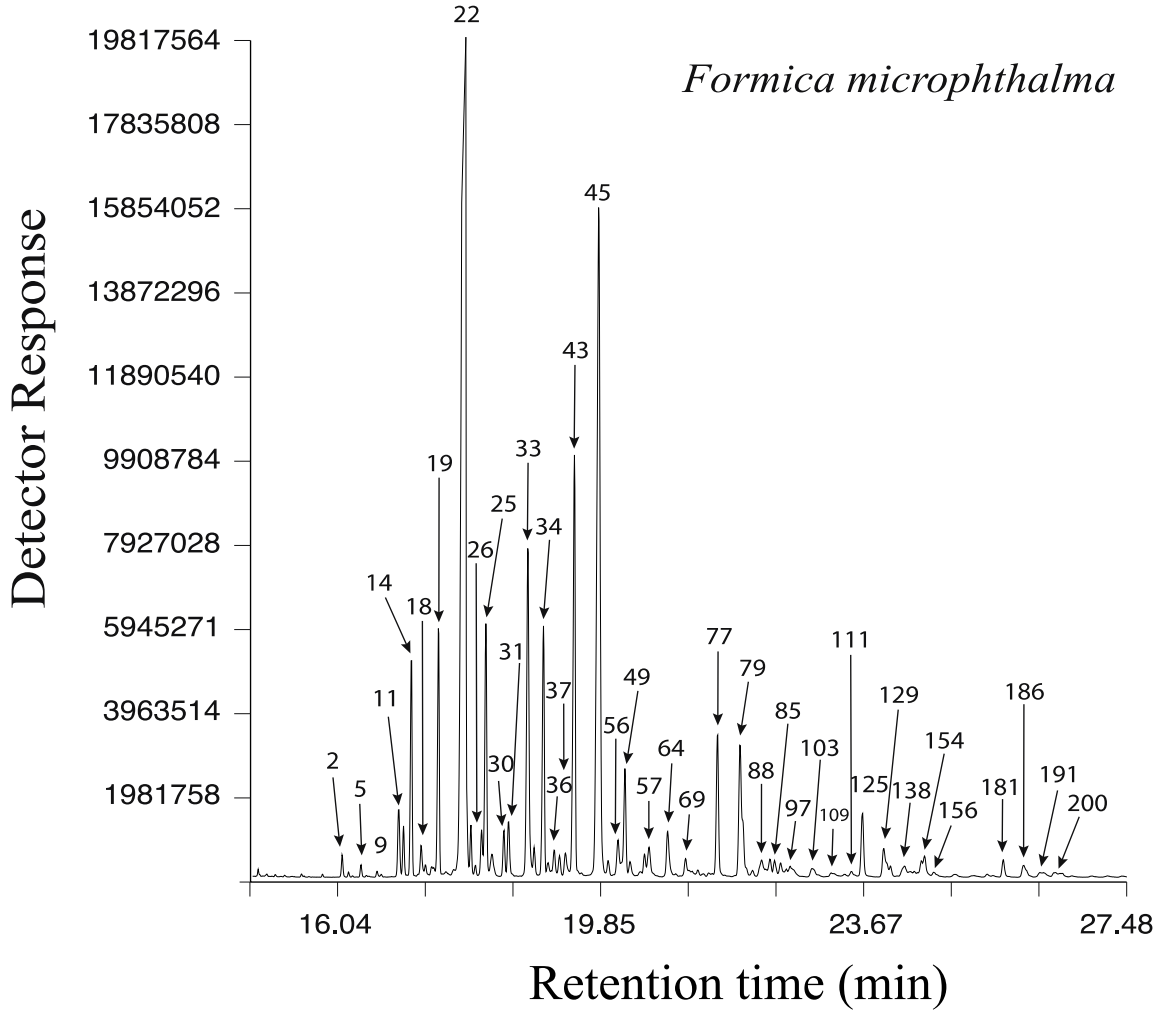
156	C_{30}	23.39	0.006±0.01	0.016±0.013	0.002±0.001	0.013±0.008	0.024±0.013	0.004±0.002	0.003±0.002
157	11 + 12 Me C_{30}	23.51	0±0	0±0	0±0	0±0	0±0	0.001±0.001	0±0
158	12 + 13 Me C_{30}	23.69	0.001±0.001	0±0	0±0	0±0	0±0	0±0	0±0
159	12 + 13 + 15 + 16 Me C_{30}	23.71	0±0	0.003±0.001	0±0	0±0	0±0	0±0	0±0
160	11 + 12 + 13 Me C_{30}	23.75	0±0	0±0	0±0	0±0	0±0	0.003±0.002	0±0
161	12 + 14 Me C_{30}	23.77	0±0	0±0	0±0	0.009±0.004	0±0	0±0	0±0
162	7 + 12 Me C_{30}	23.82	0±0	0.01±0.002	0±0	0±0	0±0	0±0	0±0
163	11 + 13 + 15 + 16 Me C_{30}	23.82	0±0	0.015±0.005	0±0	0±0	0±0	0±0	0±0
164	15 + 16 Me C_{30}	23.87	0±0	0.005±0.002	0±0	0±0	0±0	0±0	0±0
165	12 Me C_{30}	23.93	0.003±0.001	0.006±0.003	0±0	0±0	0±0	0±0	0±0
166	4 Me C_{30}	24.08	0±0	0.007±0.002	0±0	0±0	0±0	0±0	0±0
167	8 Me C_{30}	24.16	0±0	0±0	0±0	0.02±0.009	0±0	0±0	0±0
168	9 + 15 + 16 Me C_{30}	24.45	0±0	0±0	0±0	0.024±0.011	0±0	0±0	0±0
169	8,16 DiMe C_{30}	23.8	0±0	0.007±0.003	0±0	0±0	0±0	0±0	0±0
170	8,14 + 8,16 DiMe C_{30}	23.82	0±0	0±0	0±0	0.041±0.023	0±0	0±0	0±0
171	3,7 + 8,12 + 8,14 + 8,16 DiMe C_{30}	23.86	0±0	0.007±0.002	0±0	0±0	0±0	0±0	0±0
172	8,12 + 8,14 + 8,16 DiMe C_{30}	23.86	0±0	0.007±0.002	0±0	0±0	0±0	0±0	0±0
173	10,16 DiMe C_{30}	24.08	0±0	0.005±0.002	0±0	0±0	0±0	0.002±0.001	0±0
174	10,14 + 10,16 DiMe C_{30}	24.08	0.001±0.001	0.004±0.002	0±0	0.019±0.01	0±0	0±0	0±0
175	8,12 DiMe C_{30}	24.12	0±0	0.002±0.001	0±0	0±0	0±0	0±0	0±0
176	4,12 + 4,14 + 4,16 DiMe C_{30}	24.4	0±0	0.001±0.001	0±0	0±0	0±0	0±0	0±0
177	6,14 + 6,16 DiMe C_{30}	24.43	0±0	0.004±0.002	0±0	0±0	0.013±0.007	0±0	0±0
178	6,10,14 DiMe C_{30}	24.49	0±0	0.001±0.001	0±0	0±0	0±0	0±0	0±0
179	C_{31} diene	23.86	0.009±0.004	0±0	0±0	0±0	0±0	0±0	0±0
180	C_{31} monoene	24.21	0.018±0.01	0.018±0.012	0.033±0.019	0.012±0.006	0±0	0.025±0.015	0.003±0.002
181	C_{31}	24.61	0.005±0.008	0.038±0.069	0.005±0.004	0.012±0.005	0.029±0.019	0.038±0.008	0.06±0.051
182	11 + 13 Me C_{31}	24.81	0.004±0.002	0±0	0±0	0±0	0±0	0.009±0.007	0±0
183	3 + 7 Me C_{31}	24.86	0±0	0.017±0.003	0±0	0±0	0±0	0±0	0±0
184	8 + 13 + 15 Me C_{31}	24.93	0±0	0±0	0±0	0±0	0±0	0±0	0.002±0.001
185	13 + 15 Me C_{31}	24.97	0.002±0.001	0.026±0.016	0±0	0.015±0.01	0.031±0.017	0±0	0.002±0.001
186	11 + 13 + 15 Me C_{31}	24.98	0.01±0.005	0.01±0.006	0.005±0.003	0.028±0.016	0±0	0.023±0.012	0±0
187	3 Me C_{31}	25.09	0.014±0.008	0.018±0.011	0±0	0±0	0±0	0±0	0±0
188	5 Me C_{31}	25.49	0±0	0.005±0.001	0±0	0±0	0.015±0.012	0±0	0±0
189	13,17 DiMe C_{31}	25.22	0±0	0±0	0±0	0±0	0±0	0±0	0.002±0.001
190	11,15 + 11,17 DiMe C_{31}	25.3	0±0	0.013±0.005	0±0	0±0	0±0	0±0	0.002±0.001
191	11,15 DiMe C_{31}	25.31	0.02±0.024	0.054±0.055	0.001±0.001	0.095±0.035	0.015±0.012	0.029±0.023	0±0
192	7,11 + 7,13 DiMe C_{31}	25.32	0±0	0.008±0.002	0±0	0±0	0±0	0±0	0±0
193	7,11 DiMe C_{31}	25.44	0.012±0.007	0.018±0.007	0±0	0±0	0±0	0±0	0±0
194	5,13 DiMe C_{31}	25.48	0.002±0.001	0.004±0.002	0±0	0.003±0.002	0±0	0±0	0±0
195	5,17 DiMe C_{31}	25.55	0±0	0±0	0±0	0±0	0±0	0.019±0.01	0±0
196	5,15 DiMe C_{31}	25.56	0.002±0.001	0.002±0.001	0±0	0±0	0±0	0±0	0±0

197	7,15 + 7,17 DiMe C_{31}	25.6	0±0	0.025±0.009	0±0	0±0	0.071±0.028	0±0	0±0
198	5,13 + 5,15 DiMe C_{31}	25.68	0.002±0.001	0.038±0.016	0±0	0±0	0.14±0.052	0±0	0±0
199	6,12 + 6,14 DiMe C_{31}	25.77	0.005±0.002	0±0	0±0	0±0	0±0	0±0	0±0
200	13,15,17 TriMe C_{31}	25.61	0±0	0±0	0.002±0.001	0±0	0±0	0.003±0.003	0±0
201	7,11,15 TriMe C_{31}	25.89	0±0	0.023±0.01	0±0	0±0	0.022±0.013	0±0	0±0
202	5,9,13 TriMe C_{31}	26.22	0±0	0±0	0±0	0±0	0.093±0.034	0±0	0±0

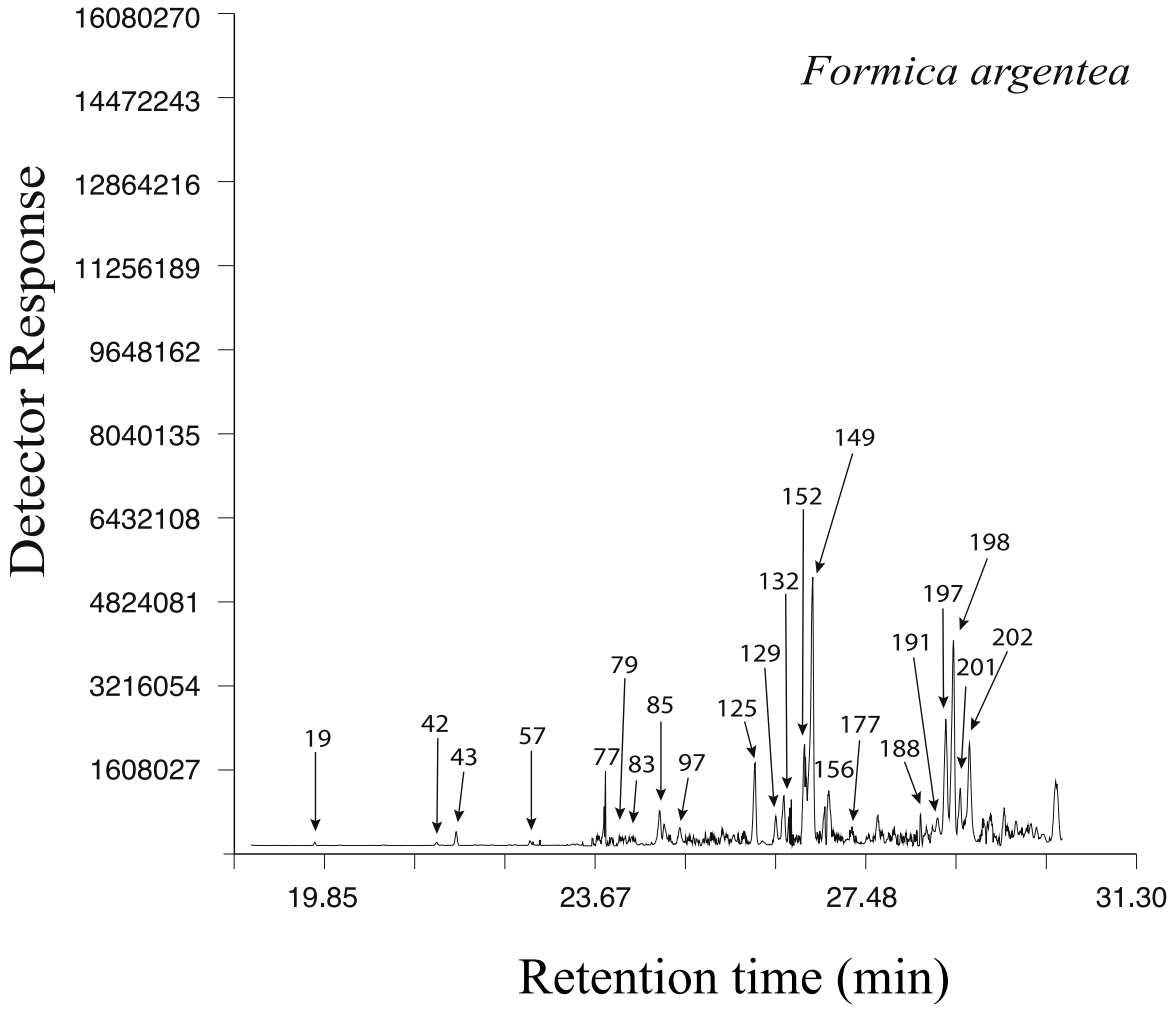
1.a


Formica accreta
Detector Response
77893784
70104408
62315028
54525648
46736268
38946888
31157510
23368132
15578754
7789376
16.04
19.85
23.67
27.48
31.30
Retention time (min)
43
45
53
57
68
71
73
77
80
83
89
90
93
94
97
115
125
129
140
141
149
156
167
168
185
191

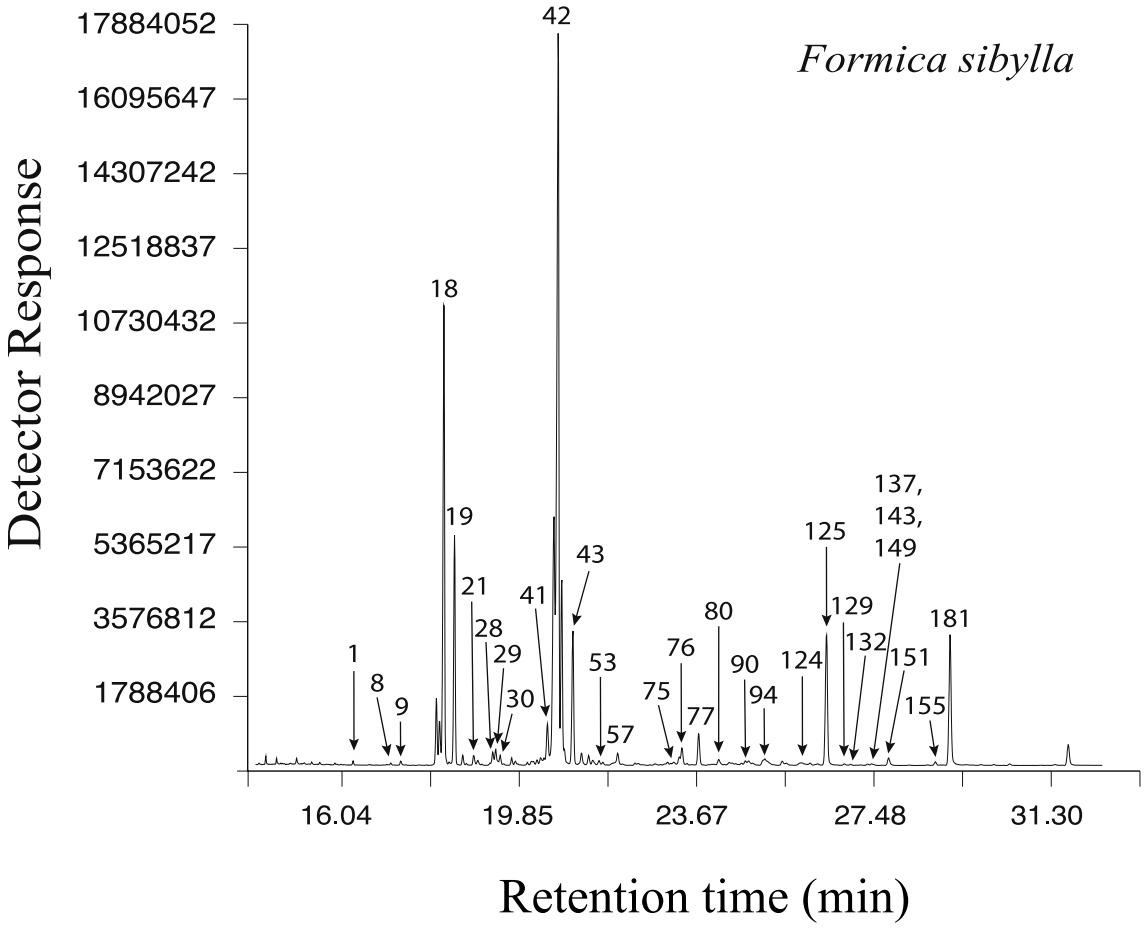
1.b


Formica microphthalma
Detector Response
19817564
17835808
15854052
13872296
11890540
9908784
7927028
5945271
3963514
1981758
16.04
19.85
23.67
27.48
Retention time (min)
22
45
43
33
19
25
34
26
14
18
31
37
49
11
56
64
77
79
111
85
129
186
103
154
2
5
30
36
57
69
88
97
109
125
138
156
181
191
200
9

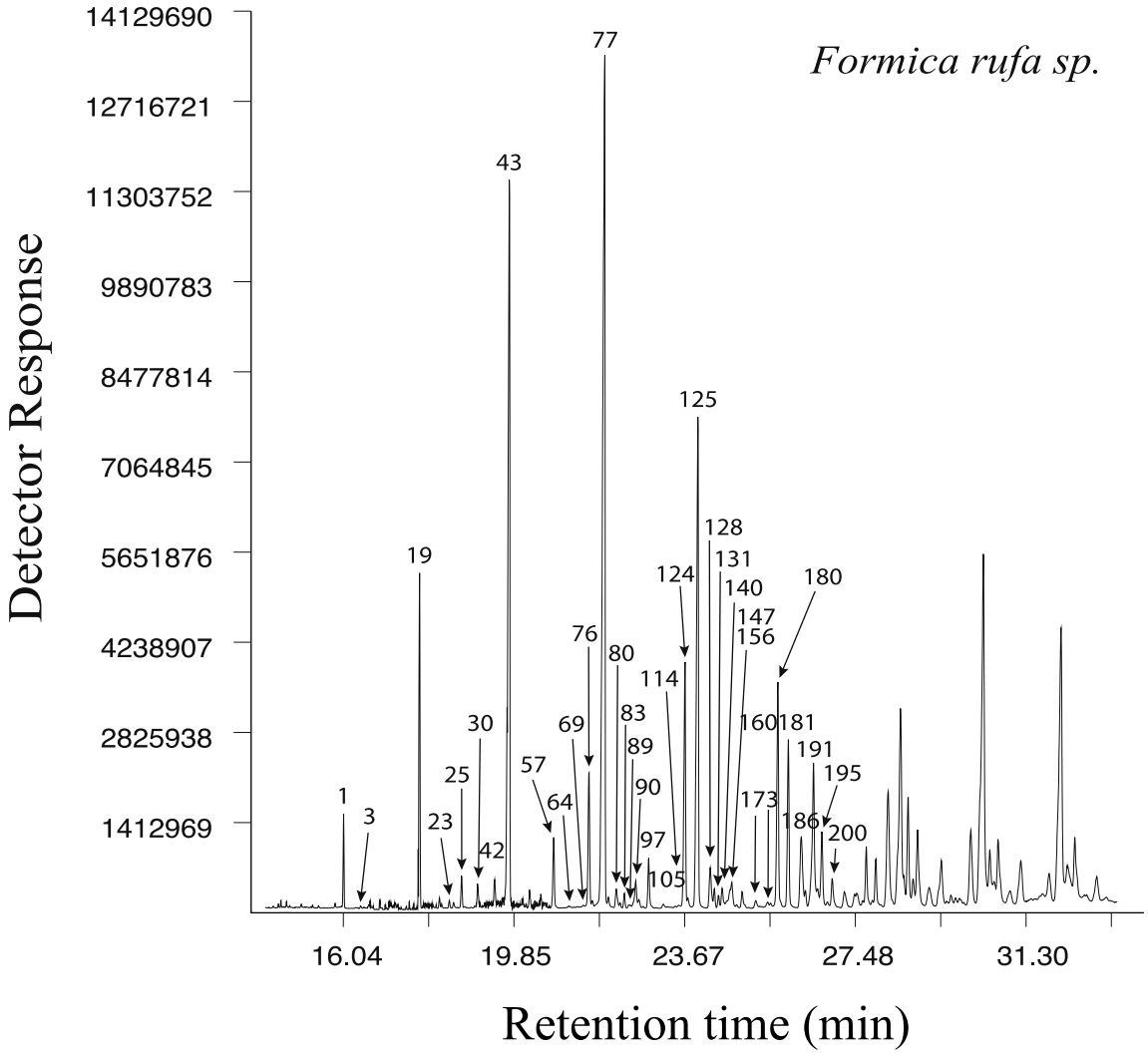
1.c


Formica argentea
Detector Response
16080270
14472243
12864216
11256189
9648162
8040135
6432108
4824081
3216054
1608027
19
42
43
57
77
79
83
85
97
125
129
132
149
152
156
177
188
191
197
198
201
202
19.85
23.67
27.48
31.30
Retention time (min)

