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Identification and Characterization of Trail Pheromones and Queen Pheromones in the Argentine Ant, *Linepithema humile* (Mayr)

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by

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Dedication

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ABSTRACT OF THE DISSERTATION

Identification and Characterization of Trail Pheromones and Queen Pheromones in the Argentine Ant, *Linepithema humile* (Mayr)

by

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Pheromones are integral to the communicative feedback loops that govern colony cohesion in social insects. These pheromones may be glandular in origin, as with trail pheromones, or may be deposited on the cuticle, like cuticular hydrocarbons. Whether glandular or cuticular, pheromones are often complex mixtures that include multiple glandular products. The first part of this dissertation identifies the volatile and cuticular chemicals found in the different castes and developmental stages of the Argentine ant, *Linepithema humile* (Mayr). Volatile headspace collections were made for live and macerated samples of queens, virgin female reproductives (gynes), males, workers, gyne larvae, and worker larvae. Queens and workers differed significantly in their relative proportions of dolichodial and iridomyrmecin, and also qualitatively in their saturated hydrocarbons (SHCs) and unsaturated hydrocarbons (UHCs). Queens were characterized by an abundance of 5-Me and 5,11-dimethylalkanes and a homologous series of long-chain UHCs. In Chapter 3, I explored the relative contributions of three worker-derived compounds to worker trail-following response and determined the absolute configuration

of ant-derived (natural) iridomyrmecin. Of the three compounds only *Z*9-16:Ald induced trail-following in worker. Natural iridomyrmecin was 94.5% (+)-iridomyrmecin, with small amounts of (+)- and (-)-isoiridomyrmecin. In Chapter 4 I showed that both saturated and unsaturated CHCs are required to inhibit workers from rearing new queens in queenless colonies, although the active queen pheromone components could not be delineated further. Finally, using four different diets ranging from nutrient-poor to highly-enriched, it was discovered that enriched and highly-enriched diets actually suppressed queen production. Queens from these colonies had significantly higher juvenile hormone levels than did queens fed standard diets.

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Chapter 1: Introduction

Chemical Communication in Social Insects

Most species of insects live solitary life styles, and rarely interact with conspecific individuals (Billen 2006). Although there are notable exceptions (e.g. gregarious locust phenotype, Pener and Simpson 2009; ladybird beetle winter aggregations, Wheeler and Cardé 2013; and bark beetle aggregations, Wermelinger 2004), most solitary insects encounter conspecific males or females briefly for the purpose of mating and never encounter one another afterward. Social insects, however, live in virtually constant contact with their nestmates. There are three characteristics of eusocial insect colonies (Michener 1974; Wilson 1975; but see Crespi and Yanega 1995): overlapping generations, cooperative care of young, and a reproductive division of labor. Eusocial insect colonies can take on a variety of forms, from primitively eusocial colonies in which there is little variation between reproductive and non-reproductive castes, typified by the relatively modest colony size of many ponerine ants, to massive colonies containing millions of individuals with many functional subdivisions, as in the leaf cutter ant genus *Atta*. Colonies exhibit a high degree of self-organization, and their behavioral plasticity enables them to negotiate variable internal and external conditions in order to reproduce and develop (Le Conte and Hefetz 2008). The foundation for these cohesive units is the coordination of behavioral and physiological systems across a large number of individuals through communication.

Communication through visual, tactile, acoustic, and magnetic modalities has been discovered for several species of eusocial insects, the predominant form of communication being chemical signaling through semiochemicals. The term semiochemical is derived from the Greek *semeion* = signal, and semiochemicals are divided into allelochemicals and pheromones. Allelochemicals function in an interspecific context and are categorized by the behavioral responses they elicit. Kairomones are alleochemicals which confer an advantage to the individual receiving the chemical signal, exemplified by the attraction of egg parasitoids to their host sex pheromone (reviewed in Vinson 1976). If the advantage is conferred upon the releaser of the signal, the allelochemical is called an allomone (Brown et al. 1970), including defensive secretions and alarm pheromones emitted by ants. Pheromones, as defined by Karlson (1958), are substances secreted by an individual that are perceived by and elicit a specific reaction from an individual of the same species.

Pheromones are divided into two categories based on the responses they elicit. Releaser pheromones elicit an immediate, specific behavioral response from the receiver and are categorized by the type of behavior they evoke (Bossert and Wilson 1963; Shorey 1973; Jackson and Morgan 1993). These include sex pheromones, dispersal or spacing pheromones, alarm pheromones, territorial pheromones, and trail/recruitment pheromones. In contrast, primer pheromones have delayed physiological and behavioral effects on the receiving individual(s), although they may have releaser effects as well (Le Conte and Hefetz 2008). These latent physiological and behavioral effects make studying

primer pheromones a challenge, mainly due to the difficulties in developing a robust bioassay that is sufficiently reproducible (Ali and Morgan 1990).