1.d


Formica sibylla
Detector Response
17884052
16095647
14307242
12518837
10730432
8942027
7153622
5365217
3576812
1788406
16.04
19.85
23.67
27.48
31.30
Retention time (min)
1
8
9
18
19
21
28
29
30
41
42
43
53
57
75
76
77
80
90
94
124
125
129
132
137,
143,
149
151
155
181

1.e


Formica rufa sp.
Detector Response
14129690
12716721
11303752
9890783
8477814
7064845
5651876
4238907
2825938
1412969
16.04
19.85
23.67
27.48
31.30
Retention time (min)
1
3
19
23
25
30
42
43
57
64
69
76
77
80
83
89
90
97
105
114
124
125
128
131
140
147
156
160
173
180
181
186
191
195
200

1.f

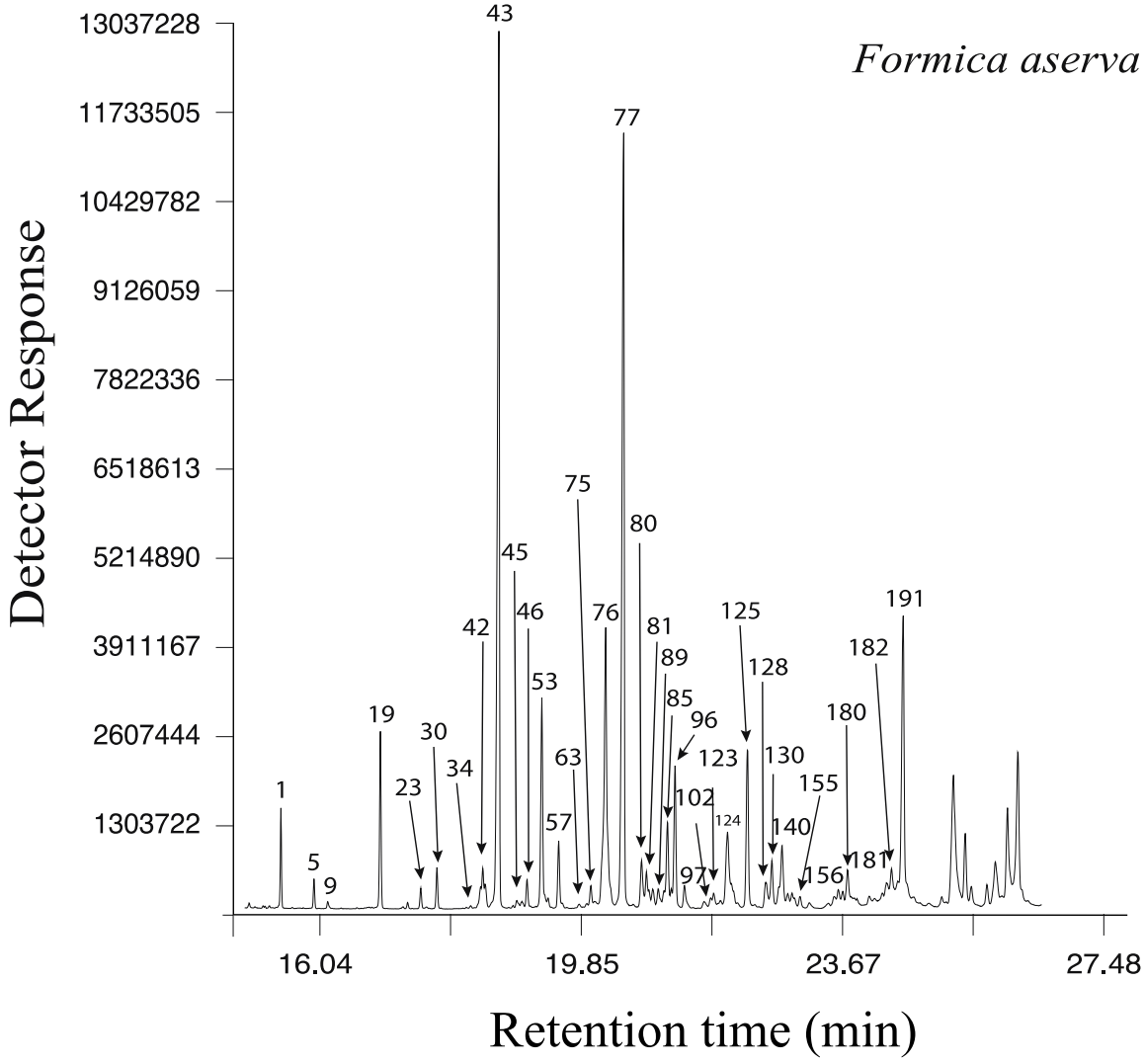
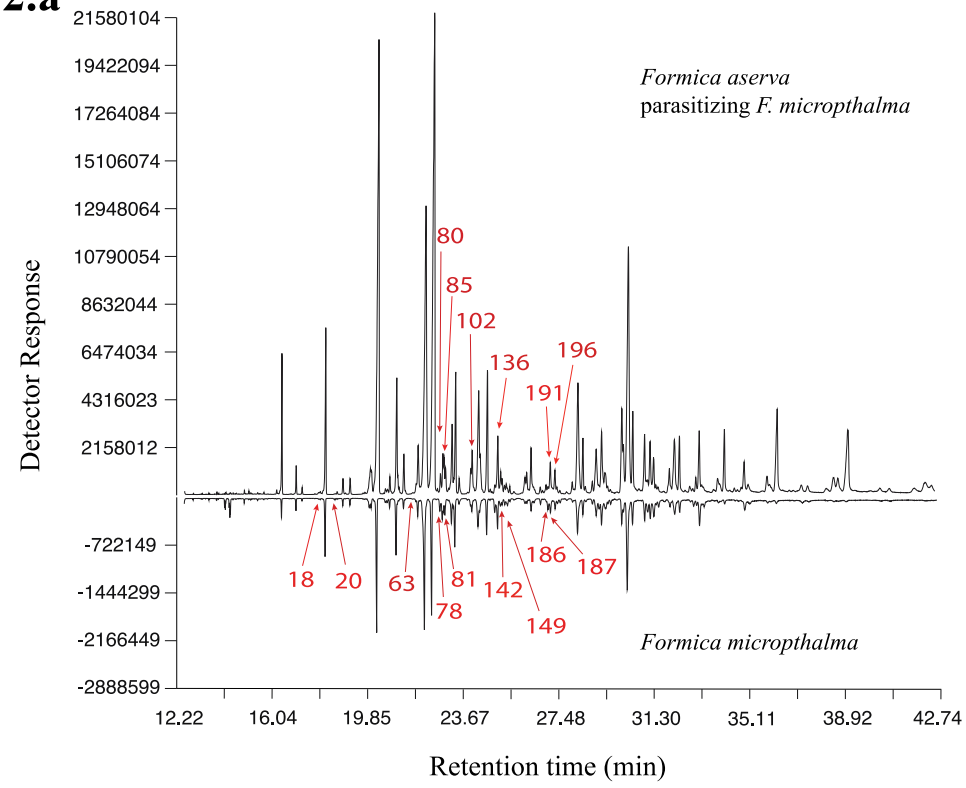
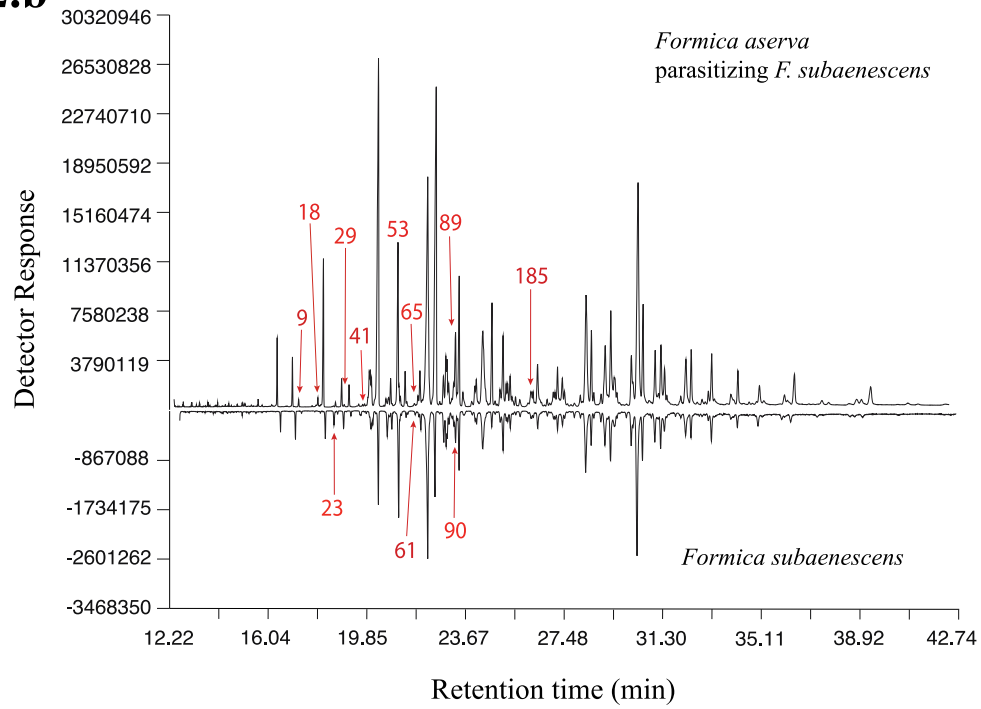


Figure 1. Representative CHC profiles of all *Formica* species with chromatograms labeled for the selected C_{21}-C_{32} identification range. Each peak label corresponds to the compound ID in Table 1. This figure excludes a representative profile from *F. subaenescens* because we did not collect chemical samples from free-living colonies of this host species.

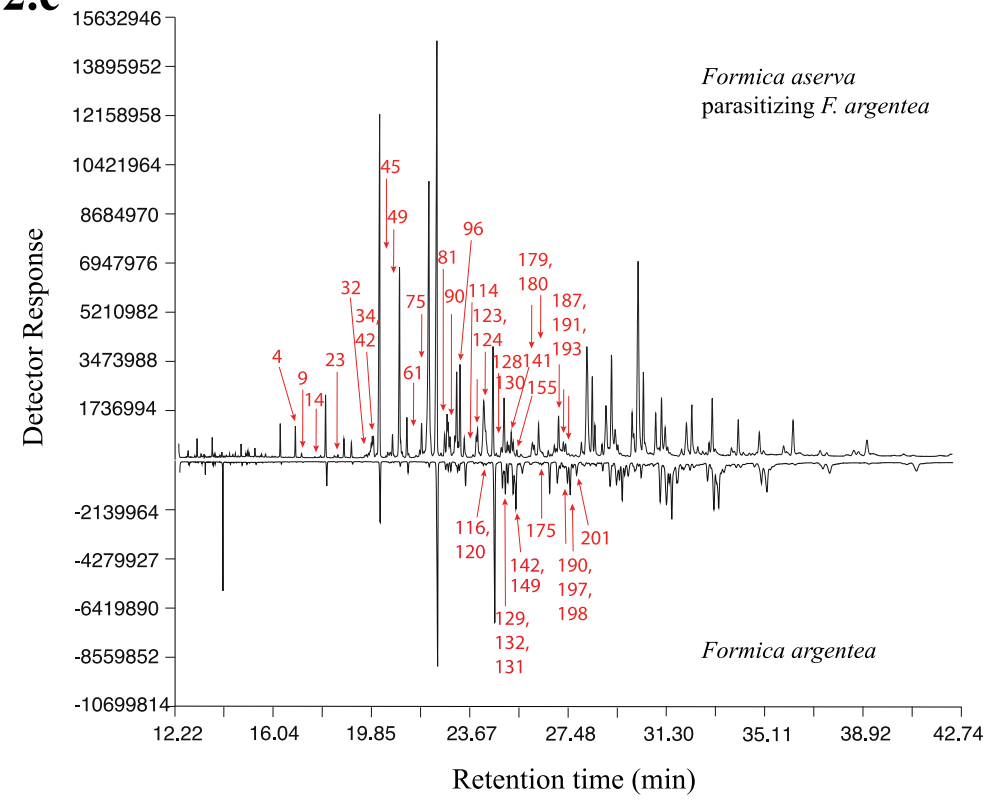
2.a


21580104
19422094
17264084
15106074
12948064
10790054
8632044
6474034
4316023
2158012
-722149
-1444299
-2166449
-2888599
Detector Response
Formica aserva
parasitizing F. micropthalma
80
85
102
136
196
191
18
20
63
81
78
142
149
186
187
Formica micropthalma
12.22
16.04
19.85
23.67
27.48
31.30
35.11
38.92
42.74
Retention time (min)

2.b


30320946
26530828
22740710
18950592
15160474
11370356
7580238
3790119
-867088
-1734175
-2601262
-3468350
Detector Response
Formica aserva
parasitizing F. subaenescens
18
29
53
89
185
9
41
65
23
61
90
Formica subaenescens
12.22
16.04
19.85
23.67
27.48
31.30
35.11
38.92
42.74
Retention time (min)

2.c


15632946
13895952
12158958
10421964
8684970
6947976
5210982
3473988
1736994
-2139964
-4279927
-6419890
-8559852
-10699814
Detector Response
12.22
16.04
19.85
23.67
27.48
31.30
35.11
38.92
42.74
Retention time (min)
Formica aserva
parasitizing F. argentea
Formica argentea
4
9
14
23
32
34,
42
45
49
61
75
81
90
96
114
123,
124
128
130
141
155
179,
180
187,
191,
193
116,
120
129,
132,
131
142,
149
175
190,
197,
198
201

2.d

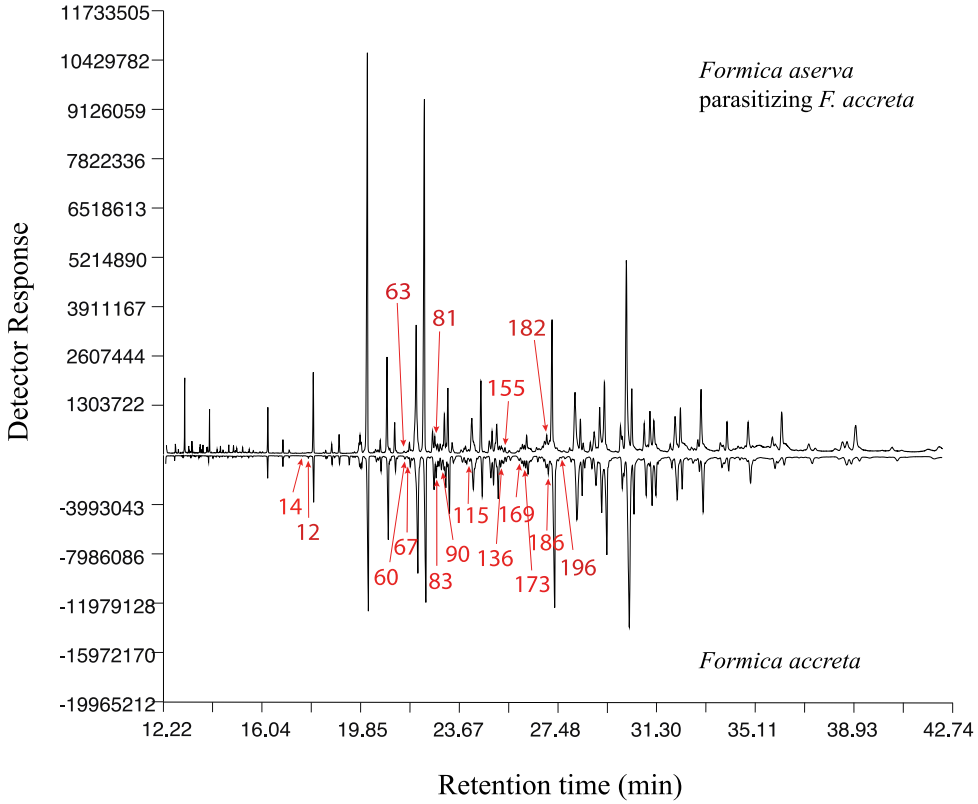

11733505
10429782
9126059
7822336
6518613
5214890
3911167
2607444
1303722
-3993043
-7986086
-11979128
-15972170
-19965212
Detector Response
12.22
16.04
19.85
23.67
27.48
31.30
35.11
38.93
42.74
Retention time (min)
Formica aserva
parasitizing F. accreta
Formica accreta
14
12
60
67
63
81
83
90
115
136
155
169
173
182
185
196

Figure 2. Representative CHC profiles of one parasite worker, *Formica aserva* (top), and one worker of its respective captive host species (bottom) with peak labels corresponding to the compound ID in Table 1. Only peaks that were different between the pairs of species are labeled in each chromatogram.

Table 2. Similarity percentages (SIMPER) of the *Formica microphthalma* species "set" including three categorical groups, *Formica aserva* (Parasite), *F. microphthalma* captives (Captive), and *F. microphthalma* free-living workers (Free). The most influential compounds contributing to the difference between the categories are listed for each pairwise comparison along with their proportional contribution ± standard deviation and their cumulative contribution to the dissimilarity we observed.

	Influential Compounds	Contribution	SD	Cumulative Contribution
	Parasite vs Captive			
1	C_{27}	0.068	0.050	0.204
2	C_{27} monoene	0.041	0.029	0.326
3	C_{25}	0.035	0.023	0.431
4	C_{29}	0.017	0.011	0.483
5	3 + 5 Me C_{25}	0.015	0.016	0.529
6	11 + 13 Me C_{25}	0.009	0.011	0.558
7	C_{29} monoene	0.009	0.007	0.585
8	3 + 7 Me C_{29}	0.009	0.006	0.612
9	C_{23}	0.008	0.005	0.636
10	3 + 7 Me C_{27}	0.008	0.004	0.659
11	C_{27} diene	0.007	0.004	0.681
12	C_{28} monoene	0.007	0.005	0.704
	Parasite vs Free			
1	9 + 11 Me C_{23}	0.113	0.090	0.172
2	C_{27}	0.096	0.049	0.318
3	C_{27} monoene	0.074	0.045	0.431
4	C_{25}	0.046	0.024	0.502
5	11 + 13 Me C_{25}	0.042	0.038	0.566
6	C_{29} monoene	0.023	0.009	0.601
7	C_{29}	0.021	0.010	0.633
8	C_{28} monoene	0.019	0.007	0.661
9	10 + 11 + 12 + 13 Me C_{24}	0.016	0.015	0.685
10	3 + 5 Me C_{27}	0.013	0.002	0.705
	Captive vs Free			
1	9 + 11 Me C_{23}	0.108	0.089	0.172
2	C_{27} monoene	0.074	0.045	0.289
3	C_{27}	0.042	0.034	0.356
4	11 + 13 Me C_{25}	0.041	0.031	0.422
5	C_{25}	0.028	0.020	0.466
6	C_{29} monoene	0.023	0.009	0.502
7	C_{28} monoene	0.019	0.008	0.532
8	3 + 7 Me C_{29}	0.018	0.006	0.561
9	3 + 5 Me C_{25}	0.016	0.015	0.586
10	10 + 11 + 12 + 13 Me C_{24}	0.016	0.015	0.612
11	C_{29}	0.016	0.016	0.637
12	3 + 5 Me C_{27}	0.013	0.004	0.657
13	3 Me C_{23}	0.012	0.005	0.676
14	13 Me C_{27}	0.011	0.009	0.694
15	C_{25} monoene	0.010	0.007	0.711

Table 3. SIMPER of the *Formica accreta* species "set" with influential compounds contributing to the difference between the categories for each pairwise comparison along with their proportional contribution ± standard deviation and their cumulative contribution to the dissimilarity we observed.

	Influential Compounds	Contribution	SD	Cumulative Contribution
	Parasite vs. Captive			
1	C_{27}	0.104	0.050	0.200
2	C_{25}	0.087	0.032	0.367
3	11,15 DiMe C_{31}	0.039	0.027	0.443
4	C_{27} monoene	0.038	0.024	0.516
5	C_{29}	0.019	0.016	0.554
6	C_{29} monoene	0.016	0.011	0.584
7	3 Me C_{25}	0.016	0.010	0.614
8	11,15 DiMe C_{29}	0.014	0.007	0.642
9	3 + 7 Me C_{29}	0.013	0.008	0.666
10	11 + 13 Me C_{27}	0.013	0.007	0.690
11	C_{28} monoene	0.011	0.007	0.712
	Parasite vs Free			
1	C_{27}	0.139	0.045	0.169
2	C_{25}	0.122	0.028	0.317
3	11 + 13 Me C_{27}	0.072	0.014	0.405
4	11,15 DiMe C_{29}	0.055	0.015	0.473
5	13 + 15 Me C_{29}	0.040	0.028	0.522
6	11,15 DiMe C_{31}	0.031	0.020	0.559
7	C_{27} monoene	0.030	0.017	0.596
8	7,11 DiMe C_{27}	0.028	0.017	0.630
9	11,15 DiMe C_{27}	0.027	0.007	0.663
10	11 + 13 + 15 Me C_{29}	0.027	0.034	0.695
11	C_{29}	0.023	0.009	0.723
	Captive vs Free			
1	C_{27} monoene	0.065	0.021	0.093
2	11 + 13 Me C_{27}	0.060	0.015	0.179
3	11,15 DiMe C_{29}	0.042	0.017	0.239
4	13 + 15 Me C_{29}	0.040	0.028	0.297
5	C_{27}	0.036	0.021	0.349
6	C_{25}	0.036	0.018	0.400
7	11,15 DiMe C_{31}	0.032	0.019	0.445
8	11 + 13 + 15 Me C_{29}	0.030	0.025	0.488
9	7,11 DiMe C_{27}	0.025	0.017	0.523
10	11,15 DiMe C_{27}	0.022	0.008	0.555
11	C_{29} monoene	0.020	0.012	0.584
12	C_{29}	0.018	0.022	0.609
13	C_{28} monoene	0.017	0.005	0.633
14	3 Me C_{25}	0.016	0.008	0.656
15	3 + 7 Me C_{29}	0.015	0.008	0.677
16	10,14 DiMe C_{28}	0.015	0.006	0.698
17	C_{25} monoene	0.012	0.024	0.715

Table 4. SIMPER of the *Formica argentea* species "set" with the most influential compounds contributing to the difference between the categories for each pairwise comparison along with their proportional contribution ± standard deviation and their cumulative contribution to the dissimilarity we observed.