Early studies on pheromone identification and characterization supported a "one compound, one response" concept (Hölldobler and Carlin 1987), but it is now clear that most insect pheromones are composed of several components that may be derived from one or multiple glands. The first identified insect pheromone, the sex pheromone of the female silkmoth *Bombyx mori* (L.) (Schneider 1969), elicited a single specific behavior. This perceived specificity of a single component, coupled with the assumption that the variation found within insect blends was a product of sample contamination or background 'noise', may have influenced the way researchers approached the identification and characterization of insect pheromones (Wilson 1970; Hölldobler and Carlin 1987). As analytical and preparative chromatographic techniques improved, so too did our knowledge of the diversity and complexity of pheromones, especially within eusocial insects. Hölldobler and Carlin (1987) outlined three reasons that make eusocial insects excellent models for studying chemical communication. First, their ecological success hinges upon their ability to communicate information about the social state of the colony, as well as information about their ever-changing environment. Second, inclusive fitness suggests selection should favor discrimination based on variation that is correlated with relatedness, the genetic underpinnings of which have been shown to affect signals involved in nestmate and caste recognition. Third, pheromones play important roles across various ecological and organization levels within and between eusocial insect societies. It is highly unlikely that each level would be characterized by a single chemical

signal. For example, the venom gland of red imported fire ant queens (*Solenopsis invicta* Buren) contains an abundant alkaloid fraction (especially *cis*-2-methyl-6-undecyl piperidine), along with a minor neutral fraction (Vander Meer and Morel 1995). Complete extracts of queen poison sacs inhibited reproductive development of virgin queens, while venom alkaloids or neutral constituents alone were not inhibitory, indicating behavioral activity requires at least some combination of both fractions (Vargo 1998). Similarly, workers of the trap jaw ant *Odontomachus brunneus* (Patton) require background hydrocarbons in addition to a queen specific alkene in order to properly recognize the presence of a queen (Smith et al. 2015). These two examples highlight the integral role complex chemical signals have in maintaining the social organization of ant colonies. Herein I will describe how these multicomponent signals affect two principle features of ant societies – trail-following/recruitment and signaling of the reproductive caste.

Trail Pheromones in Ants

Even before the advent of modern analytical methods, it had long been recognized that ants followed odor trails (Bonnet 1779; Forel 1886, 1908; Eidmann 1927; Santschi 1911, 1930). In one of the first examples of using natural extracts to test bioactivity, Goetsch (1934) showed that ants would follow trails made from the gaster contents of a freshly killed ant. Carthy (1951) was able to visualize trails laid by *Lasius fuliginosus* (Latreille) workers by dusting trails with fungal spores, and noted that ants followed these trails closely with their antennae. The classic view of trail pheromone organization established

several simple behavioral rules. Wilson (1962) showed that *Solenopsis saevissima* (Smith) workers recruiting to a newly discovered food source deposited trail pheromone as they acquired resources, and that the probability of leaving the nest was related to the amount of pheromone deposited. This allowed *S. saevissima* colonies to regulate recruitment to a resource and to cease trailing once that resource was exhausted. Hangartner (1967, 1969) found that workers of *L. fuliginosus* followed trails relative to the amount of pheromone on it, and workers deposited more pheromone when foraging to a higher-quality resource. Combining these rules detailed a system in which ants communicate resource quality and availability simply by regulating the quantity of pheromone deposited, allowing for signal parsimony (Czaczkes et al. 2015).

Other studies have discovered more nuanced pheromone systems in ants. In some species, workers may 1) prevent other workers from trailing by using 'no entry' pheromones (Robinson et al. 2005), 2) use long-lasting pheromones as an external memory which can be reactivated using more volatile pheromones (Czaczkes et al. 2015), 3) prevent overcrowding by signaling density (Grüter et al. 2012), 4) determine potential nest cavity size based on intersection with previously laid trails (Mallon and Franks 2000), and 5) recruit to a resource from a previously established trail (Flanagan et al. 2013). Indeed, trail pheromones are not used to simply recruit individuals to a resource. They are used in various contexts depending upon the ecology and biology of the species. They can be used to convey directional information (Jackson et al. 2004; Jarau 2009), for locating a nesting site (Cronin 2012), to recruit colony members to areas of conflict (Cammaerts-Tricot and Verhaeghe 1974; Hölldobler 1976), to guide the construction of

tunnels (Theraulaz et al. 1998), or even to measure the size of potential nest cavities (Mallon and Franks 2000).

Although the historical view of pheromoens has been that of "one compound, one function", pheromones are generally multicomponent signals (Holldobler 1999). An active pheromone blend may contain several substances which act in a modulatory or synergistic fashion. In the pavement ant *Tetramorium caespitum* (L.), the trail pheromone was found to be a blend of two pyrazines that were maximally active at a ratio of 3:7. Vander Meer et al. (1988) showed that two different behavioral subcategories involved in mass recruitment, recruitment and orientation to the pheromone, were mediated by different sets of compounds. The recruitment subcategory was governed by the orientation pheromone (*Z*, *E*)-α-farnesene and an unidentified homosesquiterpene. The orientation subcategory was mediated by two farnesenes and two homofarnesenes. In some species, multiple substances produced by multiple glands regulated the entire suite of recruitment behaviors. In the harvester ant *Pogonomyrmex badius* (Latreille), a long-lasting 'homing' pheromone is released in part from the Dufour's gland, while a more volatile recruitment pheromone is produced in the poison gland (Hölldobler and Wilson 1970). This recruitment signal is apparently similar across several *Pognomyrmex* species, while the Dufour's gland secretions contain species-specific hydrocarbons (Regnier et al. 1973; Hölldobler 1995).

Similarly, the trail pheromone of the Argentine ant *Linepithema humile* (Mayr) is made up of multiple substances excreted by multiple glands. The optimally active blend is composed of at least three identified compounds derived from the pygidial and Pavan's

gland (Cavill et al. 1980; Van Vorhis Key and Baker 1982; Choe et al. 2012), although other components may affect certain aspects of trail-follwoing. The first compound identified from Pavan's gland, (*Z*)-9-hexadecenal (*Z*9-16:Ald), was found to elicit trail-following in workers. Recently, Choe et al. (2012) found only two pygidial gland components, dolichodial and iridomyrmecin, from extracts of ant trails. However, it is unclear how these compounds function independently, or if they modulate or synergize the activity of one another.

Queen Pheromones

Signaling reproductive status and/or dominance is thought to be a major factor in queen-worker division of labor in many eusocial hymenoptera (Liebig et al. 2000; Le Conte and Hefetz 2008; Smith et al. 2015). Here, a sterile (facultative or obligatory) worker caste increases its inclusive fitness by allocating resources to support a highly fecund individual or caste rather than developing their own reproductive capacity. In primitively eusocial ant societies there are often no morphological differences between the primary reproductive caste and the worker caste (Liebig et al. 2000; Cuvillier-Hot et al. 2002, 2004). In these societies, primary or dominant reproductive individuals or castes are established through aggressive interactions, resulting in the formation of a reproductive hierarchy (reviewed in Kocher and Grozinger 2011). In many cases, once these reproductive hierarchies are established, there is a decrease in aggressive interactions, which may be mediated by pheromones associated with the dominant reproductive individual or caste (Cuvillier-Hot et al. 2002; Le Conte and Hefetz 2008). In many

ponerine ants, the establishment of reproductive hierarchies and accompanying ovarian activation are correlated with changes in cuticular hydrocarbon (CHC) profiles (Monnin et al. 1998; Peeters et al. 1999; Cuvillier-Hot et al. 2002; Liebig et al. 2000). In more advanced species, there are obvious morphological differences between queens and workers, and aggressive interactions between castes are rarely observed. Instead, queen pheromones are believed to have replaced physical aggression (Le Conte and Hefetz 2008), although detailed studies confirming complete pheromone identity and full biological activity are rare.

Queen pheromones regulating worker sterility have been identified and characterized in two species within the subfamily Formicinae: the black garden ant *Lasius niger* (L.), and the Iberian ant *Cataglyphis ibericus* (Emery). In *L. niger*, 3-methylhentriacontane (3-MeC_{31}) comprised the majority of the chemical profile of queens and their eggs (Holman et al. 2010a). Application of synthetic pheromone to glass beads simulating queens inhibited worker ovarian activation, affected worker aggression response, and was perceived by worker antennae (Holman et al. 2010b). Additionally, the pheromone appeared to reduce fecundity in treated queens, suggesting regulation of reproductive activity via negative feedback (Holman et al. 2012). In *C. ibericus*, contact with specific straight-chain and methyl-branched alkanes decreased the odds of workers having fully developed ovaries, and increased the likelihood of having regressed ovaries (Van Oystaeyen et al. 2014). These studies suggest that queen-specific signals do not need to be present with the background chemicals, which function in assessing group membership. In order to confirm the ability of workers to properly

recognize queen-specific signals, additional studies are required that investigate the effectiveness of extracts of the entire chemical profile. It is highly likely that signals associated with the queen are perceived in context with other chemicals, and in some ant species the queen pheromone appears to consist of a complex blend of chemical signals (Smith et al. 2015; Vargo and Hulsey 2000).

Experimental evidence for multicomponent queen pheromones has been established for several ant species. In the red imported fire ant *S. invicta*, reproductively active, inseminated queens inhibited the production of sexuals (male and female) and inhibited virgin female reproductives from shedding their wings and developing their ovaries (Fletcher and Blum 1981; Vargo and Fletcher 1986; reviewed in Vargo 1998). A robust bioassay was developed which indicated that queen corpses and queen glandular extracts were inhibitory, although not to the same degree as living queens. This activity was later found to reside in constituents of both the poison sac and the postpharyngeal gland (Vargo 1998; Vargo and Hulsey 2000). The poison sac contain a number of venom alkaloids and pyranones, while the postpharyngeal gland contents in ants are usually composed of saturated and unsaturated hydrocarbons that are similar in qualitative composition to cuticular hydrocarbons. Therefore, it appears *S. invicta* queens are able to signal their presence using mixtures of substances, which originate in two separate glands, and are very different in their chemical nature.

Recently, additional evidence for a multicomponent queen signal was reported in the trap jaw ant *O. brunneus* (Smith et al. 2015). The authors showed that the fertility signal of queens required the full complement of background chemicals in order for

accurate perception by workers. Additionally, they provided evidence that perception of an ant as a nest-mate or a foreign conspecific took precedence over fertility signaling.

In *L. humile,* pheromones are thought to regulate queen production in both field and laboratory colonies. Field populations of *L. humile* are highly polygyne, containing up to 16 queens per 1000 workers (Markin 1970). Each spring, new queens are produced following the elimination of ca. 90% of functional queens, a behavior thought to reduce the inhibitory influence of queens (Markin 1970; Keller et al. 1989; Vargo and Passera 1991; Passera et al. 1995). Laboratory colonies that have been orphaned will rear new queens from early-instar larvae, but the daily addition of queen corpses inhibits new queen production (Vargo and Passera 1991). Extracting queen corpses with pentane prior to placement in colonies does not inhibit queen production, suggesting a queen pheromone used by workers to identify the reproductive caste is removed with organic solvents (Vargo and Passera 1991). However, direct experimental evidence for extract activity is still needed to confirm this hypothesis. There is also a need for a more robust bioassay. Colony fragments in which queens have been removed will rear new queens to some degree year-round, but brood that have gone through an overwintering period are far more likely to develop into queens. If there is some way to speed up development time and increase the probability of an egg or early instar larvae developing into a queen, then bioassays could be conducted at any time and greatly speed up the screening process for putative pheromones.