	Influential Compounds	Contribution	SD	Cumulative Contribution
	Parasite vs Captive			
1	C_{27} monoene	0.081	0.033	0.130
2	C_{25}	0.053	0.022	0.216
3	C_{29}	0.053	0.029	0.301
4	C_{27}	0.049	0.034	0.380
5	5,15 DiMe C_{29}	0.030	0.018	0.428
6	C_{29} monoene	0.030	0.010	0.475
7	13 + 15 Me C_{29}	0.021	0.011	0.509
8	7,15 DiMe C_{29}	0.021	0.010	0.543
9	5,13 + 5,15 DiMe C_{31}	0.019	0.010	0.572
10	13 + 15 Me C_{31}	0.018	0.011	0.602
11	C_{28} monoene	0.018	0.008	0.631
12	7 Me C_{29}	0.016	0.010	0.656
13	3 + 7 Me C_{29}	0.014	0.005	0.678
14	C_{31}	0.013	0.007	0.698
15	3 Me C_{25}	0.012	0.023	0.718
	Parasite vs Free			
1	5,15 DiMe C_{29}	0.099	0.028	0.122
2	C_{27} monoene	0.093	0.025	0.236
3	5,13 + 5,15 DiMe C_{31}	0.070	0.023	0.321
4	C_{27}	0.069	0.039	0.406
5	C_{25}	0.066	0.024	0.486
6	5,9,13 TriMe C_{31}	0.046	0.015	0.543
7	7,15 + 7,17 DiMe C_{31}	0.035	0.012	0.586
8	7,15 + 13,15 DiMe C_{29}	0.032	0.008	0.626
9	C_{29} monoene	0.032	0.009	0.665
10	C_{29}	0.028	0.022	0.700
	Captive vs Free			
1	5,15 DiMe C_{29}	0.069	0.034	0.120
2	C_{27}	0.057	0.042	0.219
3	5,13 + 5,15 C_{31}	0.051	0.025	0.308
4	C_{29}	0.048	0.031	0.391
5	5,9,13 TriMe C_{31}	0.046	0.015	0.471
6	7,15 + 13,15 DiMe C_{29}	0.032	0.008	0.527
7	C_{27} monoene	0.027	0.053	0.574
8	7,15 + 7,17 DiMe C_{31}	0.025	0.014	0.618
9	7,15 DiMe C_{29}	0.021	0.010	0.654
10	C_{25}	0.016	0.008	0.682
11	3 Me C_{25}	0.012	0.023	0.703

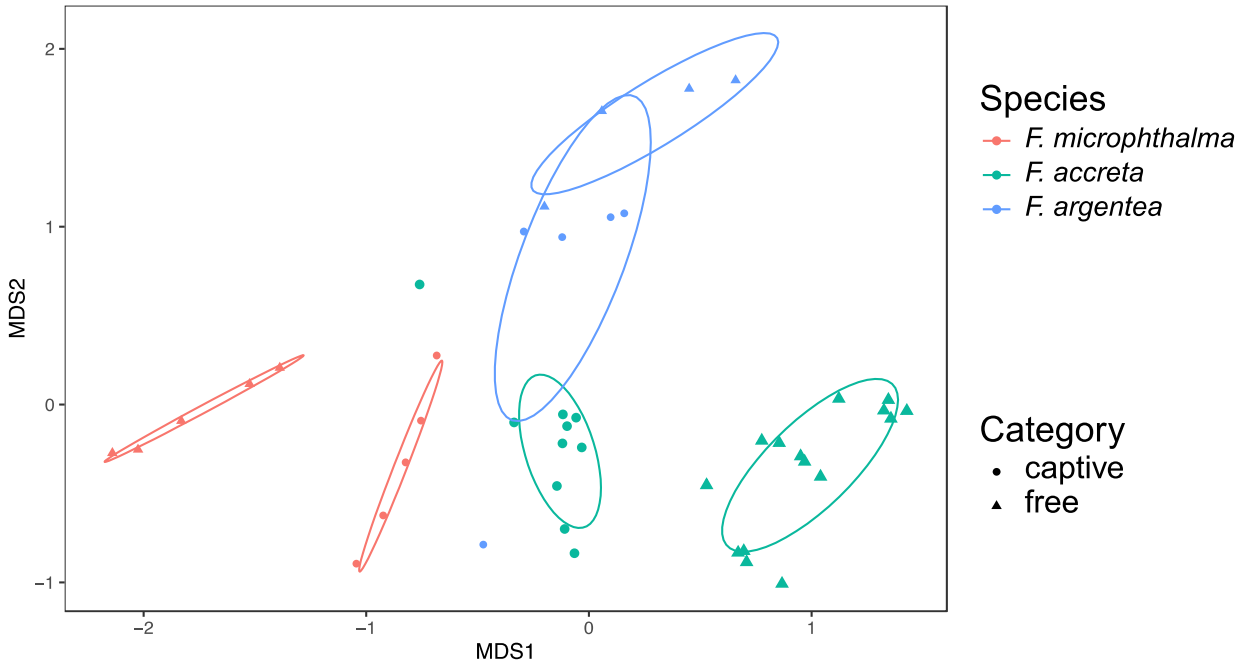


Figure 3. NMDS (Non-metric multidimensional scaling) plot of individual *Formica* workers that are captive hosts (captive; circles) and free-living hosts (free; triangles) based on the relative proportions of the detected peaks in their cuticular hydrocarbon profiles (C_{21}-C_{32}).

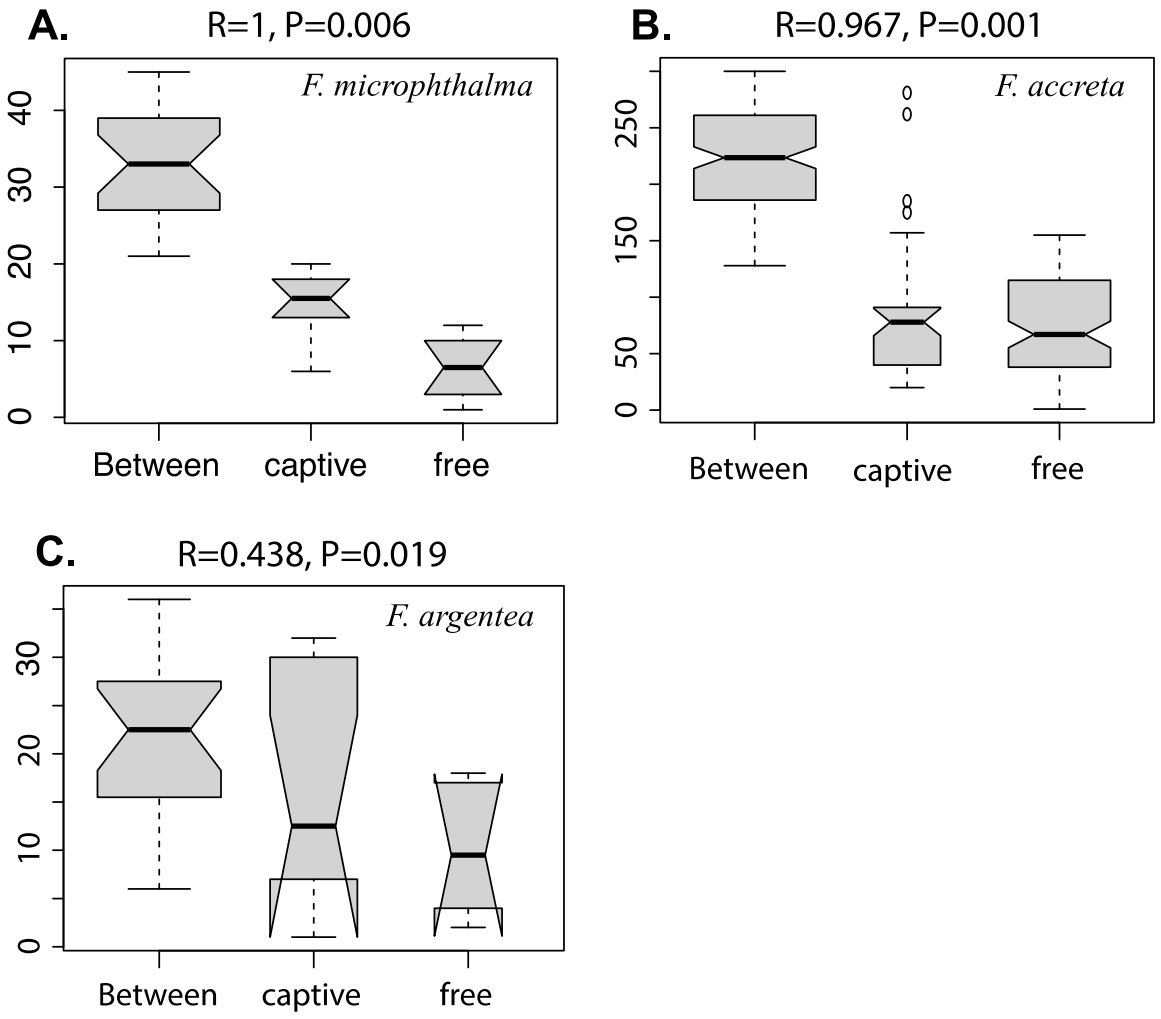


Figure 4. ANOSIM (Analysis of Similarity) results showing the degree of dissimilarity in regard to CHCs within and between the categories of *Formica*; free-living host (free) and captive host (captive). The y-axis shows the dissimilarity ranks and the R value represents the test statistic ranging from -1 to 1 with values close to one showing more dissimilarity between the groups than within them.

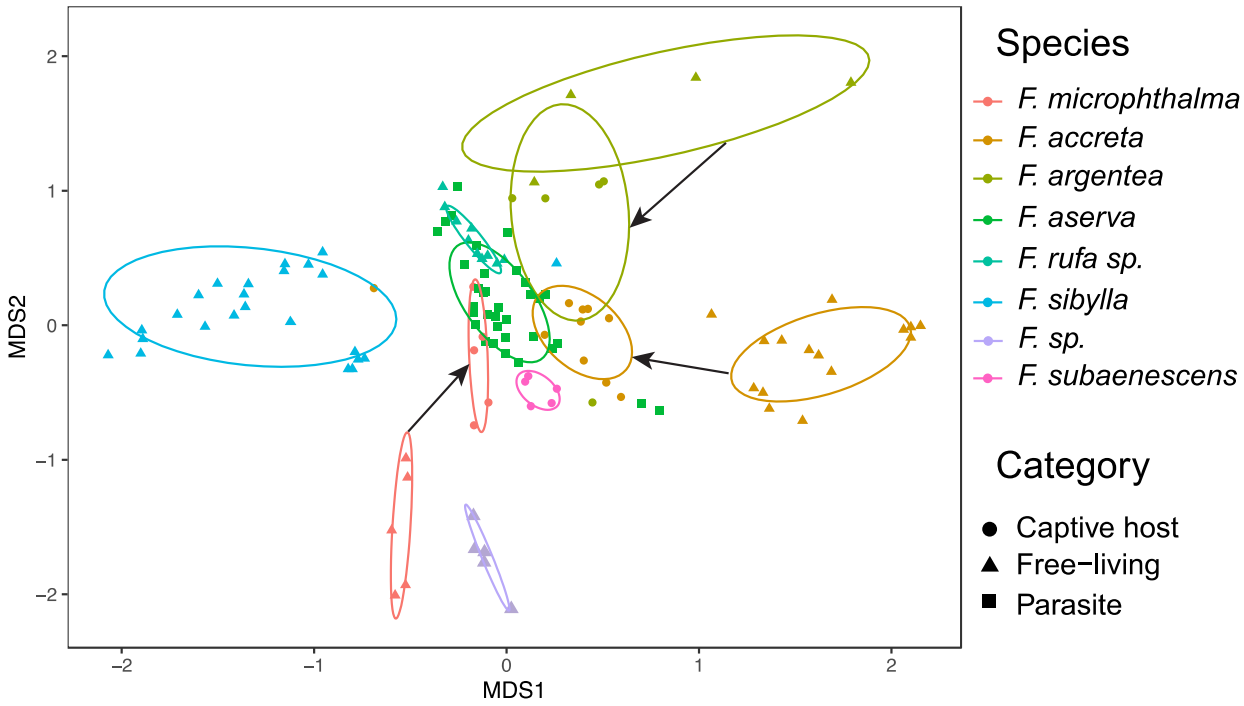


Figure 5. NMDS (Non-metric multidimensional scaling) plot of individual workers from all representative *Formica* species and categorical designations: captive hosts (circles), free-living (either host or non-host species)(triangles), and parasites (squares), based on the relative proportions of the detected peaks in their cuticular hydrocarbon profiles (C_{21}-C_{32}). Arrows show the direction with which CHC profiles change as a result of parasitism with all groups shifting to become more similar to the *F. aserva* (parasite) CHC profiles.

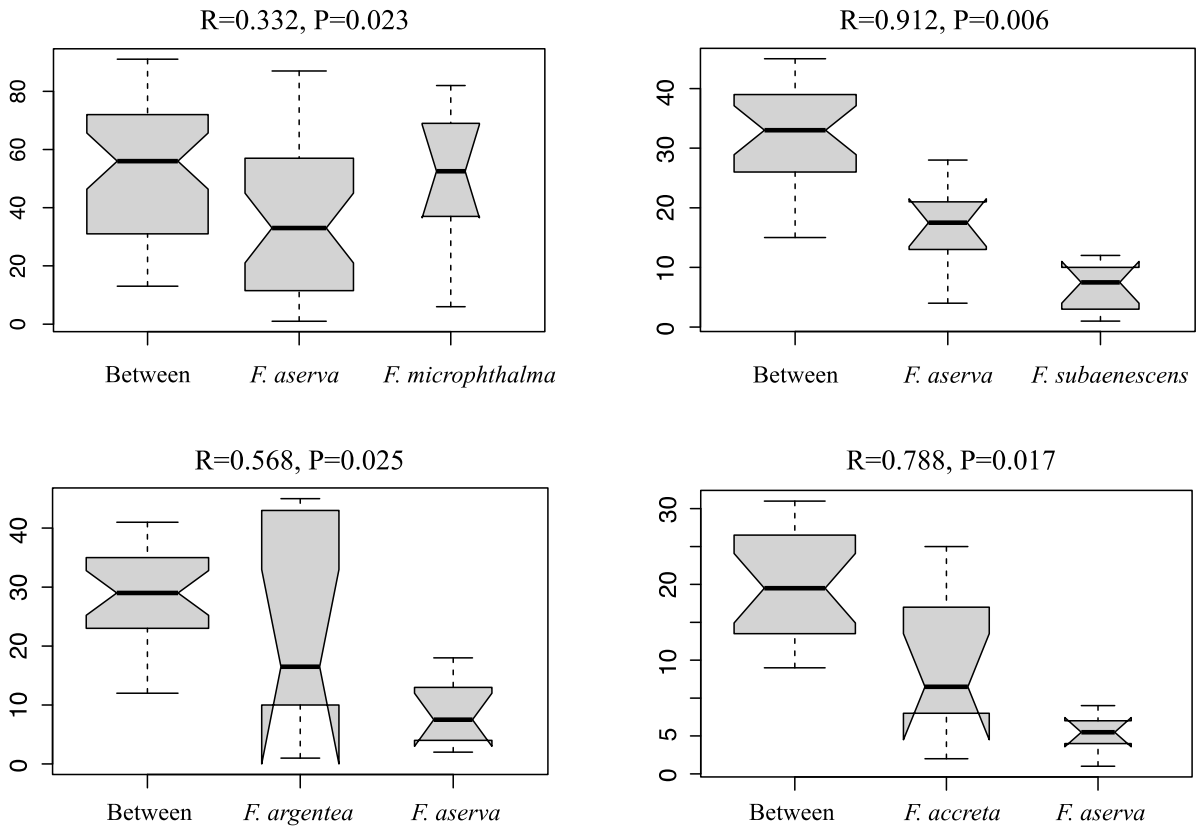


Figure 6. ANOSIM of the relative proportion of CHCs recorded for captive host species and their parasitic colony mates. Pairwise comparisons depict dissimilarity rankings between *F. aserva* workers from one colony and their representative captive species.

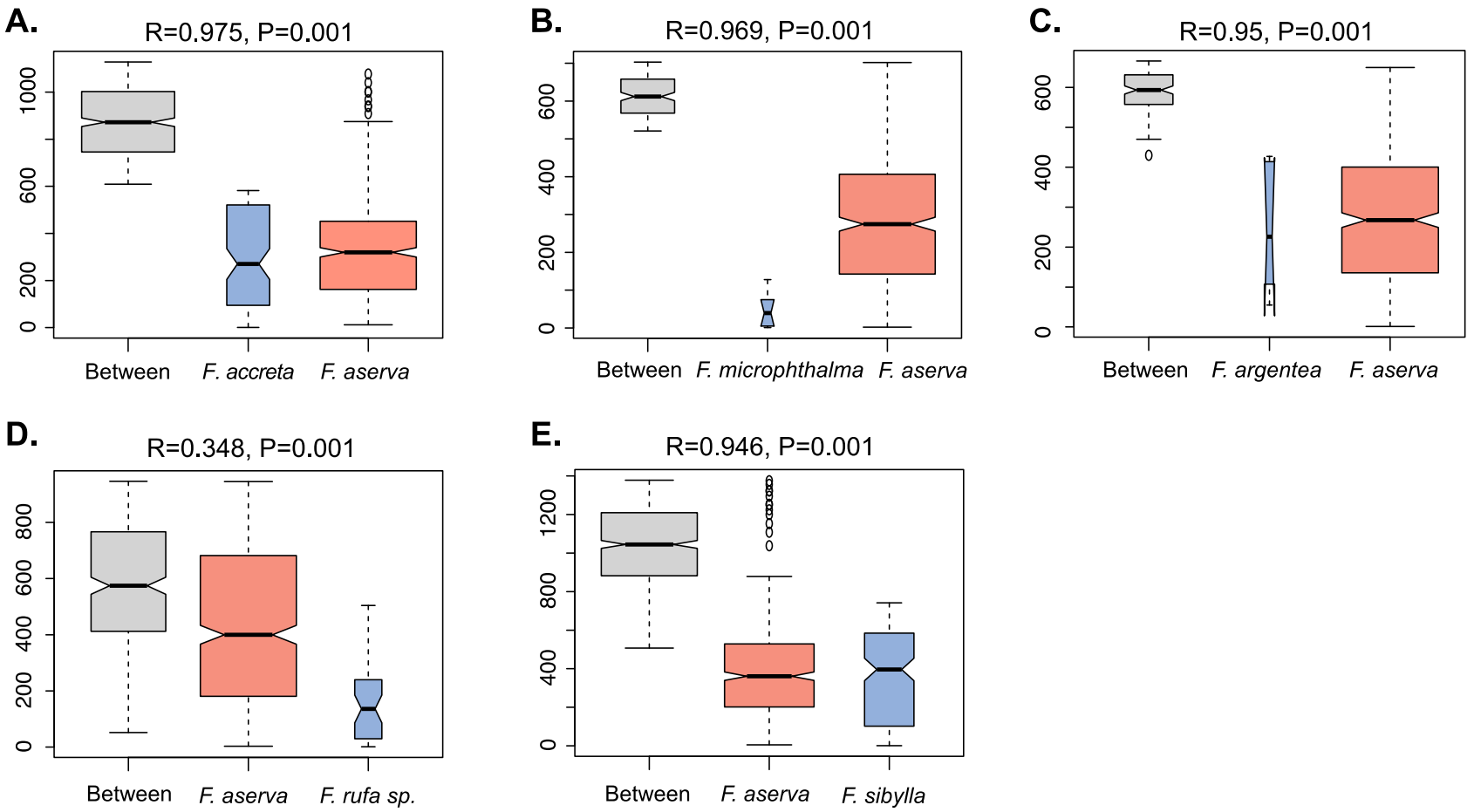


Figure 7. ANOSIM results showing the pairwise comparisons of *F. aserva* with each free-living *Formica* (host and non-host) in our study. Comparisons show the dissimilarity ranks between parasites and free-living *Formica* with each plot showing a significant difference between the CHCs of each group. The R values of all comparisons show a high degree of dissimilarity (values close to 1) with the exception of *F. rufa sp.* which appears less dissimilar from *F. aserva* (R closer to 0).