Characterizing Multicomponent Pheromones of the Argentine Ant

My research had two primary goals: 1) to identify the volatile and nonvolatile chemicals associated with all castes of the Argentine ant in order to gain a better understanding of both caste-associated and background chemicals, and 2) to isolate insect-derived extracts into smaller, testable subsets in order to identify the roles of both caste-specific and shared chemicals. Herein, I describe the identification of two multicomponent pheromone systems based on numerous caste-specific chemical analyses - the Argentine ant trail-pheromone and queen pheromone - and bioassay-guided experiments to analyze how caste-specific chemicals function alone or in the context of background chemicals. This work provides novel information on the relative importance of individual chemicals in eliciting a biological response, and describes a methodology for separating and testing complex cuticular extracts.

In Chapter 2, I analyzed and compared the volatile and cuticular chemical components of all Argentine ant castes, as well as queen and worker larvae. Previous work had focused primarily on the worker caste, and this study identified caste-specific ratios of the pygidial gland components dolichodial and iridomyrmecin, as well as differences in saturated and unsaturated cuticular hydrocarbons. I also present evidence for the first (*Z*)-14-monounsaturated hydrocarbon identified in ants.

In Chapter 3, I determine the bioactivity of three compounds believed to be involved in Argentine ant mass recruitment, either individually or in combination, and to determine the absolute stereochemistry of iridomyrmecin, which has not yet been confirmed (Choe et al. 2012). First, natural dolichodial and iridomyrmecin (collectively

referred to as iridoids) were purified from crude worker extract using a combination of preparative liquid and gas chromatography, followed by selective reduction of the iridomyrmecin fraction to remove trace amounts of dolichodial and/or *Z*9-16:Ald. Trail following by Argentine ant workers was then measured by applying individual purified iridoids or synthetic *Z*9-16:Ald to treated and measuring the time and distance workers spent following trails. A second set of trail-following assays were conducted to determine if combinations of chemicals had modulatory or synergistic roles. While we found no evidence for the involvement of the iridoids in trail following, gas chromatography-electroantennographic detection (GC-EAD) showed worker antennae responded to iridomyrmecin. Finally, the absolute configuration of Argentine ant-derived iridomyrmecin was determined by comparing the retention time and peak shape of natural iridomyrmecin to those of four synthetic stereoisomers of iridomyrmecin and isoiridomyrmecin. Natural iridomyrmecin is 94.5% (+)-iridomyrmecin, with small amounts of (+)- and (-)-isoiridomyrmecin.

In Chapter 4, I provide the first experimental evidence that *L. humile* queen extracts are able to suppress the production of new queens in orphaned laboratory colonies. The signal consisted of both saturated and unsaturated compounds, which conflicts with previous studies in which individual queen-specific chemicals were found to be inhibitory. These active fractions were then separated by chain length, recombined, and tested for activity. Although it appears no single subset of compounds is necessary for bioactivity, treatments in which the most abundant subsets were recombined failed to inhibit queen production, which suggests there is a minimum threshold of chemical

information required for workers to accurately perceive the queen signal. These results suffered from the lack of a robust and efficient bioassay, which I attempted to address in Chapter 5. Herein, I explored the effects of nutrition on queen production, juvenile hormone (JH) titer, weight, and fecundity, with the goal of identifying potential maternal effects that influence caste development. I found that queen production was negatively correlated with JH titer, and that there is an inverse correlation between weight and fecundity in queens fed an enriched or highly-enriched diet.

Overall, these studies provide new information about the multicomponent pheromone systems used by Argentine ants for trailing and for identifying queens, as well as how nutrition influences developmental fate. I also describe a bioassay-guided approach for systematically testing complex natural mixtures of putative nest-mate and caste recognition signals for bioactivity.

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Chapter 2: Volatile and non-volatile chemicals are caste-specific in the Argentine ant, *Linepithema humile* (Mayr)

Introduction

The ability to distinguish between nestmates and non-nestmates is critical to the success of social insect colonies. Efficient discrimination also prevents exploitation by an individuals' own species, as well as parasitic or cleptobiotic species (Hölldobler and Wilson 1990). In ants, discrimination of nestmates and alien individuals is mediated primarily through chemical information encoded in an individuals' cuticular hydrocarbon (CHC) profile (Hefetz 2007; Lahav et al. 1999; Suarez et al. 2002; Vander Meer and Morel 1998). These hydrocarbon profiles are composed of long-chain alkanes and alkenes, usually between 25 and 35 carbons in length. CHCs may possess modifications in the form of methyl-branching or sites of unsaturation, and these modifications often encode vital information about kinship, colony of origin, and/or reproductive capacity.

Reproductive signaling is a form of caste or nestmate recognition and is vital to the success of the colony. The ability to assess the presence and/or quality of the reproductive caste allows workers to allocate resources in a way that maximizes the inclusive fitness of all colony members. Clear associations between reproductive status and differences in CHCs have been demonstrated for over 30 ant species (Cuvillier-Hot et al. 2004; de Biseau et al. 2004; Dietemann et al. 2003; Liebig et al. 2000; Monnin et al. 1998; Smith et al. 2015; Van Oystayen et al. 2014). Direct evidence for CHCs signaling reproductive status has been found far less frequently (Van Oystaeyen et al. 2014).

Saturated hydrocarbons (alkanes) have been found to inhibit worker reproduction in several species. In these studies individual alkanes were presented without other background chemical components, and reportedly inihibited worker reproduction or ovary development. In contrast, the trap jaw ant, *Odontomachus brunneus* (Patton), an unsaturated hydrocarbon (alkene) elicited a response from workers but only in the context of the background chemical profile (Smith et al. 2015). In the red imported fire ant *Solenopsis invicta* (Buren), poison gland and postpharyngeal gland (PPG) extracts were both found to signal the presence of a mated queen (Vargo and Hulsey 2000). Together these results suggest queen signaling varies dramatically across species and is likely composed of multiple compounds that must be perceived in the appropriate chemical and/or environmental context.