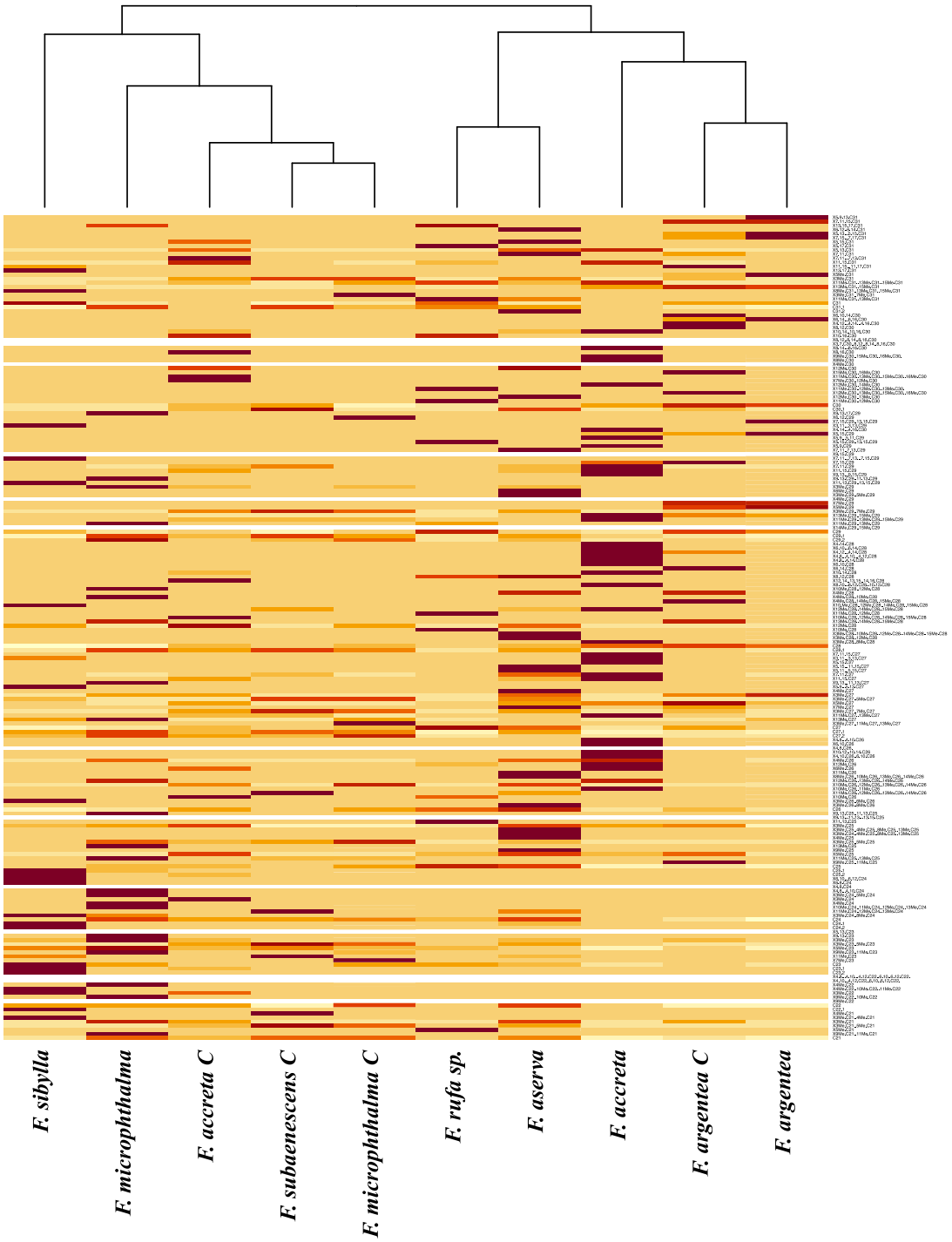


Figure 8. Heatmap representing *Formica* species including the parasite (*F. aserva*), non-host species (*F. sibylla* & *F. rufa sp.*), and free-living and captive host species (captive hosts have a "C" after their name), clustered based on their relative proportions of cuticular hydrocarbons. Compounds appear in descending order with longer chain compounds appearing at the top of the figure. Dark red bars indicate a higher amount of the respective compound; light yellow indicates a low amount, and shades in between represent a medium intensity of the proportion of CHCs present for each species.

Table 5. Average Dissimilarity between groups for each host species "set" according to SIMPER analysis.

Species	Average Dissimilarity (%)		
	Parasite + Captive	Parasite + Free-living	Free-living + Captive
F. accreta	51.7	82.1	69.8
F. microphthalma	33.1	65.6	63.0
F. argentea	62.3	81.5	57.7

Chapter 3

Sheep in Wolf's Clothing: Chemical deception strategies in host-parasite interactions of the facultative kidnapper ant, *Formica aserva*, with its *Formica* hosts

1.0 Introduction

Communication is necessary within social groups because they rely on the effective transfer of information for various aspects of group living, including delineation of group membership, coordinating tasks, and establishing and identifying the role of an individual in the society. Although there are many modes of communication, chemical communication is believed to be the most widespread modality for information transfer in animals (Candolin, 2003). The critical role of chemical communication in social insects has long attracted researchers to these highly social animals that display unique examples of group cohesion and kin recognition. As a rule, social insects use chemical signatures to form their colony identity, which they use to accept or reject individuals bearing similar or different labels from their own (Tsutsui, 2004). These colony recognition systems have evolved high levels of accuracy, thus allowing individuals to maintain the integrity of the society (Richard & Hunt, 2013), and guard against 'cheaters' that seek to exploit colony resources and to ensure that altruism is directed appropriately at colony members, who are typically relatives (van Zweden & d'Ettorre, 2010).

Cuticular hydrocarbons (CHCs) are prevalent on the bodies of insects and are believed to have evolved initially for desiccation resistance and as a barrier to microbial infection (Blomquist & Bagneres, 2010). CHCs also make up a major group of heritable lipids that are highly species- and colony-specific (Breed, 2019; Lahav et al., 1998). Additionally, these compounds act as recognition cues in various Hymenoptera including polistine wasps, social bees, termites, and ants (Howard & Blomquist 1982; Lockey, 1988). In fact, some of the first behavioral evidence for the critical role of CHCs in nestmate recognition came from studies on carpenter ants, *Camponotus spp.* (Bonavita-Cougourdan et al., 1987; Morel et al., 1988) and desert ants, *Cataglyphis niger* (Lahav et al., 1998). Many studies have since focused on colony-specific CHCs that show intra- and interspecific variation in their relative proportions on the insect body, making them good candidates for distinguishing an individual's group affiliation (Lorenzi, 2003; Martin et al., 2008). For example, in the social wasp, *Polistes dominulus*, methyl-branched alkanes and alkenes were shown to be the primary chemical classes involved in nestmate recognition, not linear alkanes (Dani et al., 2001). Similarly, Krasnec & Breed (2013) found that nestmate recognition in the ant, *Formica argentea*, is not only dependent on the presence of these same hydrocarbon classes, but also their relative abundances on the cuticle and their branching and double bond positions. These correlative studies have been bolstered by experimental tests using synthetic hydrocarbons, showing that addition of specific CHCs can trigger aggression among previously amicable nestmates (Brandt et al., 2009). Phylogenetic analyses have revealed that these compound classes can be very diverse and highly species-specific, particularly with regards to dimethylalkanes (Martin & Drijfhout, 2009).

The perception of recognition cues occurs in the peripheral nervous system where probing of the antenna leads to odorant transport and detection by olfactory receptor neurons (Wicher & Miazza, 2021; Blomquist & Vogt, 2020). Individuals learn these unique colony odors shortly after eclosion, when young workers develop a reference odor based on the chemical stimuli that surround them, which then become internalized in their higher brain centers (Ozaki & Hefetz, 2014) and used as a template against which the chemical traits of encountered individuals are compared (Sherman & Holmes, 1985). When an ant is encountered that expresses CHCs that are not in the perceiver's template, aggressive rejection typically occurs. A genetic component in nestmate recognition has been found in many social insects including in the ants *Temnothorax ambiguus* and *T. longispinosus* that, after being raised in isolation, were more

accepted by their nestmates than non-nestmates from different colonies (Kleeberg et al., 2017). In *Formica rufibarbis*, the basis of nestmate recognition was found to be almost entirely based on the individual expression of chemical cues with little influence of the physical environment (Van Zweden et al., 2010). Although the majority of these compounds are products of biosynthetic processes and, thus, products of an individual's genotype (Blomquist & Bagnères, 2010; Vander Meer & Morel, 2019) once produced they can be easily transferred to an individual's environment, which includes other members of their colony and the nest substrate (Heinze et al. 1996; Brandstaetter et al., 2008;). In fact, early olfactory imprinting on recognition cues for some social insects like polistine wasps, is based almost entirely on CHCs that are on the nest substrate that a newly eclosed worker comes in contact with shortly after emergence (Gamboa et al., 1986), revealing that the transfer of hydrocarbons from colony members to substrate can greatly impact an individual's social development and colony recognition (Pfennig et al., 1983).

Understanding the processes by which individuals produce, transfer, and acquire these crucial cues in communication is important for elucidating how certain behavioral adaptations have evolved to promote chemical congruency and cohesion within a group. In ants, the hydrocarbons on the cuticle are congruent with those that are detected in the secretions of their postpharyngeal gland (PPG) (Soroker et al., 1994), a large exocrine gland located in the head near their mouthparts (Eelen et al., 2006). The PPG is unique to the Formicidae family and has been referred to as the "gestalt organ" by Soroker et al. (1994), who found that the contents of the gland are a mixture of secreted and exogenous hydrocarbons that are distributed amongst nestmates through social interactions, resulting in a uniform odor that is uniquely characteristic of the colony (Soroker et al., 1995; Dahbi et al., 1999; Boulay et al., 2000; Lenoir et al., 2001). Soroker et al. (1994) found that trophallaxis, a common behavior in which individuals exchange food, was performed even in the absence of hunger, suggesting that this behavior is not merely for feeding, but is also crucial for the purposeful transfer of hydrocarbons between nestmates. Thus, social interactions like trophallaxis and allogrooming are thought to be the primary mode by which contents of the PPG are distributed between members of a group. Despite the presence of strict chemical barriers that delineate social insect colonies, the mechanisms by which colony members learn and distribute these odors is quite flexible. This flexibility can, at times, render a colony vulnerable to parasitic species that have evolved deceptive techniques to evade host defenses and exploit a colony's resources.

Social parasites rely on other species to provide vital resources necessary for their survival. These resources can include food, brood care, nest maintenance, and defense. Paramount to the success of social parasites is their ability to evade the chemical recognition systems of their hosts, which act as a barrier to intruders (Lenoir et al., 2001). As a result, social parasites have evolved a suite of strategies for evading or manipulating the colony recognition system of their hosts. Such strategies can include being chemically insignificant (possessing few recognition compounds) to avoid host detection, the use of propaganda and appeasement substances during invasion, chemical mimicry of a host by either biosynthesis or camouflage (Lenoir et al. 2001), and chemical integration with a host species by "labeling" captives with parasite-specific cues (Włodarczyk & Szczepaniak, 2017). This latter strategy was found exclusively in the facultative kidnapper ant, *Formica sanguinea.*

Kidnapper ants are a type of social parasite that must successfully evade host defenses at multiple stages of their life history. From the invasion tactics of foundress queens to the organization of kidnapping raids and later maintenance of a cooperative mixed-species worker

population, kidnapper ants have evolved multiple types of chemical deception to ensure smooth integration of host and parasite into a single colony. Of the kidnapping species, some are obligate parasites that are completely dependent on their host for performing essential colony tasks, whereas others are facultative, depending on host species primarily for colony founding and during the early stages of the colony's lifecycle (Hölldobler & Wilson, 1990). Facultative kidnapper ants have retained the morphological and ethological characteristics necessary for independent living, allowing them to survive without a host, although they rarely do. In general, the lack of studies investigating the chemical ecology of facultative species has produced uncertainty about the evolutionary origins of chemical deception in kidnapper ants. However, these facultative species represent a transitional stage in the evolution of social parasitism (Wilson, 1971), and thus, are invaluable for understanding the evolutionary dynamics of social parasites and their hosts.

In this study, we investigate the chemical deception strategy employed by the facultative kidnapper ant, *Formica aserva*, which it uses to manipulate the recognition cues of its captive hosts. To determine the mode of chemical deception, we constructed experimental nests containing groups of isolated *F. aserva*, and others containing only their *Formica* host species, effectively separating the heterospecific nestmates from physical contact with one another. We analyzed the CHC composition of these heterospecific nestmates before and after separation to monitor changes in the CHC profiles of parasites and their hosts once they had been removed from one another and to delineate which species accounts for the majority of compounds shared in the mixed colony signature. Additionally, we tested the behavioral response of heterospecific nestmates before and after separation to determine whether the chemical changes we observed affect colony recognition. In our previous study assessing the chemical ecology of parasite and host species at our study site (Chapter 2), we found high CHC similarity between captive hosts and parasite (*F. aserva*), apparently due to acquisition of parasite CHCs by the hosts. Based on these results, we expect that *F. aserva* will experience minor changes in their CHC profiles after separation from their hosts, whereas experimental nests of isolated hosts are expected to change more extensively. As a result of these changes in the chemical signatures of parasites and hosts, we predict that the aggression between these former colonymates will also increase after a period of separation.

Here we test three hypotheses concerning the chemical ecology of facultative kidnapper ants: 1) *Formica aserva* assimilates captive hosts by labeling them with parasite-derived CHCs, 2) *Formica* hosts will recover their species-specific cues after removal from parasite contact, and 3) heterospecific nestmates will display higher aggression towards each other after a prolonged period of physical separation.

2.0 Methods

2.1 Mixed-species nest collection

We collected *Formica aserva* nests containing *Formica* host workers from the montane habitats of Sagehen Creek Field Station and the Tahoe National Forest near Truckee, CA during August 2018. Six previously GPS-marked *F. aserva* colonies and one new colony were located in fallen logs and approximately 1000 workers + brood were excavated from each colony by hand using crowbars and axes to expose brood chambers and pockets of workers. Workers, brood, and nest substrate were placed into 5-gallon buckets lined with a thin layer of Insect-A-Slip (BioQuip, USA) at the rim of each bucket to prevent the workers from escaping. The

colonies were transported to the lab and workers and brood were immediately removed from the remaining nest substrate using aspirators and forceps. Insects were then placed into clean tubs lined with Insect-A-Slip and placed on top of bricks in a second tub that functioned as a moat with soapy water. Each tub contained nesting tubes, which consisted of 28 mL round bottom glass tubes (Fisherbrand, USA) filled with 10 mL of water and a cotton ball that was inserted until saturated then covered by red film to block light. The ants were provided with a standard ant diet as food (Bhatkar and Whitcomb, 1970). The colonies were acclimated to their lab nests for 72 hours before chemical and behavioral data collection began.

2.2 Mitochondrial identification of Formica *hosts*

We collected seven colonies of the kidnapper ant, *F. aserva*, that were using one of three different host species: *F. subaenescens*, *F. microphthalma*, or *F. accreta*. We relied, primarily, on the molecular identification of *Formica* host species although conspicuous morphological characters were still used to further support these identifications. We extracted total genomic DNA from the whole bodies of two *Formica* host workers from each of the lab nests we established from field colonies. We used the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following a modified version of the manufacture's protocol for ant DNA (By: M. Branstetter; edited by: M. Tonione). Samples were stored in molecular grade, 100% ethanol (Fisher Scientific, Waltham, MA, USA). We used a forward primer, LCO1490 5' GGTCAACAAACATAAAGATATTGG 3', and reverse primer, HCO2198 5' TAAACTTCAGGGTGACCAAAAAATCA 3', to amplify a ~650bp fragment of the *cytochrome oxidase I* (CO1) mitochondrial gene by performing Polymerase Chain Reactions (PCR) in a total volume of 30µl containing 11.75µl of Master Mix (5X Reaction buffer, 1.2mM of $MgCl_2$, 300µM of dNTP, 0.5 µM of each primer, and 0.04 units of GoTaq Flexi DNA Polymerase (Promega, San Luis Obispo, CA)), 2µl of DNA, and 16.25µl of Ultrapure H_2O. The PCR conditions for the CO1 gene are as follows: 95°C for 4 min, 38 cycles of 95°C for 30 s, 45°C for 45 s, and 72°C for 45 s; and a final extension at 72°C for 5 min. We verified gene amplification on 1% agarose gels and purified the PCR products with ExoSAP-IT (Applied Biosystems, USA) in a 6µl reaction volume containing 1µl of reagent and 5µl of post-PCR product following the manufacturer's temperature protocol. Purified samples were sent to the UC Berkeley Sequencing Facility and sanger sequencing was performed from both directions.

We edited and aligned our sequences using Geneious Pro v.9.0. (Biomatters Ltd, Aukland, New Zealand). We created assemblies of our sequences and the CO1 sequences from *Serviformica* species produced by Torres et al. (2018) by mapping all sequences to one of our *Formica* sequences as a reference, producing a ~680bp alignment. We used IQ-TREE to build a maximum likelihood tree inferred from our combined alignment of *Formica spp.*. We used a *Camponotus atrox* sequence derived from GenBank (CO1: KT159775) and a *Polyergus breviceps* sequence from Torres et al. (2018) as outgroups to root our phylogeny. We determined the best nucleotide substitution model by calculating Akaike information criterion (AIC) and Bayesian information criterion (BIC) in IQ-TREE (Nguyen et al., 2015; Hoang et al., 2017) and ran our analysis using the Juke-Cantor (JC) model with default parameters including an Ultrafast bootstrap analysis with 1000 alignments and an approximate likelihood ratio test (aLRT) with 1000 replicates (Anisimova & Gascuel, 2006). The resulting maximum likelihood tree was used to identify our unknown *Formica* species based on their positions within the clades produced by the *Formica spp.* from Torres et al. (2018) (Figure S1).

2.3 Species-separation experiment

After the 72-hour acclimation period, a subset of 50 *Formica aserva* workers and 50 *Formica* host workers were collected from each colony and transferred to separate experimental nests. The experimental nests consisted of square containers (4-5/16" x 4-5/16" x 1-3/8", Pioneer Plastics Inc., USA) with ventilated lids, Insect-A-Slip lining, nesting tubes, and food. We collected behavioral data at time zero (immediately following collection from lab nest) and at 45 days after heterospecific separation into experimental nests. Chemical data were collected at time zero and 45 days post-separation.

To assess the colony recognition behaviors of host and parasite workers before and after separation, we constructed aggression assay arenas where heterospecific nestmates would be introduced to each other under a Canon digital SLR (Canon, USA) for recording the behaviors. We made the aggression assay arenas by connecting two 6-well tissue culture plates (Falcon, USA) so that each of the wells aligned with the opposite well on the other plate when they were joined together and closed like a book. Immediately following the separation of parasite and host workers into different experimental nests, six *F. aserva* workers and six of their *Formica* host nestmates were collected, one by one, and placed into the individual wells of the aggression arena, with *F. aserva* workers placed into the wells of the first plate and the *Formica* host workers placed into the wells of the second plate. Then, when closed together, the host worker from one side would fall into the opposite well containing an *F. aserva* worker. We allowed the ants to acclimate in the wells for three minutes before introducing them to their heterospecific nestmate in the opposite well.

Once introduced, we recorded the behaviors of *F. aserva* and their hosts for three minutes. We used an aggression score scale (Tsutsui et al., 2000) (Table 6) to characterize the level of aggression displayed by the workers based on a series of commonly observed behaviors that range from amicable (i.e. allogrooming, trophallaxis) to aggressive (i.e. biting, lunging, pulling). After three minutes in the arenas, the parasite and host workers were collected separately and freeze-killed in -20° C freezer for later chemical analysis.

2.4 Chemical extraction

Chemical extractions were performed using the same, optimized methods described in Chapter 2 with slight modifications due to the accessibility of specific laboratory equipment that was not available during the field extractions in our previous study. Freeze-killed specimens from the before and after separation trials were removed from -20°C and placed in 2mL screw-top GC vials (Agilent Technologies, Santa Clara, California, USA) that were filled with 200µL of chromatography grade hexanes (HPLC grade, Fisher Scientific, Fair Lawn, New Jersey, USA) and then immediately swirled for 10 minutes on a Thermolyne Roto Mix (Marshall Scientific, Hampton, New Hampshire, USA). Sample extracts were filtered through a silica gel column (200 mg silica in a glass pipette with a glass wool plug) and eluted with 1000 µL of hexane into another 2 µL GC vial which was then evaporated under a flow of nitrogen gas (Praxair, Inc., Danbury, Connecticut, USA). The dried extracts were resuspended in 100 µL of hexane and carefully swirled along the walls of the vial to collect all remaining extracts, then subsequently transferred into a 250 µL GC insert (Agilent Technologies, Santa Clara, California, USA) placed inside the original GC vial. This step was repeated with another 100 µL hexane for a total of 200 µL of hexane-suspended extract within the GC insert. Suspended extracts were evaporated for a second time under a flow of nitrogen gas and then resuspended in 20 µL of hexane with

7.5ng/ μL of n-dodecane (EMD Millipore Corp., Billerica, Massachusetts, USA) as an internal standard.

2.5 GC-MS analysis of cuticular hydrocarbons

Prepared CHC extracts were analyzed using an Agilent 7890A Gas Chromatograph (GC) paired with a 5975C Mass Spectrometer (MS) operating in electron impact ionization mode. Five μL of each sample was injected with an autosampler (Agilent 7683 series) in splitless mode with an inlet temperature of 325°C. Chemical compounds were separated using a capillary column (DB-5MS, 30 m x 0.32 mm x 0.25 μm, Agilent J&W GC columns, Santa Clara, California, USA) using a temperature program optimized for *Formica* CHCs: 50°C for 5 min, 40°C/min to 200°C and then 5°C/min to 320°C for 10 min. The MS was programmed to scan from 40-600 amu.

2.6 CHC identification

CHC identifications were performed using the same methods described in Chapter 2. A standard series of n-alkanes (C_{21}-C_{40}) was run in the GC-MS under the same temperature conditions as our samples before large sample batches and after GC-MS machine maintenance. These n-alkane series were used to detect fluctuations in retention times so that retention indices could be calculated and later used in library preparation for automated peak identification. The n-dodecane standard was also run about one for every 10 samples to monitor any fluctuations in chemical detection as well as provide a measure for standardizing peak identification.

Peak area integration and calculation was initially performed using the data analysis software "Enhanced Chemstation", G1701EA Version E.02.02 (Agilent Technologies, Santa Clara, California, USA) which was set to automatically integrate peaks with an area reject of 0, an initial peak width of 0.017, and an initial threshold of 13. Shoulder detection was turned off. All automatically detected peaks were visually assessed in a separate data analysis software "OpenChrom", Community Edition 1.3.0 (Dalton), which was used to manually adjust integration parameters to better fit our CHC data. We prepared a custom-built library of CHCs for our specific *Formica* using the adjusted area calculation under each peak, retention times, retention indices (calculated from our n-alkane series calibration based on when and under what GC-MS conditions the specific sample was run), diagnostic ions, and mass spectra. Our custom CHC library file was used to automate compound identification with the OpenChrom software. The final samples for this study were chosen based on the quality of their GC-MS output. Representative profiles from each group and species were manually spot-checked for accuracy and peak identifications were corrected across samples.

2.7 Statistical Analyses

We used R packages *vegan* and *ggplot2* to run a Nonmetric Multi-dimensional Scaling (NMDS) analysis on the 164 hydrocarbons identified across *F. aserva* and host samples. We built a matrix containing all samples, their categorical grouping (parasite/host), their treatment (before/after separation), and all recorded CHCs and calculated the relative proportions of each compound for individual samples. These proportions were plotted using the NMDS analysis to determine if there was categorical grouping of CHCs depending on whether samples were collected before or after separation. We followed this analysis by performing an analysis of similarity test (ANOSIM), using the R package *vegan* and function *anosim,* to test the significance of pairwise comparisons of the data. We used similarity percentages breakdown

(SIMPER) to calculate the average percent contribution of individual compounds to the dissimilarity observed between groups which produced a list of the most influential compounds accounting for the overall dissimilarity. We constructed bar graphs of the mean relative proportions of all compounds, highlighting the most influential, for each of the species (host and parasite) using the before and after samples within those species using the R packages *dplyr* and *ggplot2*. In order to assess the degree of change in the influential compounds before and after separation from heterospecific nestmates, we calculate the relative difference of each compound using the before and after CHC proportions and plotted these results using the same R packages. Additionally, one-way Analyses of Variance (ANOVA) and *post-hoc* tests were performed for the mean aggression scores for each colony, pre and post separation, using Microsoft Excel, version 16.23.

3.0 Results

3.1 Chemical profiles of parasite and host nestmates before and after separation

We identified a total of 164 hydrocarbons from the bodies of *Formica aserva* workers and their three host species before and after separation (Table 1). The same ambiguity in peak identification that occurred after C_{32} in our samples from Chapter 2 was also true for these samples, so we focused our analyses on compounds in the C_{21}-C_{32} chain length range. This focal range was selected based on the relatively high quality of the mass spectra and the evenness in representation of compounds that each species in this study exhibited within the range.

We first used SIMPER analysis to quantify dissimilarity in CHC profiles of *F. aserva* versus host species, both before and after separation. We found that, before separation, parasites and their captives were, on average, 42% different in regards to their CHC composition. After separation, parasites differed from their host species to varying degrees; *F. aserva* and their host species, *F. microphthalma*, became 62% different from one another after separation and *F. aserva* using *F. accreta* became 51% different (Table 2). The greatest difference post-separation was observed in the *F. aserva/F. subaenescens* species pair, which became 85% different from each other (Table 2). It should be noted that, due to the low sampling of *F. aserva* colonies using *F. subaenescens* as hosts (N=1 colony) compared to *F. accreta* and *F. microphthalma* hosts (N=2 colonies; N=4 colonies, respectively) as well as the poor quality GC-MS reads we obtained for some of the replicates of this host species, we were only able to use one representative CHC profile for the before treatment and one representative profile for the after treatment in our NMDS and SIMPER analyses and no significance testing could be conducted. The before and after *F. subaenescens* samples we included were chosen based on the high quality GC-MS output we received, which included good peak detection and clear mass spectra. Therefore, these samples were good representatives for the qualitative assessment of differences between samples of the before and after separation treatments.

Consistent with the dissimilarity percentages from our SIMPER analyses, the non-metric multidimensional scaling (NMDS) showed disparities in host and parasite CHCs before and after separation. We observed a close relationship between parasite workers and their captive host workers before separation with host samples clustering by species close to the *F. aserva* parasitic samples (Figure 1). This finding complements the results of the NMDS analyses in Chapter 2, which showed a similar grouping of captive (host) and parasite workers based on the similarity of their CHC composition. After 45 days of separation, host species became more chemically distant from the recorded CHCs of their homo- and heterospecific nestmates prior to separation.

The relative abundance of compounds represented in parasite and host profiles showed distinct differences before and after separation (Figures 2, 3, & 4). *Formica microphthalma* hosts exhibited the greatest increase in novel compounds after separation and an overall higher degree of cue diversity as a result (Figure 2a) while the *F. accreta* and *F. subaenescens* hosts showed noticeable changes post-separation but not to the same degree (Figures 3a & 4a). *Formica aserva* workers showed distinct changes to the relative abundance of influential compounds in their profile after removal from hosts but these compounds were those that were present in both before and after groups and, overall, *F. aserva* did not experience the same emergence of novel compounds post-separation as did the host species (Figures 2b, 3b, & 4b). We determined the compounds we believe to be unique to each species based on the presence of cues both during and after separation. All species (parasite and host) shared a series of specific cues with their heterospecific nestmates during cohabitation that were subsequently found to be absent within the opposite species' profile once these nestmates had been separated. The unique CHCs found across all host species were monomethyl- and dimethyl alkanes and only one of these "unique" compounds was found in common between *F. microphthalma* and *F. accreta* hosts (Peak 36: 3-Me$C_{24)}$ (Figures 2a, 3a, 4a). Interestingly, we observed distinct differences in the chemical classes that were considered to be unique to *F. aserva* relative to the unique compounds of host species. Half of the parasite-specific compounds from *F. aserva* profiles were monoenes and dienes while the other half comprised monomethyl mixtures and linear alkanes (Figures 2b, 3b, 4b). Across all *F. aserva* colonies, regardless of host species, parasite workers shared two "unique" compounds C_{27} & C_{29} dienes in common.

3.2 Species related differences before and after separation

We performed pairwise comparisons of *F. aserva* and their *F. microphthalma* hosts using ANOSIM to test the significance of the CHC differences within and between these species before and after being separated from their heterospecific nestmates. We were unable to perform these same tests on parasites and their *F. accreta* and *F. subaenescens* hosts because we collected fewer parasite colonies containing these host species, resulting in a limited sampling that was not sufficient for statistical testing.

The results of our pairwise comparison between *F. aserva* and *F. microphthalma* showed that host workers were significantly different from parasites after separation (R=0.727, P=0.001; Figure 5a). This dissimilarity was greater than what was observed prior to separation (R=0.594, P=0.03; Figure 5b) based on comparisons of the R test statistic, which represents the degree of dissimilarity between the groups, with values close to one indicating more dissimilarity.

Not only were the *F. microphthalma* host CHCs found to be more dissimilar from parasite CHCs after separation, but the profiles of these same hosts also became significantly different from their initial CHC composition (prior to separation), when they were cohabitating with *F. aserva* (R=0.446, P=0.004) (Figure 6). The overall dissimilarity percentages that we calculated for all three of the host species in this study revealed that the *F. microphthalma* and *F. subaenescens* hosts showed higher CHC dissimilarity in comparisons of before versus after separation (*F. microphthalma*=60% different; *F. subaenescens*=69% different) whereas *F. accreta* hosts were only about 35% different from their pre-separation CHCs. However, as mentioned above, limited sampling of *F. accreta* and *F. subaenescens* hosts may contribute to these results. Regardless, our qualitative assessment of host cue divergence post-separation suggests that the CHCs of captives are influenced by the presence of parasite workers.

The qualitative comparisons of *F. aserva* CHCs before and after separation showed spatial distinction in our NMDS (Figure 1), but this difference appeared to be far less than what was observed in the host species. We also found that *F. aserva* profiles became significantly different from the original colony profile after separation from their host species (R=0.332, P=0.008) (Figure 7) but the magnitude of this difference was less than we observed for the host profiles (refer to R statistic in Figure 5a). In sum, all samples in our study experienced shifts in their CHC composition after being separated into homospecific experimental groups, but the CHCs of host species appeared to change more than the CHCs of the *F. aserva* parasites.

3.3 Influential compounds in the observed differences of before and after CHCs

We identified the most influential compounds contributing to the differences between the before and after separation treatments of parasites and their hosts by calculating similarity percentages (SIMPER), which uses Bray-Curtis dissimilarities to compare CHCs between the groups (Tables 3, 4, 5). We also calculated the relative difference of each influential compound based on relative proportions before and after separation for each species (Figure 8). We found that the class of compounds and the degree to which they increased or decreased was different between the parasite workers and their host species counterparts.

Of the influential compounds recorded in the three host species, only methyl-branched alkanes showed an increase post-separation (Figure 8) with one exception being the slight increase in C_{23} (a linear alkane) post-separation for *F. accreta* hosts. These methyl-branched hydrocarbons were either absent or minor components in parasite profiles. Meanwhile, the influential linear alkanes and alkenes in host profiles were all found to decrease after separation.

In contrast to host changes, the influential compounds in parasite profiles were predominantly linear alkanes and alkenes, with the exception of one methyl-branched hydrocarbon, which was present in all parasite profiles regardless of host species (3-MeC_{25}). Similar to their hosts, parasites experienced a decrease in most of their influential linear alkanes post-separation while conversely showing an increase in all influential alkenes as well as 3-MeC_{25} after removal from hosts. An inverse relationship in the relative difference of shared influential compounds between parasites and their hosts exists for all shared alkenes between parasite and host workers as well as 3-MeC_{25}. That is, all compounds that decreased in hosts after removal increased in the parasites after separation.

All species showed a greater magnitude of difference in the compounds that increased after separation compared with those that decreased. Across all of the host species, methyl-branched alkanes increased: *F. microphthalma* ($mean_{rel\ diff} \pm sd = 484.41 \pm 98.64$) (Table 4), *F. accreta* ($mean_{rel\ diff} \pm sd = 224.85 \pm 147.22$) (Table 3), and *F. subaenescens* ($mean_{rel\ diff} \pm sd = 327.03 \pm 91.35$) (Table 5). The compounds that decreased, including the alkenes, linear alkanes, and 3-MeC_{25}, were those that were also found in parasite profiles. These compounds decreased at lower rates ($mean_{rel\ diff} \pm sd = -60.98 \pm 23.57$) than the rate of increase for the influential methyl-branched hydrocarbons across all of the host species. This trend was consistent for *F. aserva* samples as well which showed a larger percent increase in the influential compounds that increased post separation ($mean_{rel\ diff} \pm sd = 187.35 \pm 82.92$) in comparison with those that decreased ($mean_{rel\ diff} \pm sd = 33.95 \pm 15.58$).

3.4 Nestmate aggression before and after separation

The results of the aggression assays between host and parasite before and after separation showed distinct behavioral differences displayed by both *F. aserva* workers and host workers in

each of the seven colonies that we assayed. Before separation, parasite and host workers interacted in the arenas amicably, exhibiting behaviors that were non-aggressive, such as antennation and trophallaxis (score of 1; Table 6). After 45 days of separation, heterospecific nestmates displayed a range of behaviors that were either avoidant or aggressive (biting, lunging, pulling; scores 2-4; Table 6) and the mean aggression scores between parasites and hosts post-separation for colonies using *F. microphthalma* were (mean ± sd) 2.67 ± 1.01, colonies using *F. accreta* were 2.17 ± 1.27, and colonies using *F. subaenescens* were 3.0 ± 1.1. The results of our one-way ANOVA comparing the effect of pre- and post-separation worker aggression revealed a significant difference in at least two of the colony's aggression scores as a result of heterospecific removal ($F_{(1,82)} = 84.563$, $p<0.0001$). *Post hoc* t-tests revealed that all of the experimental colonies, with the exception of Colony 13 using *F. accreta* hosts, showed a significant increase in the mean aggression scores between former nestmates after separation (Table 7). To test whether the level of aggression differed across host species after separation, we ran another one-way ANOVA comparing the mean aggression scores of the different host species with their *F. aserva* nestmates. We did not find a significant difference in the level of aggression displayed based on the host species ($F_{(2,4)} = 0.58$, $p = 0.602$) suggesting that the chemical changes that occurred across all species after being isolated from heterospecific contact had the same behavioral effect for the host and parasite workers.

4.0 Discussion

4.1 Parasites assimilate captive hosts by labeling them with their own recognition cues

The results of this experiment complement and extend the results reported in Chapter 2. In Chapter 2, we found that captive *Formica* hosts have CHC profiles that are more similar to their *F. aserva* parasites than they do to free-living conspecifics, and that *F. aserva* CHC profiles are fairly uniform across different colonies, even when cohabitating with different host species. These results suggest that *F. aserva* is producing CHCs that are acquired by the host workers in captivity, but the hosts have little or no effect on the *F. aserva* profiles. In the present study, we performed an experiment to test whether CHC profiles and/or colony recognition behavior changes when hosts and parasites are separated and maintained in single-species colonies. If *F. aserva* has evolved a strategy to assimilate host workers by promotion of a colony signature that is dominated by parasite-specific cues for recognition, we would expect the hosts to lose parasite-specific CHCs when kept in isolation, and these chemical changes could translate into changes in in colony recognition behavior. After separation, were able to identify specific changes in the presence and abundance of hydrocarbons extracted from parasites and their hosts, which allowed us to delineate the specific chemical deceptive strategy that *F. aserva* has evolved to maintain cohesion with their captives.

Although the chemical differences we observed in the host species of this experiment help to explain the differences we observed between captive (parasitized) and free-living host species in wild populations, the degree to which the parasitic CHC profile changed after removal from their hosts was essential in testing our first hypothesis: that *F. aserva* assimilates host workers by labeling them with parasite-specific CHCs. Here, comparison of the host CHC profiles before and after separation revealed that, after separation, the host CHC profiles changed more than the *F. aserva* profiles. This finding suggests that the mixed colony gestalt we observed is more representative of *F. aserva*-specific cues than it is of their hosts. Thus, parasite workers appear to dominate the mixed colony signature with their own cues by disproportionately

labeling captive workers with parasite odors, partially masking species- and colony-specific odors produced by the host species.

Our first hypothesis is further supported by our quantitative analyses of the relative proportions of compounds within parasite and host profiles before and after separation. These show that the number and abundance of methyl-branched alkanes on the host increase after separation from parasites, while the more parasite-specific cues, including many alkenes, decrease. The parasites, on the other hand, did not possess any of the influential branched hydrocarbons that were found to increase on hosts after removal, suggesting that those branched hydrocarbons are likely species- and/or colony-specific cues of their hosts that had been dampened by larger quantities of cues transferred during physical interaction with parasite workers.

Examples of similar, parasite-centric, deceptive strategies have been found for other facultative social parasites, including the paper wasp, *Polistes biglumis*, whose foundress queens often usurp conspecific nests as an alternate strategy to independently colony founding (Cervo & Dani, 1996). Lorenzi et al. (2011) found that this behavior has resulted in higher proportions of methyl-branched alkanes on foundresses queen bodies, which they use to mark their usurped host nests. Newly eclosed workers of those host nests will imprint on the parasite queen's cues and internalize those cues as their own colony signature, ensuring the acceptance of the parasitic queen (Lorenzi et al., 2011). Similarly, a recent study on the European facultative kidnapper ant, *F. sanguinea*, revealed that parasite workers mask the recognition odors of their captives through the mechanical transfer of parasite-specific cues onto colony members, rather than mimicking the specific odors of those captives (Włodarczyk & Szczepaniak, 2017). Włodarczyk (2016) also found that *F. sanguinea*, through the promotion of their own recognition cues, effectively reduced the CHC variation within the mixed species colony, which would otherwise be overwhelmed by the disparate cues produced by hosts species originating from a variety of different colonies. Our own analyses revealed an increase in CHC diversity in hosts after separation which compliments what Włodarczyk (2016) found in *F. sanguinea*, as well as our previous results from the chemical data we collected from wild colonies of host species, namely, free-living host species have higher cue diversity than captive hosts.

Understanding the overall evolution of these chemically deceptive techniques in kidnapper ants requires specific knowledge about the similarities and differences between facultative and obligate kidnapper ants. The techniques used by facultative parasites to assimilate their hosts while also reducing species-related odor differences appear to be somewhat different than what has been observed in obligate kidnapper ants. Obligate species invade and maintain cohesion with their hosts primarily through the mimicry of host specific cues (Savolainen & Deslippe, 1996; Mori et al., 2001) This mimicry can be achieved in two ways: 1) by manually acquiring host cues via close contact and eliciting social interactions that promote CHC transfer between heterospecifics and 2) by evolving similar biosynthetic pathways for producing and expressing the same CHCs that are involved in the nestmate recognition of their hosts (Lenoir et al., 2001). Although some species, like inquiline (workerless) social parasites, demonstrate a high degree of chemical mimicry of their hosts (Dettner & Liepert, 1994), obligate kidnapper ants, such as *Polyergus breviceps*, have been shown to only partially mimic their hosts while maintaining some degree of their own genus-specific cues (Torres & Tsutsui, 2016). The same has been reported for other *Polyergus* species as well as for the obligate kidnapper ant, *Rossomyrmex minuchae* (Habersetzer & Bonavita, 1993; Liu et al., 2003; Errard et al., 2006). Although obligate kidnappers do not perfectly match the cues of their hosts, maintaining some of

their own species-specific cues in their chemical signature, the degree of cue matching that occurs between obligate parasites and their hosts is still believed to be driven by coevolutionary mechanisms as a result of their complete dependence on host species (Dettner & Liepert, 1994). In contrast, facultative kidnapper ants, like *F. aserva*, appear to maintain cohesion in the nest by making their captives appear more like themselves rather than chemically appearing more like their hosts. This strategy may be sufficient for facultative parasites, like *F. aserva*, which represent an intermediate stage in the evolution of kidnapping behavior, as the selective pressure for more complex strategies like chemical mimicry in host deception may be weaker due to their intermediate dependence on a host species for survival (Guillem et al., 2014).

Systems in which the social parasite achieves colony integration by labeling hosts with parasite odors should be strongly affected by the relative numbers of host versus parasites in the colony. Since *F. aserva* appears to be dominating the CHC profile of the mixed nest through the manual distribution of their own odors on captive hosts, then the relative abundance of parasite and host workers in the parasitic nest would surely affect the success of this strategy, which is likely dependent on physical contact. It is possible, then, that there is an asymmetric transfer of CHCs between parasites and their hosts with a greater influence of the parasite's odor due to the greater number of *F. aserva* workers within parasite colonies. Consistent with this, colonies of the facultative kidnapper ant, *F. sanguinea*, have been reported to have more parasite workers than captive workers (Włodarczyk & Szczepaniak, 2017). Similarly, we also observed a high parasite to host ratio within the *F. aserva* colonies at our site. Comparative studies of facultative and obligate kidnapper ants have shown that kidnapper to host ratios within colonies are higher for facultative social parasites than for obligate social parasites (Savolainen & Deslippe, 1996). This makes sense when considering the far greater dependence of obligate parasites on their hosts, who perform almost all of the colony tasks (e.g. foraging, brood care, nest maintenance, defense) and thus must be present at large numbers in the colony. Facultative parasites, on the other hand, have retained the behavioral and morphological phenotypes required to live independently of hosts, which reduces their need to acquire and maintain a large host population. Captives in these nests also mainly perform intranidal tasks, which may result in the unequal exchange of CHCs between parasites and their hosts (Kharkiv, 1997; Mori et al., 2000). The different species composition observed within facultative and obligate kidnapper colonies may also explain why our study, along with others, found lower CHC variation in mixed nests while obligate kidnapper ant colonies appeared to be more variable in their CHC composition (Torres & Tsutsui, 2016). The larger population of host workers in the colonies of obligate social parasites (especially if they originate from a large number of different free-living colonies) likely translates into a greater diversity of colony recognition cues. Thus, the ability of the social parasites to overwhelm host colony recognition cues is likely more limited in colonies of obligate social parasites compared to facultative species, such as *F. aserva*. In this light, it would be interesting for future studies to perform observational or genetic studies to compare raid number and host population diversity in facultative versus obligate kidnapper ants.

4.2 Captive workers recover their species- and colony-specific CHCs after separation from parasite workers

We found that the CHC profiles of all of the host species in this study diverged from their parasitic nestmates after a prolonged period of separation. Although we observed some specific differences between the hydrocarbon profiles of cohabitating hosts and parasites before separation, as was also observed in our previous study with wild colonies, the chemical

differences between the two groups increased after separation. Thus, physical contact with parasite workers appears to be crucial for homogenizing the colony odor and increasing uniformity among members of the group.

In parallel with this finding, we also found that the CHC profiles of hosts were divergent before and after separation. Host CHCs that were extracted initially, at the time of cohabitation with the parasite, were generally less diverse than the CHC profiles after separation. This was especially true for *F. microphthalma*, which showed a striking increase in the presence and abundance of unique hydrocarbons after separation from the parasite. We also found that, after removal, hosts experienced increases in the presence and abundance of influential methyl-branched alkanes, which were not found to be present or influential in parasite profiles. Thus, it is possible that the species-specific hydrocarbons expressed by hosts were no longer dampened by close contact with their parasitic nestmates, resulting in the increase of host-specific cues and decrease in parasite-specific cues after separation. These data also suggest the intriguing possibility that *F. aserva* may be altering the CHC profiles of their captive hosts by altering the expression of genes in the CHC biosynthesis pathways. Although speculative, this hypothesis would be relatively easy to test in future studies by quantifying expression of the relevant genes in free-living versus captive host workers.

4.3 Formica aserva *and their hosts exhibit higher heterospecific aggression as a result of prolonged physical separation*

The behavioral changes we observed in our separation experiment appear to be a manifestation of the underlying changes in colony recognition cues. Hosts and parasites that were separated became more aggressive toward one another, which suggests that the host compounds that were detected post-separation were likely host-specific compounds that were potentially critical as recognition cues.

Additionally, we found that aggression was asymmetric with parasite workers initiating the majority of aggressive events. This suggests that the species-specific CHCs that appeared on hosts after separation are likely the same CHCs that triggered a rejection response from the parasite. A similar study testing the effect of the discriminatory behaviors of the facultative kidnapper ant, *Formica sanguinea*, and their hosts showed that species-separation resulted in more frequent rejection of hosts from their former nestmates while the hosts accepted their parasitic nestmates from artificially pure parasite colonies (i.e. not in contact with host species) (Włodarczyk, 2016). Thus, physical contact with parasitic nestmates is necessary for hosts to be re-accepted after removal while hosts are generally more apt to accept former nestmates regardless of separation. Since the parasite workers in our study experienced less drastic changes to their chemical profiles as a result of separation, then it makes sense that the separated hosts would still recognize and accept their former nestmates.