In Argentine ants, *Linepithema humile* (Mayr), queens play an integral role in the production of female sexuals. In laboratory colonies, queenless colony fragments produce new queens from early instar larvae, presumably by altering larval nutritional status (Vargo and Passera 1991, 1992). The daily addition of queen corpses to queenless laboratory colonies prevents queen production, while the addition of queen corpses rinsed with pentane does not prevent queen production, suggesting removable chemical signals are mediating this behavior. This queen signal is known to differ from worker CHCs in the presence of 5-methylalkanes and several alkenes (de Biseau et al. 2004), although these alkenes are obscured partially by queen alkanes (Neff, unpublished data). Moreover, nothing is known about the volatile constituents of Argentine ant queens and how these might play a role in queen recognition.

The aim of this study was to identify the major CHC and volatile constituents from every adult caste, as well as two juvenile stages (gyne and worker larvae), in order to 1) determine which compounds might be involved in reproductive signaling, and 2) identify caste-specific chemicals that have a potential signaling function. We analyzed saturated hydrocarbons (SHCs) and unsaturated hydrocarbons (UHCs) of *L. humile* queens, virgin female reproductives (gynes), and males. The proportions of eighteen alkanes present in all samples were compared between castes. We identified double bond positions in alkenes by epoxidation of concentrated natural queen extracts. Volatile chemical profiles were analyzed by aerating whole insects and trisected body parts, in order to compare the chemicals released by live insects to the total body content. Our results reveal qualitative and quantitative differences in saturated and unsaturated CHCs, and volatile constituents between castes of Argentine ants. These results provide a baseline for future studies exploring the roles of caste-specific compounds as signals that mediate behavioral interactions among colony members.

Methods

Ant collection and maintenance

Ants were collected by excavation from citrus groves under biological control or from a landscaped area near the Insect Quarantine Facility located on the UC Riverside campus. Ants were excavated from soil and transferred to 0.61 m x 0.91 m wooden boxes housed lined with Fluon to prevent escape. Heat lamps were placed over the soil and ants moved eggs, brood, and queens into moistened plaster of Paris disks (9 cm diameter). The ants

were transferred and maintained in large Sterilite® containers on a 12:12 LD cycle (24°C, 80% RH) and fed an artificial diet, American cockroaches (*Periplaneta americana* L.), and water. In addition to insect protein (*P. americana*), colonies were given 0.1 ml of a nutrient-rich diet three time per week. The diet consisted of 5 g protein and vitamin-rich powder (Optimum Nutrition Inc., Aurora, IL), 2 g royal jelly (eBeeHoney, Ashland, OH), and 35 ml water.

Separation and analysis of CHCs

Queens, gynes, males, workers, or 4th instar gyne or worker larvae (20, 20, 50, 100, 20, and 60, respectively) were freeze-killed and immersed in 2 ml hexanes for 10 min. Extracts were concentrated to 500 µl under a stream of N_2. Columns prepared from Pasteur pipettes plugged with glass wool were loaded with 250 mg of silica gel impregnated with silver nitrate (10% w/w, SigmaAldrich, St. Louis, MO) and oven-dried overnight at 120°C. After cooling, each column was rinsed with hexane and loaded with crude ant extract from one of the aforementioned groups. The column was eluted sequentially with 4 ml hexanes (saturated fraction; SHCs), 2 ml of 5% cyclohexene in hexanes (unsaturated fraction; UHCs), and 2 ml ethyl ether (polar fraction). Fractions were evaporated to 50 µl, and 1 µl of each sample was spotted on a silica gel TLC plate. Plates were developed using a 9:1 hexane – EtOAc solvent system and visualized by heating plates following immersion in phosphomolybdic acid in EtOH. Exactly 1 µl of each sample was analyzed by GC-MS. For GC-MS, electron impact mass spectra (70 eV) were taken with an Agilent 5975C mass selective detector interfaced to an Agilent 7890A

gas chromatograph fitted with a DB-5 column (30 m x 0.32 mm inner diameter, Agilent Technologies, Santa Clara, CA). Extracts were injected in splitless mode using a temperature program of 50°C for 1 min and then 10°C min to 300°C with a 30 min hold. The temperature of the injector and transfer lines was 280°C. Compounds were identified by comparing their retention indices to those of straight-chain alkane standards, by their characteristic fragmentation patterns (Carlson et al. 1998; Lenoir et al. 1997; Monnin et al. 1998), and by reference to previously identified compounds from *L. humile* queens and workers (Brophy et al. 1983; Cavill and Houghton 1973; de Biseau et al. 2004; Sunamura et al. 2009).

Epoxidation of alkenes

The unsaturated fraction obtained from 100 mated *L. humile* queens was evaporated to dryness in an 8 ml glass vial to which a small magnetic stir bar, and 0.2 mg *m*-CPBA in 2 ml methylene chloride were added. The resulting mixture was stirred for 3 min an ice bath. The methylene chloride was evaporated to dryness and the residue was partitioned between 0.8 M aqueous NaOH and 2 ml hexanes. The hexane fraction was concentrated to 100 µl and purified by small-scale column chromatography. Columns consisted of Pasteur pipettes plugged with glass wool loaded with 250 mg of silica gel. The column was first eluted with 4 ml hexanes to remove unreacted hydrocarbons. The epoxidized products were eluted with a 9:1 hexanes: ethyl acetate solvent system, concentrated to 50 µl, and analyzed by GC-MS as previously described. To verify our results synthetic

standards of identified alkenes were derivatized and their retention times and fragmentation patterns compared with the insect-produced compounds.