Although our findings help to generally inform which chemically deceptive strategy facultative kidnapper ants are using to assimilate their hosts, future studies testing the effect of specific compounds on the discriminatory behaviors of *F. aserva* and others are necessary for deciphering the cues that are most fundamental to the nestmate recognition of facultative parasites and, thus, crucial for maintaining amicable behaviors between parasites and their captives.

In conclusion, it is apparent, based on our results, that the environmental context within which host workers emerge informs their chemical identity, primarily based on the exogenous

cues from their parasitic colony mates. Despite the substantial effect that environmental context has on an individual's development of their colony identity, these effects are not heritable and so, when removed from these stimuli, the host species in our study recovered their inherited cues as central to their chemical identity. Thus, our analyses of the CHC composition from host workers before and after separation from *F. aserva* showed a rebounding effect of host-specific cues in the absence of parasitic influence. The asymmetric change in CHCs that we observed in *F. aserva* and their *Formica* hosts following physical separation also resulted in an asymmetric display of aggression by the parasites towards their former nestmates. The culmination of these results support our hypotheses concerning the chemical deception strategies of facultative parasites; the chemical profiles of captive hosts are strongly influenced by physical contact with parasitic nestmates which ultimately benefits the cohesion and integrity of the mixed-species nests.

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Table 1. Mean relative abundances ± standard deviations of 164 cuticular hydrocarbons (CHCs) collected from *Formica aserva* and their three host species before (T_0) and after (45 days) separation into homospecific groups. CHCs are presented in the order of their retention times. The (+) indicates the presence of co-eluting compounds, for example, peak 51 is potentially a mixture of 9,13 DiMe C_{25} *and* 11,13 DiMe C_{25}.

			Formica aserva CHCs						Host CHCs					
			Using *F. microphthalma*		Using *F. accreta*		Using *F. subaenescens*		*Formica microphthalma*		*Formica accreta*		*Formica subaenescens*	
Peak Number	Compound	RT	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	C_{21}	12.47	0.011 ±0.003	0.016 ±0.008	0.009 ±0.005	0.02 ±0.006	0.005 ±0	0.018 ±0	0.017 ±0.003	0.003 ±0.001	±0.003	±0.002	0.018 ±0	0.002±0
2	9 + 11 Me C_{21}	12.84	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	±0	±0	0 ±0	0±0
3	9 Me C_{21}	12.87	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0.001	0.002 ±0.002	±0	±0	0 ±0	0±0
4	3 + 5 Me C_{21}	12.95	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0.001	±0	±0	0 ±0	0±0
5	5 Me C_{21}	13.02	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	±0	±0	0 ±0	0.001±0
6	3 Me C_{21}	13.17	0.003 ±0.002	0.009 ±0.006	0.002 ±0.002	0.005 ±0.001	0.002 ±0	0.008 ±0	0.011 ±0.007	0.007 ±0.003	±0.003	±0.001	0.005 ±0	0.001±0
7	5 Me C_{22}	13.43	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.003 ±0.002	±0	±0	0 ±0	0±0
8	4 Me C_{21}	13.43	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.004 ±0.002	0 ±0	±0	±0	0 ±0	0±0
9	5,11 DiMe C_{21}	13.29	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0.001	±0	±0	0 ±0	0±0
10	5,x DiMe C_{21}	13.44	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	±0	±0	0 ±0	0±0
11	C_{22}	13.46	0.002 ±0.001	0.003 ±0.001	0.002 ±0.002	0.003 ±0.001	0.002 ±0	0.004 ±0	0.005 ±0.003	0.005 ±0.003	±0.001	±0.001	0.003 ±0	0.002±0
12	10 Me C_{22}	13.81	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0.001	0 ±0	±0	±0	0 ±0	0±0
13	9 + 10 + 11 + 12 Me C_{22}	13.83	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.003 ±0.001	±0	±0	0 ±0	0±0
14	9 + 10 Me C_{22}	13.88	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.004 ±0.003	±0	±0	0 ±0	0±0
15	6 Me C_{22}	14.08	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.005 ±0.002	±0	±0	0 ±0	0±0
16	4 Me C_{22}	14.15	0.001	0.001	0 ±0	0.001	0 ±0	0.001	0.002	0.004	±0	±0	0 ±0	0±0

			±0.001	±0.001		±0.001		±0	±0.001	±0.004				
17	3 Me C_{22}	14.24	0.001 ±0.001	0.001 ±0.001	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0.002 ±0.001	0.001 ±0.001	±0	±0	0 ±0	0±0
18	4,12 Me C_{22}	14.4	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0.001	±0	±0	0 ±0	0±0
19	C_{23} monoene	14.26	0 ±0	0.001 ±0.001	0 ±0	0.001 ±0.001	0 ±0	0.001 ±0	0 ±0	0.002 ±0.001	±0	±0	0 ±0	0±0
20	C_{23}	14.55	0.038 ±0.006	0.055 ±0.017	0.037 ±0.013	0.073 ±0.021	0.034 ±0	0.082 ±0	0.039 ±0.025	0.058 ±0.057	±0.004	±0.003	0.03 ±0	0.007±0
21	9 + 11 Me C_{23}	14.96	0.001 ±0.001	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0.01 ±0.009	0.06 ±0.064	±0.001	±0.003	0 ±0	0.002±0
22	5 Me C_{23}	15.09	0.001 ±0.001	0.001 ±0.001	0.001 ±0.001	0.001 ±0.001	0 ±0	0.001 ±0	0.002 ±0.002	0.005 ±0.005	±0.001	±0.001	0 ±0	0.001±0
23	3 + 4 Me C_{23}	15.25	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0±0
24	3 Me C_{23}	15.36	0.003 ±0.002	0.008 ±0.005	0.002 ±0.001	0.005 ±0.001	0.001 ±0	0.007 ±0	0.011 ±0.007	0.019 ±0.012	±0.002	±0.001	0.004 ±0	0.001±0
25	9,13 DiMe C_{23}	15.26	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0.001	±0	±0	0 ±0	0±0
26	9,11 DiMe C_{23}	15.29	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	±0	±0	0 ±0	0±0
27	5,13 DiMe C_{23}	15.6	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.008 ±0.004	±0	±0	0 ±0	0±0
28	C_{24}	15.7	0.008 ±0.001	0.009 ±0.003	0.009 ±0.003	0.01 ±0.002	0.008 ±0	0.014 ±0	0.008 ±0.005	0.01 ±0.009	±0.001	±0.001	0.007 ±0	0.004±0
29	10 + 11 + 12 + 13 Me C_{24}	16.09	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.004 ±0.002	0.011 ±0.007	±0	±0.001	0 ±0	0±0
30	11 + 12 + 13 Me C_{24}	16.19	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.017 ±0.009	±0	±0	0 ±0	0.001±0
31	6 Me C_{24}	16.21	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0.001	±0	±0	0 ±0	0±0
32	10 + 12 + 13 Me C_{24}	16.28	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0±0
33	5 Me C_{24}	16.28	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0.001±0
34	4 + 6 Me C_{24}	16.33	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0.001	0 ±0	0±0
35	4 Me C_{24}	16.4	0.001 ±0.001	0.004 ±0.005	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0.003 ±0.003	0.009 ±0.006	±0	±0.001	0 ±0	0.001±0
36	3 Me C_{24}	16.59	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	0.004 ±0.002	±0.003	±0.001	0 ±0	0.001±0
37	4,12 DiMe C_{24}	16.78	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.006 ±0.002	±0	±0	0 ±0	0±0
38	4,8 + 4,10 + 4,12 DiMe C_{24}	16.78	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	±0	±0	0 ±0	0±0

39	C_{25} diene	16.33	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0.001 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0±0
40	C_{25} monoene	16.63	0.002 ±0.001	0.008 ±0.004	0.002 ±0.002	0.011 ±0.007	0.01 ±0	0.03 ±0	0.01 ±0.006	0.003 ±0.002	±0.002	±0.001	0.007 ±0	0±0
41	C_{25}	17	0.245 ±0.017	0.199 ±0.062	0.257 ±0.019	0.245 ±0.029	0.279 ±0	0.219 ±0	0.123 ±0.09	0.09 ±0.067	±0.011	±0.029	0.122 ±0	0.027±0
42	11 + 13 Me C_{25}	17.35	0.001 ±0.001	0.001 ±0.001	0 ±0	0.001 ±0.001	0.001 ±0	0.001 ±0	0.008 ±0.007	0.046 ±0.032	±0.002	±0.004	0.002 ±0	0.007±0
43	3 + 7 Me C_{25}	17.45	0 ±0	0.003 ±0.001	0 ±0	0.002 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0±0
44	5 + 7 Me C_{25}	17.49	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0.002	0 ±0	0±0
45	3 + 5 Me C_{25}	17.55	0.002 ±0.002	0.006 ±0.004	0 ±0	0.003 ±0.002	0 ±0	0.005 ±0	0.01 ±0.007	0 ±0	±0.002	±0	0 ±0	0±0
46	5 Me C_{25}	17.55	0.002 ±0.001	0 ±0	0.001 ±0.001	0.003 ±0.002	0.002 ±0	0 ±0	0 ±0	0.009 ±0.004	±0.003	±0.002	0.004 ±0	0.008±0
47	4 Me C_{25}	17.71	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0.001	0 ±0	±0	±0	0 ±0	0±0
48	3 Me C_{25}	17.87	0.028 ±0.017	0.06 ±0.03	0.015 ±0.009	0.034 ±0.004	0.014 ±0	0.041 ±0	0.102 ±0.048	0.077 ±0.016	±0.006	±0.009	0.054 ±0	0.018±0
49	11,13 DiMe C_{25}	17.69	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.004 ±0.002	±0	±0	0 ±0	0±0
50	4,8 + 4,10 + 5,13 DiMe C_{25}	17.7	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.003 ±0.001	±0	±0	0 ±0	0±0
51	9,13 + 11,13 DiMe C_{25}	17.71	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.004 ±0.002	±0	±0	0 ±0	0±0
52	9,13 + 11,13 + 13,15 DiMe C_{25}	17.93	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.004 ±0.002	±0	±0	0 ±0	0±0
53	C_{26}	18.2	0.024 ±0.002	0.017 ±0.007	0.025 ±0.001	0.019 ±0.002	0.021 ±0	0.02 ±0	0.012 ±0.01	0.009 ±0.006	±0.001	±0.007	0.017 ±0	0.006±0
54	10 + 12+ 13 + 14 Me C_{26}	18.57	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.007 ±0.003	±0	±0.004	0 ±0	0±0
55	13 + 14 Me C_{26}	18.59	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	0 ±0	±0	±0	0 ±0	0±0
56	10 + 11 + 12 + 13 + 14 Me C_{26}	18.6	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.011 ±0.006	±0	±0.003	0 ±0	0±0
57	10 + 11 + 13 + 14 Me C_{26}	18.6	0.001 ±0.001	0 ±0	0.001 ±0.001	0.001 ±0.001	0 ±0	0 ±0	0.004 ±0.002	0 ±0	±0.002	±0.003	0 ±0	0±0
58	12 + 13 + 14 Me C_{26}	18.68	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.007 ±0.004	±0	±0	0 ±0	0.01±0
59	6 Me C_{26}	18.74	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	±0	±0.001	0 ±0	0.002±0
60	10 + 11 + 12 Me C_{26}	18.76	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	0 ±0	±0	±0	0 ±0	0±0
61	4 Me C_{26}	18.92	0.001	0 ±0	0.001	0 ±0	0 ±0	0 ±0	0 ±0	0.006	±0.001	±0.004	0 ±0	0.005±0

			±0.001		±0.001					±0.003					
62	4 + 9 Me C_{26}	18.98	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0.001	0 ±0	±0	±0	0 ±0	0±0
63	3 Me C_{26}	19.07	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.004 ±0.002	±0	±0.005	0 ±0	0±0
64	4,10 + 6,10 DiMe C_{26}	19.12	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0.007±0
65	4,8 + 4,12 + 4,14 DiMe C_{26}	19.31	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0.006±0
66	C_{27} diene	18.85	0.003 ±0.001	0.01 ±0.006	0.002 ±0.002	0.006 ±0.002	0.003 ±0	0.009 ±0	0.009 ±0.006	0.002 ±0.001	±0.001	±0	0.003 ±0	0±0
67	C_{27} monoene	19.19	0.043 ±0.014	0.145 ±0.075	0.035 ±0.032	0.097 ±0.011	0.054 ±0	0.126 ±0	0.176 ±0.094	0.029 ±0.019	±0.023	±0.015	0.133 ±0	0±0
68	C_{27}	19.57	0.401 ±0.046	0.227 ±0.093	0.426 ±0.062	0.269 ±0.014	0.409 ±0	0.234 ±0	0.142 ±0.121	0.064 ±0.033	±0.008	±0.054	0.188 ±0	0.04±0
69	11 + 13 Me C_{27}	19.9	0.002 ±0.002	0.003 ±0.002	0.002 ±0.001	0.004 ±0.002	0.002 ±0	0.003 ±0	0.013 ±0.01	0.081 ±0.049	±0.005	±0.015	0.013 ±0	0.061±0
70	13 Me C_{27}	19.94	0.003 ±0.002	0.004 ±0.002	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.021 ±0.007	±0	±0	0 ±0	0±0
71	3 + 7 Me C_{27}	19.98	0.002 ±0.002	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.007 ±0.005	0 ±0	±0	±0	0 ±0	0±0
72	7 Me C_{27}	20.02	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0	0 ±0	0 ±0	0.022 ±0.007	±0	±0	0.005 ±0	0±0
73	5 Me C_{27}	20.12	0.003 ±0.002	0.003 ±0.001	0.001 ±0.001	0.002 ±0.001	0 ±0	0 ±0	0.008 ±0.005	0.013 ±0.008	±0.004	±0.003	0.013 ±0	0.025±0
74	3 + 5 Me C_{27}	20.19	0.004 ±0.002	0.01 ±0.005	0 ±0	0 ±0	0 ±0	0 ±0	0.014 ±0.007	0.031 ±0.01	±0	±0	0 ±0	0±0
75	4 + 9 Me C_{27}	20.27	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0.001	0 ±0	±0	±0	0 ±0	0±0
76	3 Me C_{27}	20.45	0.033 ±0.01	0.039 ±0.009	0.024 ±0.01	0.028 ±0.004	0.016 ±0	0.025 ±0	0.045 ±0.002	0.024 ±0.011	±0.002	±0.01	0.046 ±0	0.06±0
77	11,15 DiMe C_{27}	20.27	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0.001	0.014 ±0.011	±0.005	±0.012	0 ±0	0±0
78	9,13 + 9,15 + 11,15 + 13,15 DiMe C_{27}	20.27	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.006 ±0.002	±0	±0	0 ±0	0±0
79	9,13 DiMe C_{27}	20.27	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0.007±0
80	7,11 DiMe C_{27}	20.34	0 ±0	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0.016 ±0.011	±0.007	±0.011	0 ±0	0±0
81	3,13 + 3,15 DiMe C_{27}	20.84	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0.129±0
82	C_{28} monoene	20.59	0.011 ±0.005	0.034 ±0.019	0.012 ±0.006	0.026 ±0.006	0.01 ±0	0.025 ±0	0.054 ±0.029	0.035 ±0.013	±0.002	±0.016	0.048 ±0	0±0
83	C_{28}	20.79	0.013 ±0.001	0.009 ±0.004	0.014 ±0.003	0.01 ±0.002	0.011 ±0	0.01 ±0	0.008 ±0.005	0.017 ±0.007	±0.002	±0.005	0.018 ±0	0±0

84	5 Me C_{28}	21.13	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0.001	0 ±0	±0	±0	0 ±0	0±0
85	10 + 12 + 14 + 15 Me C_{28}	21.14	0.001 ±0.001	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0.005 ±0.003	0 ±0	±0.007	±0.011	0 ±0	0±0
86	12 Me C_{28}	21.15	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0±0
87	12 + 13 + 14 + 15 Me C_{28}	21.15	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.004 ±0.002	0.006 ±0.002	±0	±0	0 ±0	0±0
88	12 + 14 + 15 Me C_{28}	21.16	0 ±0	0 ±0	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	±0.004	±0	0 ±0	0±0
89	10 + 12 Me C_{28}	21.2	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0.013	0 ±0	0±0
90	13 + 14 + 15 Me C_{28}	21.3	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.002	±0	±0	0 ±0	0±0
91	4 Me C_{28}	21.48	0 ±0	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	±0.002	±0	0 ±0	0±0
92	6 Me C_{28}	21.25	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0.001	±0.002	0 ±0	0±0
93	8,12 DiMe C_{28}	21.19	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.041 ±0.02	±0	±0	0.022 ±0	0.099±0
94	8,12 + 8,14 Me C_{28}	21.21	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.044 ±0.022	±0	±0	0 ±0	0±0
95	5,11 DiMe C_{28}	21.39	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0.016	0 ±0	0±0
96	10,14 DiMe C_{28}	21.51	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.006 ±0.003	±0	±0.008	0 ±0	0±0
97	5,9 + 10,14 Me C_{28}	21.51	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.013 ±0.006	±0	±0	0 ±0	0±0
98	4,10 + 6,10 Me C_{28}	21.63	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0.004	0 ±0	0±0
99	6,10 + 6,14 DiMe C_{28}	21.66	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.008 ±0.004	±0	±0	0 ±0	0.015±0
100	4,14 DiMe C_{28}	21.87	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.005 ±0.003	±0	±0	0 ±0	0±0
101	4,8 + 4,10 + 4,12, + 4,14 DiMe C_{28}	21.87	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.005 ±0.002	±0	±0	0 ±0	0±0
102	4,8 + 4,12 + 4,14 DiMe C_{28}	21.88	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0.013±0
103	C_{29} diene	21.37	0.004 ±0.003	0.016 ±0.011	0.004 ±0.003	0.01 ±0.003	0.002 ±0	0.017 ±0	0.009 ±0.008	0 ±0	±0.001	±0	0 ±0	0±0
104	C_{29} monoene	21.67	0.008 ±0.003	0.034 ±0.028	0.008 ±0.008	0.022 ±0.007	0.014 ±0	0.026 ±0	0.041 ±0.029	0.018 ±0.016	±0.003	±0.003	0.036 ±0	0±0
105	C_{29}	22.09	0.097 ±0.013	0.058 ±0.028	0.105 ±0.043	0.075 ±0.019	0.095 ±0	0.047 ±0	0.041 ±0.033	0.021 ±0.016	±0.001	±0.013	0.057 ±0	0.015±0
106	13 + 15 Me C_{29}	22.4	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.004	0 ±0	±0	±0	0 ±0	0±0

107	11 + 13 + 15 Me C_{29}	22.45	0.002 ±0.001	0.002 ±0.001	0.001 ±0.001	0.003 ±0.002	0.002 ±0	0.002 ±0	0.002 ±0.002 0.01 ±0.008	0.061 ±0.042	±0.023	±0.025	0.054 ±0	0.074±0
108	3 + 7 Me C_{29}	22.55	0.005 ±0.003	0.004 ±0.002	0.003 ±0.002	0.005 ±0.003	0.003 ±0	0.01 ±0	0.035 ±0.022	0.011 ±0.005	±0	±0	0.01 ±0	0±0
109	7 Me C_{29}	22.55	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.007 ±0.004	0.026 ±0.009	±0	±0	0 ±0	0±0
110	5 Me C_{29}	22.67	0.001 ±0.001	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0.006 ±0.003	0.009 ±0.003	±0	±0	0 ±0	0.006±0
111	6 Me C_{29}	22.88	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0±0
112	3 Me C_{29}	22.91	0.007 ±0.002	0.01 ±0.006	0.006 ±0.002	0.007 ±0.002	0.004 ±0	0.004 ±0	0.008 ±0.006	0.005 ±0.003	±0.008	±0.007	0.017 ±0	0±0
113	4,8 + 4,16 DiMe C_{29}	22.2	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.006 ±0.003	±0	±0	0 ±0	0±0
114	9,13 DiMe C_{29}	22.41	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.005 ±0.003	±0.019	±0.031	0 ±0	0.016±0
115	11,15 DiMe C_{29}	22.77	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.053 ±0.033	±0.017	±0.06	0 ±0	0±0
116	7,11 + 9,11 + 11,x DiMe C_{29}	22.87	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.005 ±0.003	0 ±0	±0	±0	0 ±0	0±0
117	7,11 DiMe C_{29}	22.88	0.001 ±0.001	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0.005 ±0.006	0.017 ±0.008	±0.003	±0	0.004 ±0	0±0
118	7,11 + 7,13 DiMe C_{29}	22.9	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0.013±0
119	7,15 DiMe C_{29}	22.91	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.008 ±0.003	±0	±0	0 ±0	0±0
120	9,11 DiMe C_{29}	22.92	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.029 ±0.014	±0	±0	0 ±0	0±0
121	5,13 + 5,15 DiMe C_{29}	23.03	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.033 ±0.011	±0	±0	0 ±0	0±0
122	5,13 DiMe C_{29}	23.03	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0.039±0
123	5,15 DiMe C_{29}	23.05	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.01 ±0.005	0.024 ±0.012	±0	±0	0 ±0	0±0
124	X Me C_{29}	23.25	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.013 ±0.006	±0	±0	0 ±0	0±0
125	5,9 + 5,13 + 5,15 DiMe C_{29}	23.06	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.031 ±0.013	±0	±0	0 ±0	0±0
126	3,11 + 7,11 DiMe C_{29}	23.18	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	±0	±0	0 ±0	0±0
127	C_{30} diene	22.72	0 ±0	0.002 ±0.002	0 ±0	0.002 ±0.002	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0±0
128	C_{30} monoene	23.14	0.001 ±0.001	0.004 ±0.002	0.001 ±0.001	0.003 ±0.002	0 ±0	0.003 ±0	0.005 ±0.003	0.011 ±0.009	±0.005	±0.006	0 ±0	0.005±0