Collection of volatile compounds

Volatile compounds were collected from the following living castes and developmental stages: 3 mated queens, 3 gynes, 15 males, 15 workers, 10 4^{th} instar gyne larvae, and 30 2^{nd} to 3^{rd} instar worker larvae. Ants were anesthetized with CO_2 and placed inside 127 mm x 20 mm diameter double-ended clean glass chambers and held in place with soxhlet-extracted glass wool. For crushed and trisected body aerations, the same numbers of individuals were used. Ants were anesthetized and trisected with a razor blade before being placed inside aeration chambers. Body parts were then crushed using a clean Pasteur pipette. Chambers were swept with air (20 ml/min) purified by passage through activated charcoal filters, with volatiles collected on 50-200 mesh thermally-desorbed activated charcoal traps held in place by glass wool plugs placed 2 cm above the insects within the chamber. Volatiles were collected for 24 h and recovered from traps by elution with 1 ml methylene chloride and concentrated to ~10 µl under a constant stream of N_2. Exactly 1 µl of each sample was injected into an HP 6890 gas chromatograph equipped with a DB-17 column (30 m x 0.25 mm i.d. x 0.25 µm film) coupled with an HP 5973 mass selective detector. Helium was used for the carrier gas. Injections were made in splitless mode, and the oven temperature was programmed from 50°C for 1 min then 10°C per min to 280°C. Injector and detector temperatures were 150°C and 250°C, respectively. Transfer line temperature was 275°C. Identifications of dolichodial and

iridomyrmecin were confirmed by comparing their mass spectra to those of synthetic standards (a gift from J. Hofferberth, Kenyan College, OH) and by comparison with volatile constituents previously reported for *L. humile* (Cavill and Houghton 1974a). Identification of 2,5-dimethyl-3(3-methylbutyl)pyrazine was confirmed by comparison with previously reported pyrazines in *L. humile* and by analyizing fragmentation patterns (Cavill and Houghton 1974b).

Data analysis

Eighteen alkanes were selected in the GC traces of queens, gynes, and workers for between-caste comparison. Males were excluded due to low sample size. The selected compounds were manually integrated and the relative abundance of each compound converted into percent of selected compounds. We used Kruskal-Wallis ANOVA with Conover-Inman post-hoc test ($\alpha = 0.05$) (Systat 2013) to compare differences between castes for both individual and classes of compounds. The 3 + 5,X dimethyl alkanes were analyzed separately because they co-eluted and the proportion of each could not be determined accurately in all groups.

Results

Eighteen alkanes found in queens, gynes, and workers were selected for between-caste comparisons (Fig. 2.1, Table 2.1). Representative chromatograms for each caste are found in Figures S2.3-S2.7. There were significant differences in the percentages of saturated hydrocarbon classes between castes (Fig. 2.1; Kruskal-Wallis ANOVA, H =

37.0, df = 2, $P < 0.01$), although this difference was borderline when comparing gyne and worker CHCs (Conover-Inman post-hoc comparisons; Gyne vs. Worker: $P < 0.05$; Queen vs. Gyne: $P < 0.01$; Queen vs. Worker: $P < 0.01$). When the abundance of the 18 compounds between castes was compared, all compounds except for *n*-C_{28} ($P = 0.45$) and *n*-C_{33} ($P = 0.08$) were significantly different between castes (Fig. S2.1). Post-hoc tests on compounds that were different between castes showed queens were not different from gynes in their proportions of 13- + 11-MeC_{31} ($P = 0.80$), 5,X-diMeC_{33} ($P = 0.50$; X = 17, 15, 13, 11), or from workers in the proportion of n-C_{27} ($P = 0.96$). Gynes and workers were not different in the abundance of 5-MeC_{27} ($P = 0.08$), 17- + 11-MeC_{33} ($P = 0.15$), 5,13,17-triMeC_{33} ($P = 0.06$), or 5,X-diMeC_{35} ($P = 0.06$; X = 17, 15, 13, 11).

There were significant differences between castes for *n*-alkanes, monomethyl alkanes, dimethyl alkanes, and trimethyl alkanes (H = 24.0, 22.7, 16.6, 21.9, respectively; $P < 0.01$ for all groups; Fig. 2.1). Queens had the highest abundance of *n*-alkanes (26.5 ± 4.0%) compared with gynes and workers (6.6 ± 3.6%, 12.6 ± 2.0%, respectively). Queens also had the highest abundance of monomethyl alkanes at 45.6 ± 4.4% relative to gynes (10.6 ± 1.5%) and workers (1.1%). This increase was in large part due to the high abundance of 5-Me alkanes present in queen profiles, which constituted 40.1 ± 6.3% of their total alkanes. Gynes and workers had similar levels of both dimethyl- and trimethylalkanes (22.0 ± 4.1% and 25.2 ± 1.8%; 52.2 ± 9.0% and 50.5 ± 3.4%, respectively) compared with queens (8.9 ± 2.5% and 5.8 ± 2.0%). The co-eluting 3-Me + 5,X-diMe homologues, containing carbon backbones of 27, 29, and 31, were

considerably greater in abundance in queens than in gynes and workers, with workers having significantly less than gynes ($P < 0.01$). Based on overall abundance in CHC

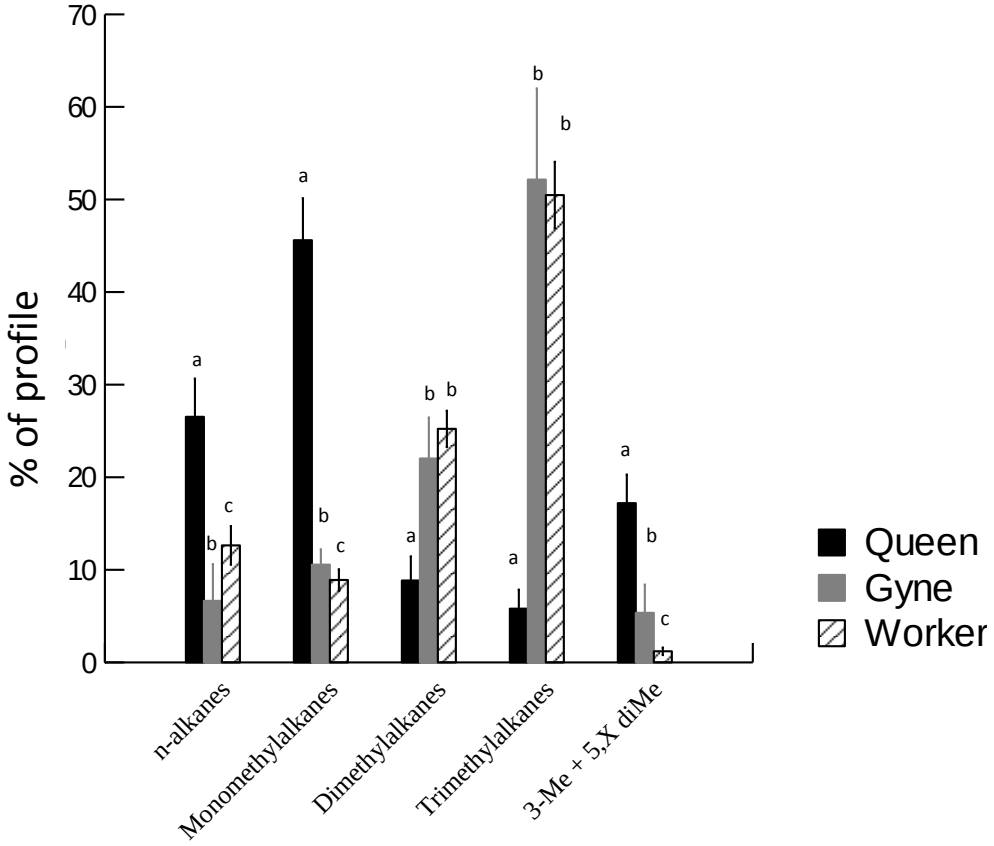

Figure 2.1 Percent (mean ± SE) of saturated alkanes, as a percent of total saturated CHCs, in the CHC profiles of different *Linepithema humile* castes. X = 17, 15, 13, 11 for 5,X dimethyl group. 3-Me + 5,X diMe were analyzed separately due to their co-elution in queens. Letters above bars indicate significant differences between castes (Kruskal-Wallis ANOVA; $P < 0.01$). All castes were significantly different except for Gyne-Worker percentages of dimethylalkanes ($P = 0.13$) and trimethylalkanes ($P = 0.35$; Conover-Inman pairwise comparisons).

Table 2.1 Percent relative abundance (mean ± SD) of methyl-branched alkanes that discriminate adult queens from gynes and workers.

Peak[a] no.	Compound	KI[b]	Queens[c] (*n* = 14)	Gynes[c] (*n* = 7)	Workers[c] (*n* = 9)
11	5-MeC27	2750	1.97 ± 0.77a	0.10 ± 0.08b	0.04 ± 0.02b
14	5-MeC28	2848	0.24 ± 0.21	0	0
15	4-MeC28	2855	0.85 ± 0.34	0	0
22	5-MeC29	2957	19.74 ± 4.00a	1.10 ± 0.60b	0.17 ± 0.16c
23	3-Me ; 5,17- + 5,11-diMeC29	2984	11.59 ± 2.28a	3.03 ± 2.00b	0.39 ± 0.11c
26	5-MeC30	3047	0.30 ± 0.19	0	0
27	4-MeC30	3055	0.75 ± 0.21	0	0
33	5-MeC31	3152	14.75 ± 3.57a	0.68 ± 0.39b	0.11 ± 0.12c
34	3-Me ; 5,17- + 5,11-diMe C31	3176	4.50 ± 0.94a	1.67 ± 0.82b	0.54 ± 0.21c
46	5-MeC33	3349	3.11 ± 0.88	0	0

[a] Peak numbers correspond to those found in Table S2.1.
[b] Kovat's values were determined based on compound retention time on a DB-5MS column.
[c] Males were excluded due to low sample number (*n* = 3). Compounds found in all castes were analyzed by Kruskal-Wallis ANOVA ($P < 0.01$ for all shared compounds). Letters next to values indicate significant differences using Conover-Inman multiple comparisons test with Bonferroni adjustment.

profiles of various castes, mated queens are characterized by 5-Me and 5,X-diMe alkanes with 27, 29, and 31 varbon chains (Table 2.1). Queens also possessed small amounts of even-numbered 5-Me and 4-Me alkanes, also with carbon chains between 27 and 32, that were not observed in other castes. Conversely, alkanes of gynes, workers, and males alkanes were dominated by higher molecular weight compounds with carbon backbones between 33 and 37 (71.0%, 88.7%, and 89.3%, respectively). This can be attributed largely to the presence of peaks 48, 49, 54, 56, 60 and 62 (Table S2.1). Two dimethylalkanes (compounds 44 and 51) were significantly higher in abundance in gynes, workers, and males than in queens. Both gyne and worker larvae had much simpler profiles compared with adults of the same caste. Alkanes of gyne larvae consisted primarily of *n*-alkanes with 25, 27, and 29 carbon backbones (92.4%), with smaller amounts of *n*-heptacosane and *n*-hentriacontane (6.0% combined). Gyne larvae were unique in their alkane composition, having what appeared to be homologus 3,23-dimethylalkanes with 31, 33, and 35 carbon backbones (Fig. S2.8). Worker larvae alkanes were mostly 27 and 29-carbon *n*-alkanes (64.1%) but differed from gynes in their abundance of 3-MeC_{27} and 3-MeC_{29}, which comprised 20.3% of their alkanes (Figs. S2.14, S2.15).