129	C_{30}	23.31	0.003 ±0.001	0.002 ±0.001	0.003 ±0.002	0.002 ±0.001	0.002 ±0	0.003 ±0	0.004 ±0.002	0.007 ±0.004	±0.001	±0.003	0.024 ±0	0±0
130	12 + 13 Me C_{30}	23.6	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0±0
131	12 Me C_{30}	23.66	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0.002	±0	0 ±0	0±0
132	12 + 13 + 14 Me C_{30}	23.66	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0±0
133	11 + 12 + 13 Me C_{30}	23.67	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.004 ±0.002	0 ±0	±0	±0	0 ±0	0±0
134	13 + 15 + 16 Me C_{30}	23.67	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.006 ±0.002	±0	±0	0 ±0	0±0
135	4,14 + 4,16 Me C_{30}	23.35	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.024 ±0.012	±0	±0	0 ±0	0.134±0
136	8,14 + 8,16 DiMe C_{30}	23.6	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.023 ±0.012	±0	±0	0 ±0	0±0
137	8,10 + 8,14 + 8,16 Me C_{30}	23.66	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0.005	0 ±0	0±0
138	4,12 DiMe C_{30}	23.68	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0±0
139	8,12 + 8,14 + 8,16 DiMe C_{30}	23.7	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.003 ±0.002	0 ±0	±0	±0	0 ±0	0±0
140	8,12 DiMe C_{30}	23.71	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0	0 ±0	0 ±0	0.026 ±0.013	±0	±0	0.022 ±0	0.069±0
141	10,14 + 10,16 DiMe C_{30}	23.97	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.009 ±0.007	±0.001	±0.008	0 ±0	0±0
142	8,12,16 TriMe C_{30}	23.99	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0.012±0
143	C_{31} diene	23.87	0.001 ±0.001	0.006 ±0.004	0 ±0	0 ±0	0 ±0	0.004 ±0	0.005 ±0.003	0 ±0	±0	±0	0 ±0	0±0
144	C_{31} monoene	24.13	0.004 ±0.001	0.014 ±0.009	0.002 ±0.002	0.009 ±0.002	0.003 ±0	0.011 ±0	0.018 ±0.011	0.008 ±0.005	±0.003	±0.004	0.008 ±0	0±0
145	C_{31}	24.54	0.004 ±0.001	0.003 ±0.001	0.005 ±0.002	0.004 ±0.003	0.003 ±0	0.003 ±0	0.005 ±0.005	0.005 ±0.005	±0.003	±0.004	0.004 ±0	0±0
146	11 + 13 Me C_{31}	24.88	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	0 ±0	±0	±0	0 ±0	0±0
147	13 + 15 Me C_{31}	24.89	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0	0 ±0	0 ±0	0.019 ±0.012	±0	±0	0.017 ±0	0.021±0
148	11 + 13 + 15 Me C_{31}	24.89	0.002 ±0.001	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0.008 ±0.005	0.019 ±0.01	±0.008	±0.009	0 ±0	0±0
149	13 Me C_{31}	24.99	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0±0
150	7 Me C_{31}	25	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.003 ±0.002	0 ±0	±0	±0	0 ±0	0±0
151	3 + 7 Me C_{31}	25.02	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.015	±0	±0	0 ±0	0±0

										±0.005				
152	3 Me C_{31}	25.08	0.002 ±0.001	0.002 ±0.001	0 ±0	0.007 ±0.004	0 ±0	0 ±0	0.018 ±0.009	0 ±0	±0	±0	0 ±0	0±0
153	5 Me C_{31}	25.14	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	±0	±0	0 ±0	0±0
154	11,13 DiMe C_{31}	25.21	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.001 ±0.001	0 ±0	±0	±0	0 ±0	0±0
155	11,15 DiMe C_{31}	25.23	0.002 ±0.001	0 ±0	0.001 ±0.001	0.002 ±0.002	0 ±0	0 ±0	0.008 ±0.004	0.031 ±0.023	±0.001	±0.023	0 ±0	0.006±0
156	7,13 DiMe C_{31}	25.32	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	0 ±0	±0	±0	0 ±0	0±0
157	7,11 + 7,13 DiMe C_{31}	25.34	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0.004±0
158	7,11 DiMe C_{31}	25.35	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.005 ±0.002	±0	±0	0 ±0	0±0
159	5,9 DiMe C_{31}	25.43	0 ±0	0 ±0	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0.004	±0	0 ±0	0±0
160	5,13 DiMe C_{31}	25.46	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.017 ±0.006	±0	±0	0 ±0	0±0
161	5,13 + 5,15 DiMe C_{31}	25.47	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0.004±0
162	5,15 DiMe C_{31}	25.47	0.001 ±0.001	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.002 ±0.001	0.008 ±0.005	±0	±0.003	0 ±0	0±0
163	7,11,15 TriMe C_{31}	25.63	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0.002	0 ±0	0±0
164	3,13 + 3,15 DiMe C_{31}	25.78	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	±0	±0	0 ±0	0.036±0

Table 2. Average dissimilarity in CHC composition between heterospecific nestmates before (T_0) and after (45 days) separation

	Average Dissimilarity (%)	
Species	Parasite w/ host BEFORE (T_0)	Parasite w/ host AFTER (45 days)
F. accreta + parasite	36.1	50.8
F. micropthalma + parasite	48.4	61.5
F. subaenescens + parasite	43.0	84.8

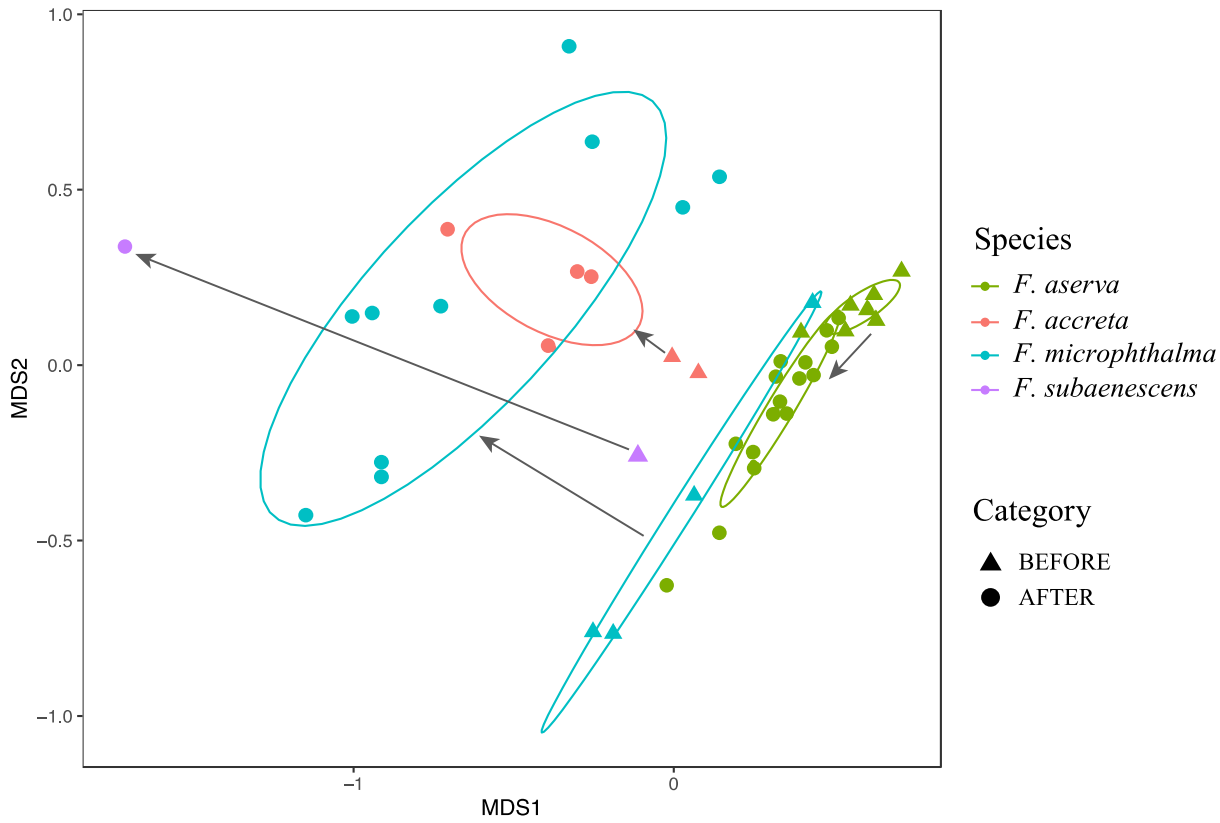


Figure 1. NMDS (Non-metric multidimensional scaling) plot of individual *Formica* workers from before (triangles) and after (circles) separation from heterospecific nestmates based on the relative proportions of the detected peaks in their cuticular hydrocarbon profiles (C_{21}-C_{32}).

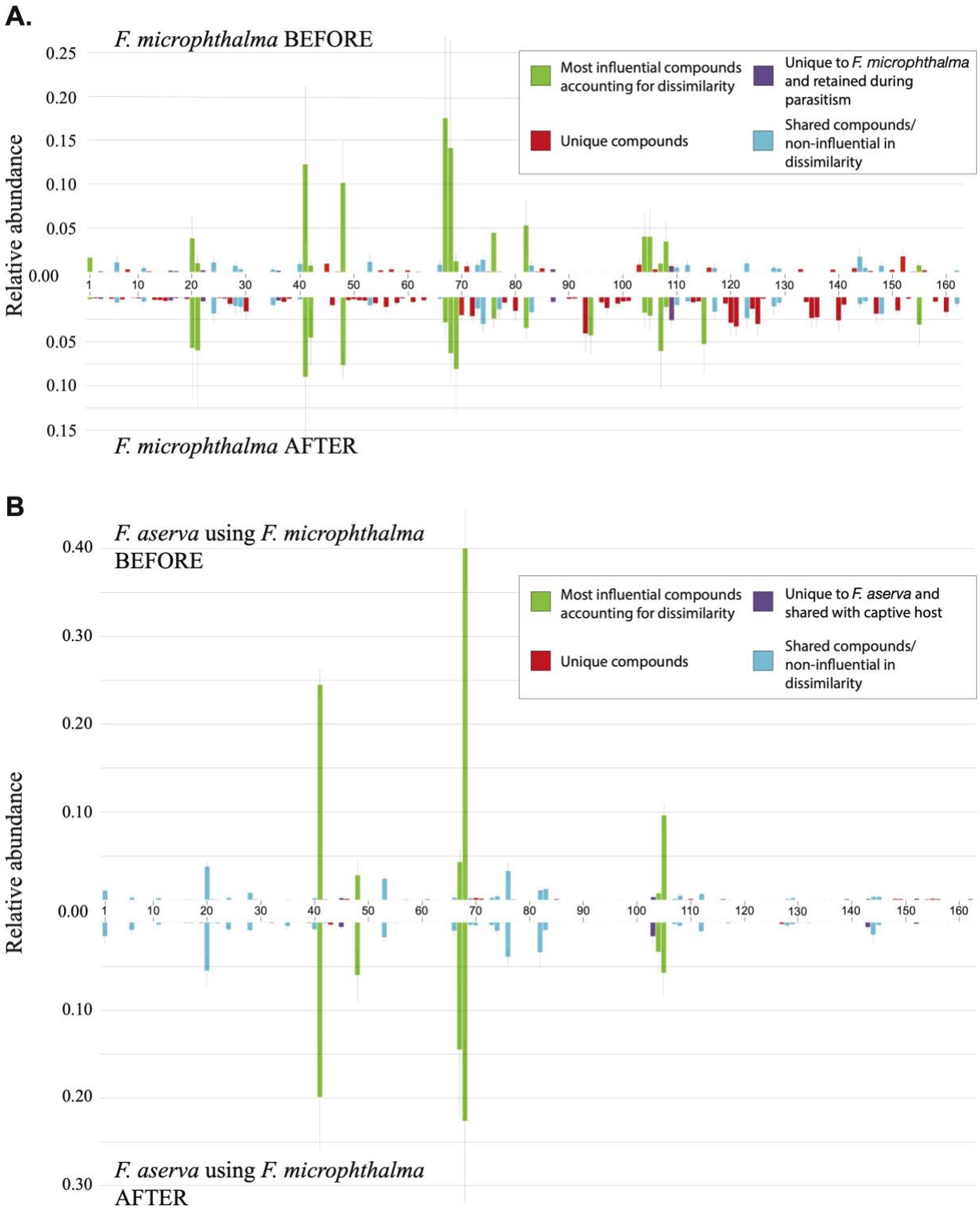


Figure 2. Relative proportions of CHCs before and after separation for *F. microphthalma* hosts (A) and their *Formica aserva* nestmates (B). Mirrored bar plots display CHC profiles before separation (top) and after separation (bottom) for each species (host and parasite) and each bar represents a detected peak with its corresponding peak number on the x-axis. Green bars

represent compounds that are the most influential in contributing to the difference observed between the before and after treatments within species, red bars represent unique compounds that are not shared between the before and after CHCs within species, and purple bars indicate the species- and colony- specific compounds that are shared between heterospecific nestmates during cohabitation.

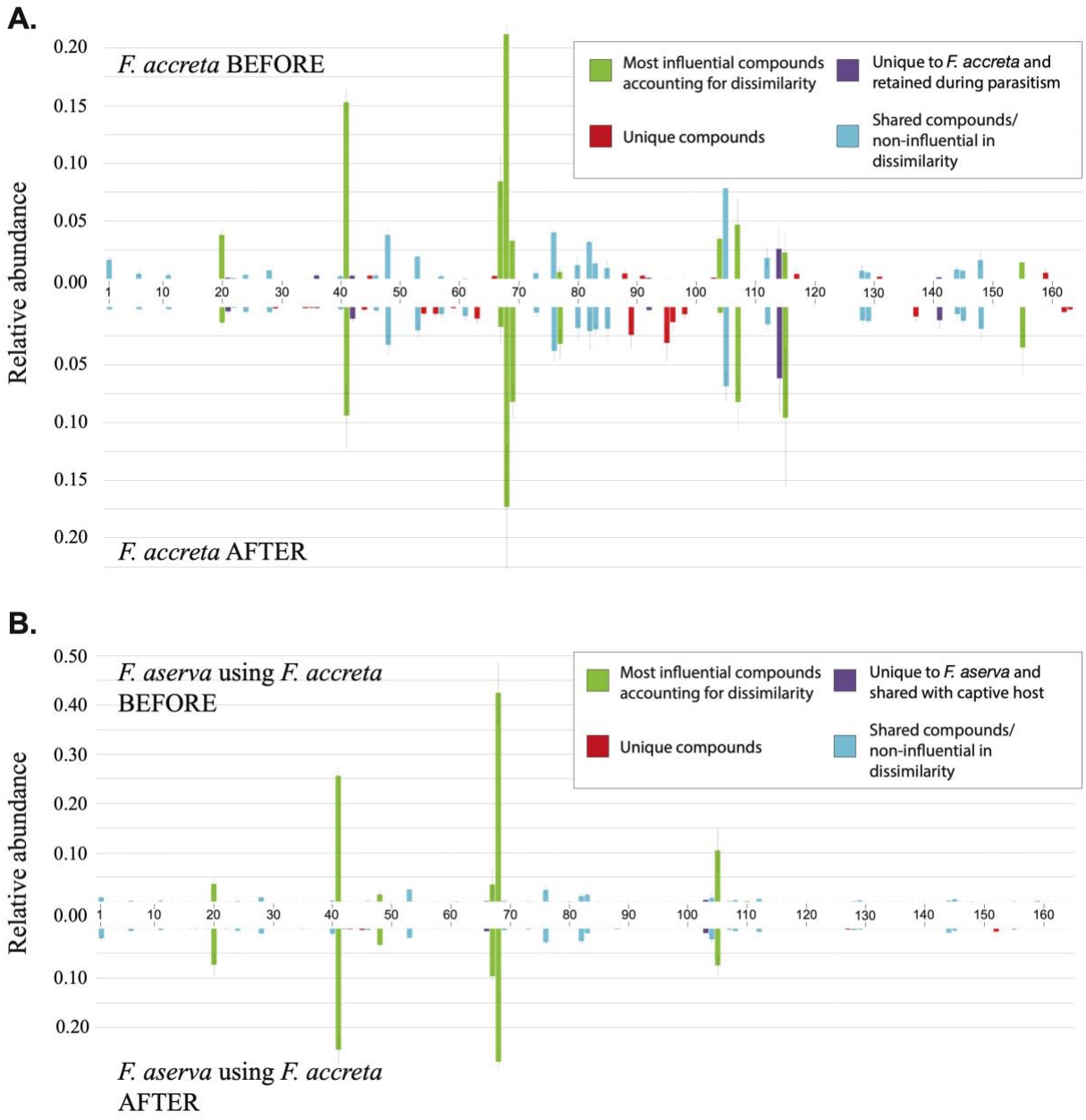


Figure 3. Relative proportions of CHCs before and after separation for *F. accreta* hosts (A) and their *Formica aserva* nestmates (B). Mirrored bar plots display CHC profiles before separation (top) and after separation (bottom) for each species (host and parasite) and each bar represents a detected peak with its corresponding peak number on the x-axis. Green bars represent compounds that are the most influential in contributing to the difference observed between the before and after treatments within species, red bars represent unique compounds that are not shared between the before and after CHCs within species, and purple bars indicate the species- and colony- specific compounds that are shared between heterospecific nestmates during cohabitation.

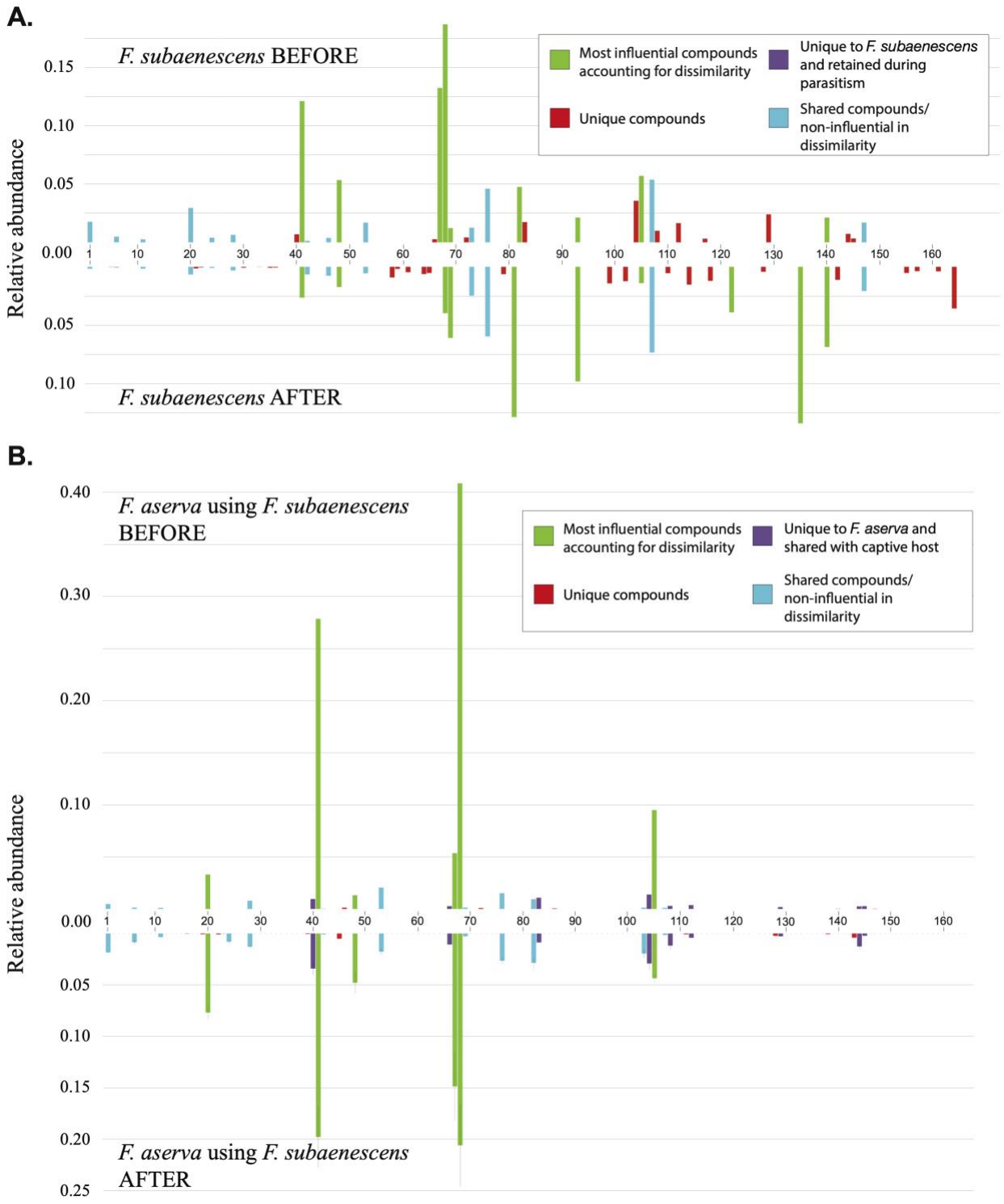


Figure 4. Relative proportions of CHCs before and after separation for *Formica subaenescens* hosts (A) and their *Formica aserva* nestmates (B). Mirrored bar plots display CHC profiles before separation (top) and after separation (bottom) for each species (host and parasite) and each bar represents a detected peak with its corresponding peak number on the x-axis. Green bars represent compounds that are the most influential in contributing to the difference observed

between the before and after treatments within species, red bars represent unique compounds that are not shared between the before and after CHCs within species, and purple bars indicate the species- and colony- specific compounds that are shared between heterospecific nestmates during cohabitation.

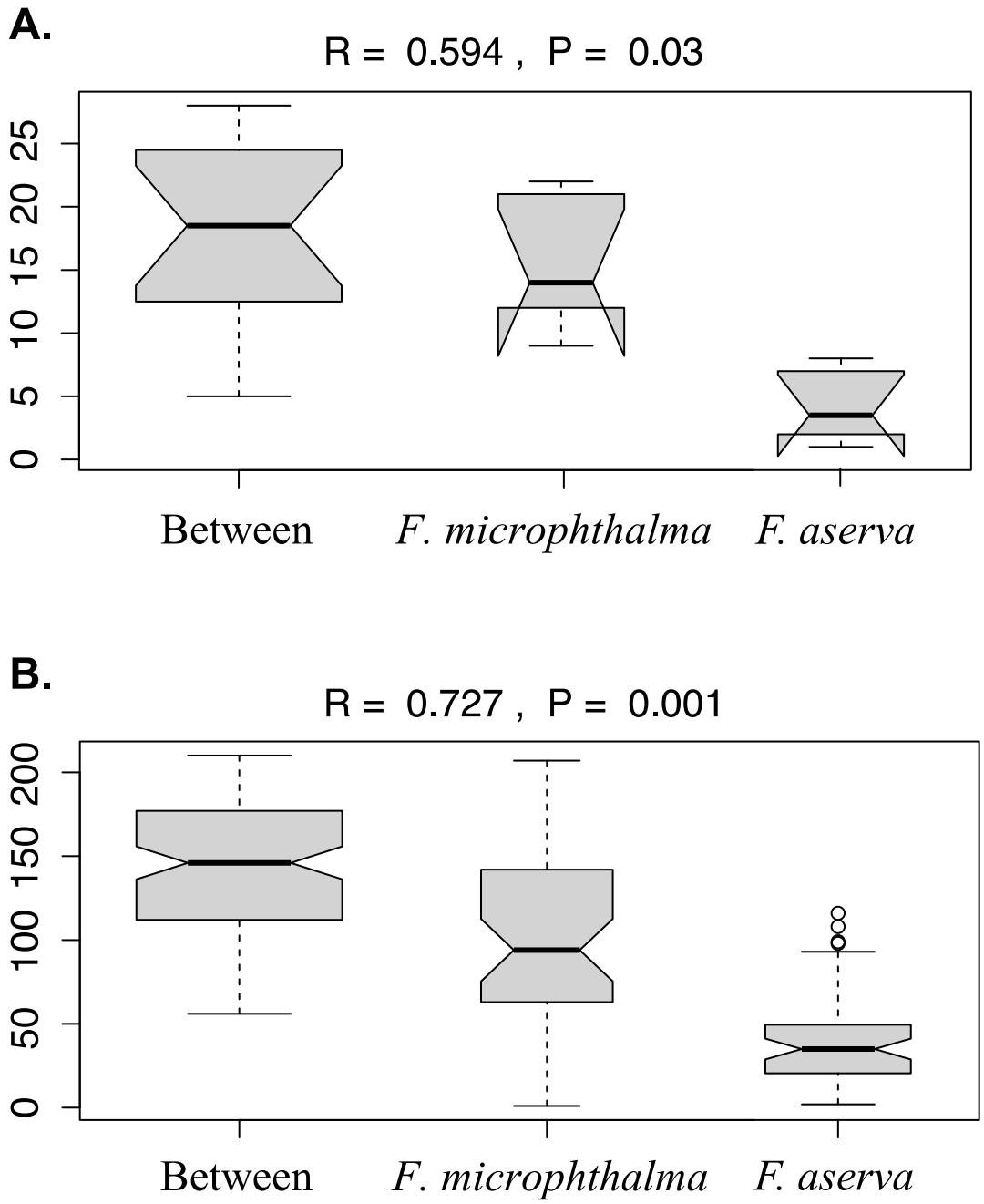


Figure 5. ANOSIM (Analysis of Similarity) showing the degree of dissimilarity in regards to CHCs within and between species before (A) and after (B) separation for *F. aserva* (parasite) and *F. microphthalma* (host).

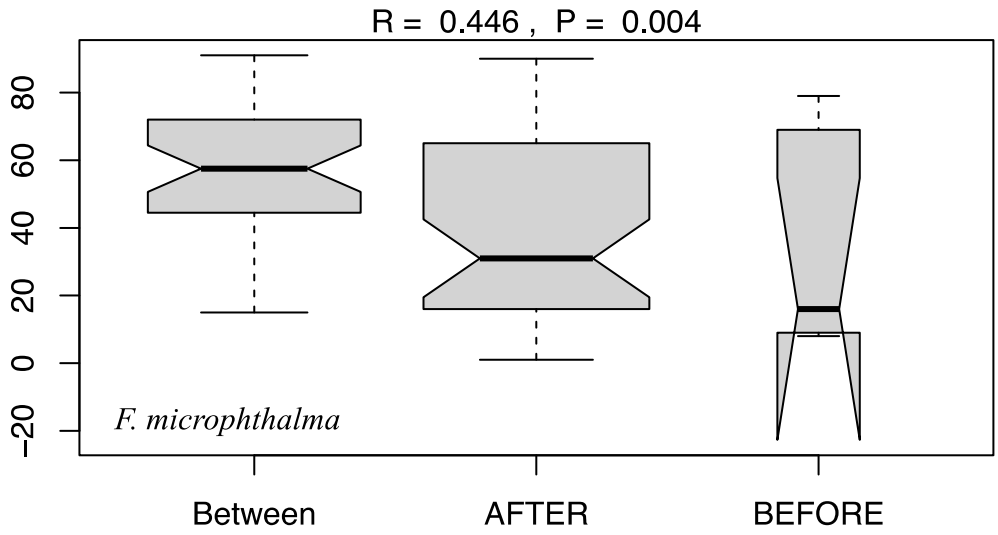


Figure 6. ANOSIM results showing the dissimilarity in CHC composition between *F. microphthalma* (host) workers before and after being removed from contact with their parasitic nestmates.

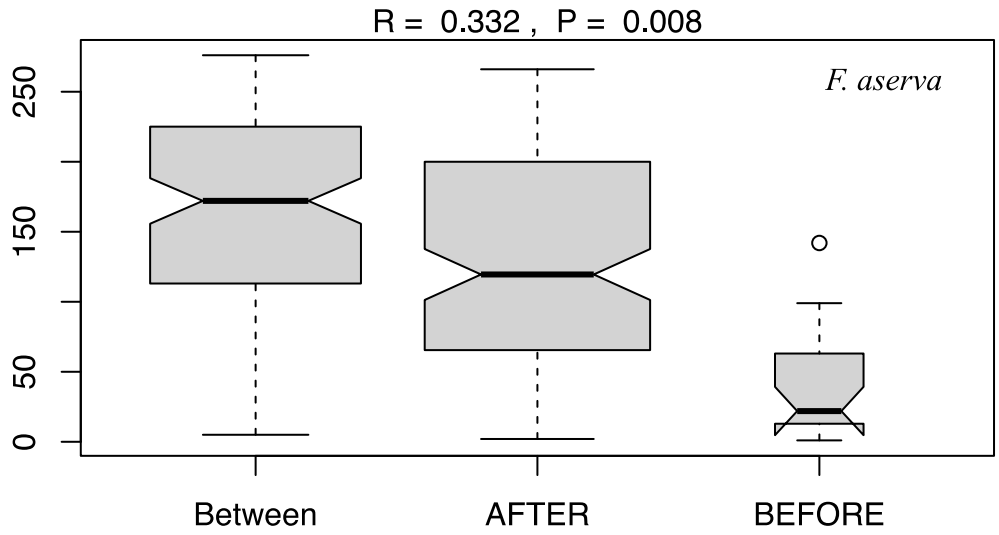


Figure 7. ANOSIM results showing the dissimilarity in CHC composition between *F. aserva* (parasite) workers before and after being removed from contact with their captive hosts.

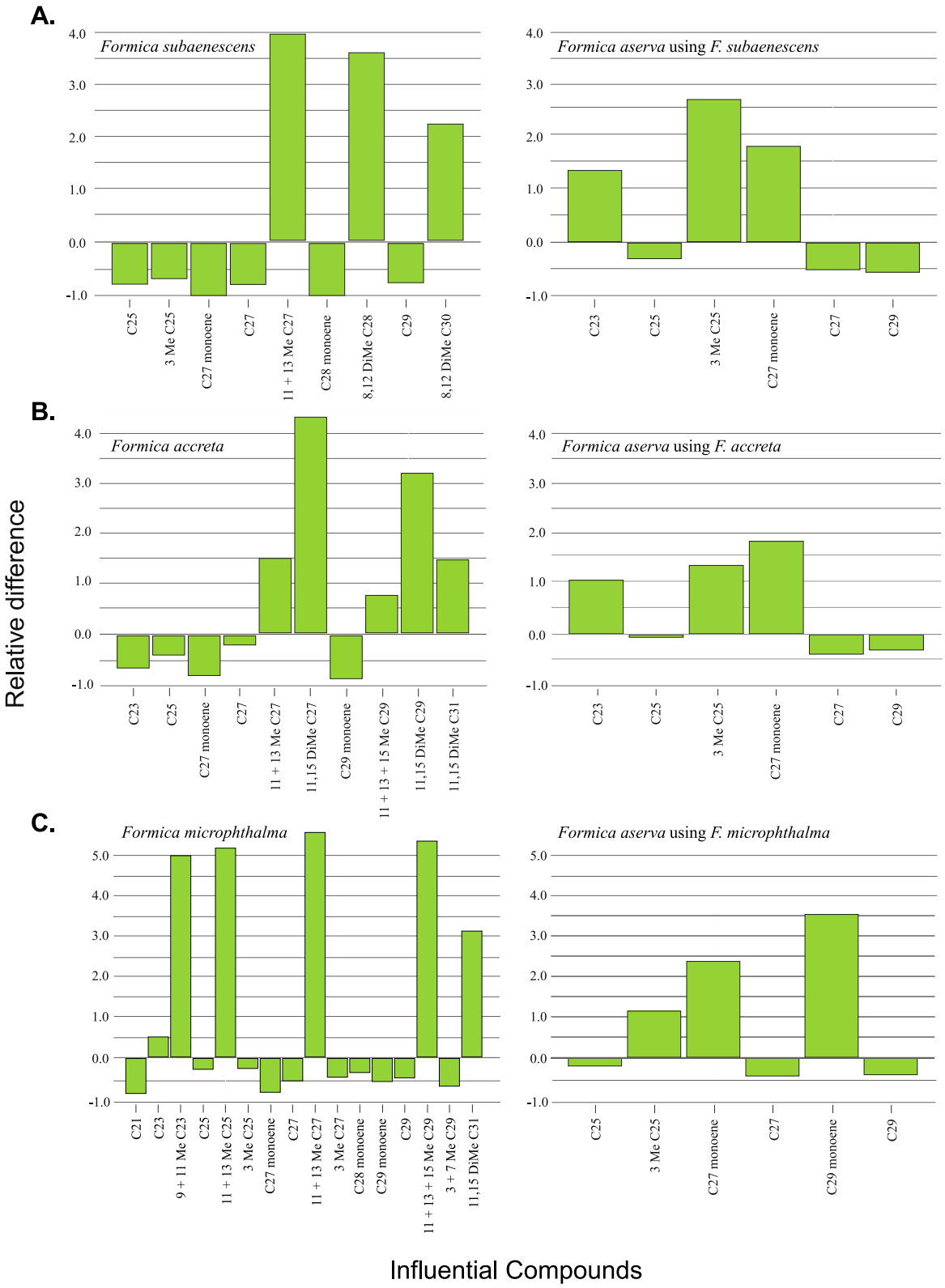


Figure 8. Relative differences of the influential compounds accounting for the observed dissimilarity within *Formica* host species and *F. aserva* parasitizing different hosts based on

CHC samples taken from workers while cohabitating with heterospecific nestmates and samples taken after a prolonged period of separation (45 days). Relative difference is presented as proportions with positive values indicating an increase in the compound after separation and negative values indicating a decrease in the compound after separation.

Table 3. Influential compounds contributing to the dissimilarity observed between before and after CHCs of *F. accreta* hosts and their *F. aserva* nestmates and the relative difference in the abundance of these compounds as a result of separation.

Peak Number	Influential Compounds	Proportion Contribution (±SD)	Relative Difference (%)	Shared with heterospecific nestmates
	Formica accreta			
20	C_{23}	0.013 ± 0.002	-64.66	X
41	C_{25}	0.030 ± 0.014	-38.56	X
67	C_{27} monoene	0.036 ± 0.011	-79.73	X
68	C_{27}	0.023 ± 0.022	-18.35	X
69	11 + 13 Me C_{27}	0.025 ± 0.007	149.35	
77	11,15 DiMe C_{27}	0.015 ± 0.006	432.95	
104	C_{29} monoene	0.017 ± 0.002	-86.04	
107	11 + 13 + 15 Me C_{29}	0.019 ± 0.014	75.15	
115	11,15 DiMe C_{29}	0.034 ± 0.025	320.38	
155	11,15 DiMe C_{31}	0.014 ± 0.006	146.44	
	Formica aserva			
20	C_{23}	0.019 ± 0.011	101.84	X
41	C_{25}	0.012 ± 0.011	-4.59	X
48	3 Me C_{25}	0.010 ± 0.004	130.13	
67	C_{27} monoene	0.031 ± 0.013	176.04	X
68	C_{27}	0.079 ± 0.024	-36.72	X
105	C_{29}	0.019 ± 0.015	-28.75	

Table 4. Influential compounds contributing to the dissimilarity observed between before and after CHCs of *F. microphthalma* hosts and their *F. aserva* nestmates and the relative difference in the abundance of these compounds as a result of separation.

Peak Number	Influential Compounds	Proportion Contribution (±SD)	Relative Difference (%)	Shared with heterospecific nestmates
	Formica microphthalma			
1	C_{21}	0.008 ± 0.002	-86.792	
20	C_{23}	0.022 ± 0.021	49.276	
21	9 + 11 Me C_{23}	0.029 ± 0.030	499.384	
41	C_{25}	0.041 ± 0.034	-26.792	X
42	11 + 13 Me C_{25}	0.021 ± 0.015	518.129	
48	3 Me C_{25}	0.023 ± 0.012	-24.656	X
67	C_{27} monoene	0.075 ± 0.040	-83.831	X
68	C_{27}	0.050 ± 0.046	-55.308	X
69	11 + 13 Me C_{27}	0.032 ± 0.022	556.96	
76	3 Me C_{27}	0.013 ± 0.006	-46.298	
82	C_{28} monoene	0.017 ± 0.008	-34.82	
104	C_{29} monoene	0.017 ± 0.012	-57.121	X
105	C_{29}	0.014 ± 0.013	-47.915	X
107	11 + 13 + 15 Me C_{29}	0.028 ± 0.020	535.487	
108	3 + 7 Me C_{29}	0.013 ± 0.010	-68.659	
155	11,15 DiMe C_{31}	0.011 ± 0.011	312.109	
	Formica aserva			
41	C_{25}	0.030 ± 0.025	-18.76	X
48	3 Me C_{25}	0.016 ± 0.014	114.04	X
67	C_{27} monoene	0.040 ± 0.028	236.93	X
68	C_{27}	0.062 ± 0.044	-43.51	X
104	C_{29} monoene	0.012 ± 0.012	352.9	X
105	C_{29}	0.016 ± 0.009	-40.39	X

Table 5. Influential compounds contributing to the dissimilarity observed between before and after CHCs of *F. subaenescens* hosts and their *F. aserva* nestmates and the relative difference in the abundance of these compounds as a result of separation.

Peak Number	Influential Compounds	Proportion Contribution (±SD)	Relative Difference (%)	Shared with heterospecific nestmates
	Formica subaenescens			
41	C_{25}	0.048 ± NA	-78.19	X
48	3 Me C_{25}	0.019 ± NA	-67.59	X
67	C_{27} monoene	0.067 ± NA	-100	X
68	C_{27}	0.074 ± NA	-78.78	X
69	11 + 13 Me C_{27}	0.025 ± NA	396.61	
82	C_{28} monoene	0.024 ± NA	-100	
93	8,12 DiMe C_{28}	0.039 ± NA	360.9	
105	C_{29}	0.022 ± NA	-75.43	X
140	8,12 DiMe C_{30}	0.024 ± NA	223.59	
	Formica aserva			
20	C_{23}	0.022 ± 0.004	132.43	
41	C_{25}	0.041 ± 0.015	-28.99	X
48	3 Me C_{25}	0.018 ± 0.006	264.4	X
67	C_{27} monoene	0.048 ± 0.017	177.39	X
68	C_{27}	0.102 ± 0.020	-49.62	X
105	C_{29}	0.026 ± 0.003	-54.18	X

* We only had one *F. subaenescens* sample for each of the before and after separation analyses so the % contribution represents actual values, not an average.

Table 6. Aggression scores based on the behavioral interactions observed in aggression assays of heterospecific nestmates

Aggression Score	Behavioral Interaction	Example
1	Touch	Physical contact including prolonged antennation
2	Avoid	Quick retreat of one or more ants following physical contact
3	Aggression	Lunging, biting, and pulling legs or antennae
4	Fight	Prolonged aggression between individuals

*Aggression scores and example behaviors obtained from Tsutsui et al. (2000)

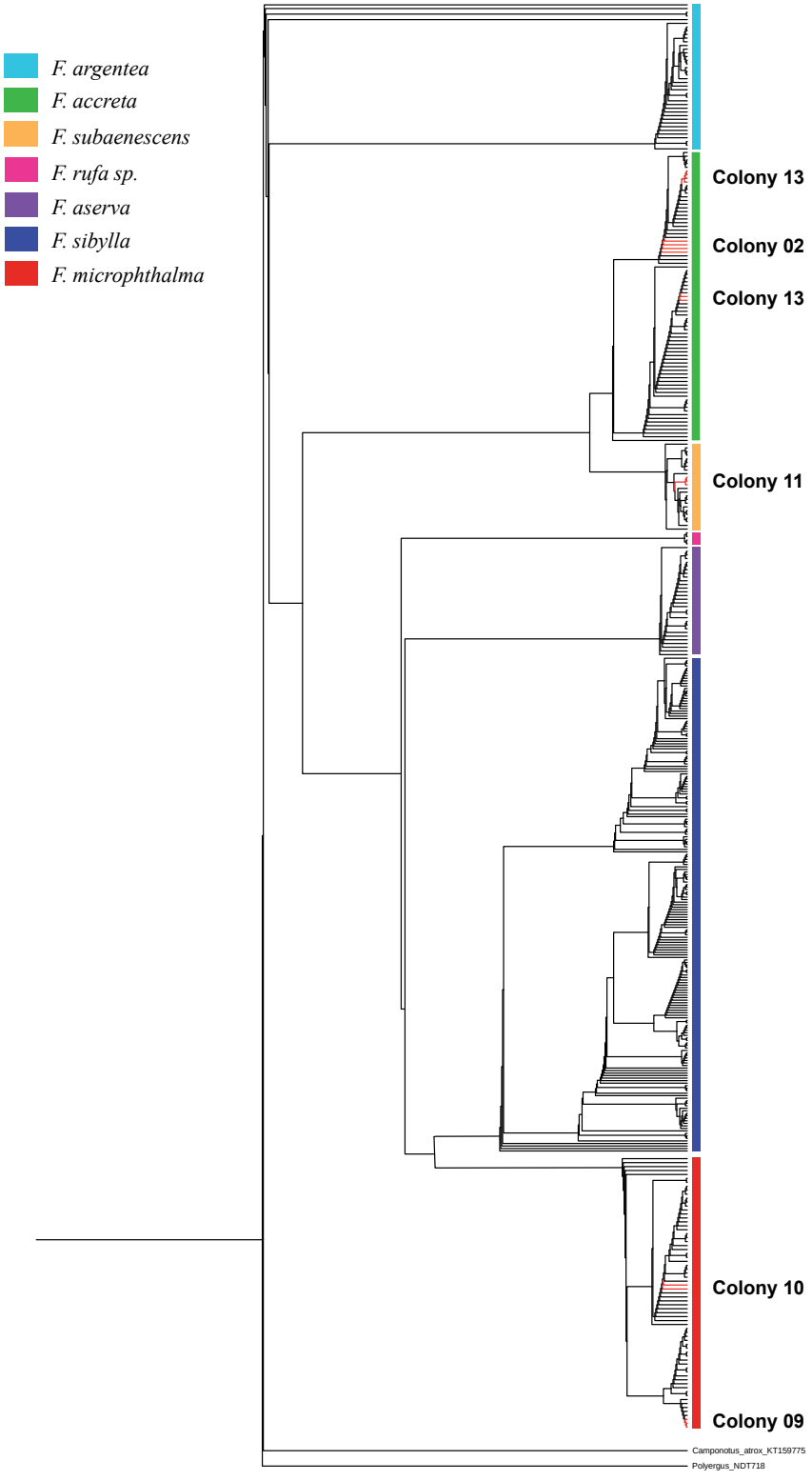
Table 7. Mean aggression scores of *F. aserva* and their cohabitating host species, derived from the seven experimental colonies in our study, before separation (T_0) and 45 days after separation.

Colony	*F. aserva* vs host species	Mean Aggression BEFORE (T_0) (N=6)	Mean Aggression AFTER (45 days) (N=6)	p-value
SAG.02	*Formica accreta*	1.0	3.0	0.004**
SAG.03	*Formica microphthalma*	1.0	3.0	<0.001***
SAG.09	*Formica microphthalma*	1.0	2.167	0.05*
SAG.10	*Formica microphthalma*	1.0	3.0	<0.001***
SAG.11	*Formica subaenescens*	1.0	3.0	0.004**
SAG.13	*Formica accreta*	1.0	1.334	0.19
SAG.14	*Formica microphthalma*	1.0	2.5	0.016*

*Aggression scores based on Table 6 and developed by Tsutsui et al. (2000)

APPENDIX 1:

Chapter 3 supplementary figures



S1. Maximum likelihood consensus tree based on a 680bp alignment of sequence fragments from the CO1 mitochondrial gene. Bars are color coded by species and red branches represent the individual host samples from the *F. aserva* colonies used in this study.