There were marked differences in alkene composition between castes as well. Queens had large amounts of (*Z*)-14- and (*Z*)-9- alkenes with 29, 31, and 33 carbon backbones, which were not present in workers, males, or larvae of either gynes or workers (Figs. S2.9, S2.10). Workers, males, and both larval castes possessed only short-

chain alkenes with 14, 16, 18, 19, and 20-carbon backbones (Figs. S2.12, S2.13). These long-chain alkenes were present in small amounts on gyne cuticles (Fig. S2.11).

Volatile constituents varied qualitatively and quantitatively between different castes and body segments, and also between live and crushed samples (Table S2.3; Figs. S2.16-S2.30). Volatile compounds included saturated and monounsaturated hydrocarbons, the iridoids dolichodial and iridomyrmecin, and an alkyl pyrazine. In general, compounds 63, 65, and 67 were primarily found in aerations of crushed heads, with compounds 64 and 66 being the main components of crushed gaster aerations (Table S2.2). Crushed thoraces of all castes yielded only trace amounts of iridoids, likely due to their close association with workers prior to removal from stock colonies, along with several small unidentified peaks. Aerations of live ants showed queens produced significantly more dolichodial than iridomyrmecin, while workers produced more iridomyrmecin than dolichodial (Figs. 2.3, 2.4). Aerating crushed queens yielded similar results (Fig. S2.17). Aerations of crushed queen heads yielded almost nothing, with only trace amounts of iridoids noticeable against the background. Aerations of live and crushed workers showed an opposite trend, with workers having low levels of dolichodial and comparatively high amounts of iridomyrmecin compared with queens and gynes. Crushed worker heads contained an alkyl pyrazine, *n*-heptadecane and (*Z*)-9-nonadecene. Aerations of live and crushed males showed a near-complete absence of iridoids, which were occasionally present in trace amounts and were likely acquired during contact with workers during the collection process. An alkyl pyrazine was the primary constituent and was found along with *n*-heptadecane and (*Z*)-9-nonadecene in aerations of crushed head

capsules. Worker larvae volatile compounds were not found in any appreciable concentration. There were trace amounts of iridoids and the pyrazine in both live and crushed samples, with *n*-heptadecane as the base peak in both cases.

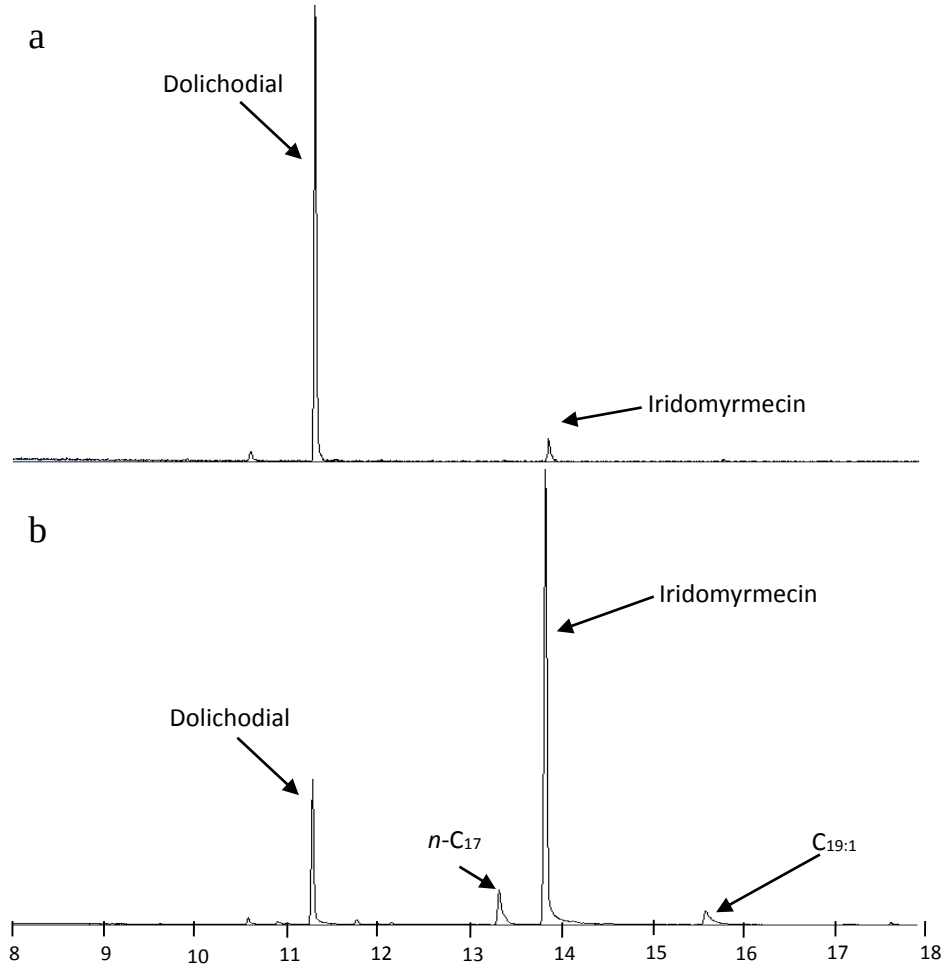

Figure 2.2 GC traces showing differences in the volatile constituents obtained by aerating a) live queens and b) live workers (DB-5MS column; $n = 8$ for both groups).

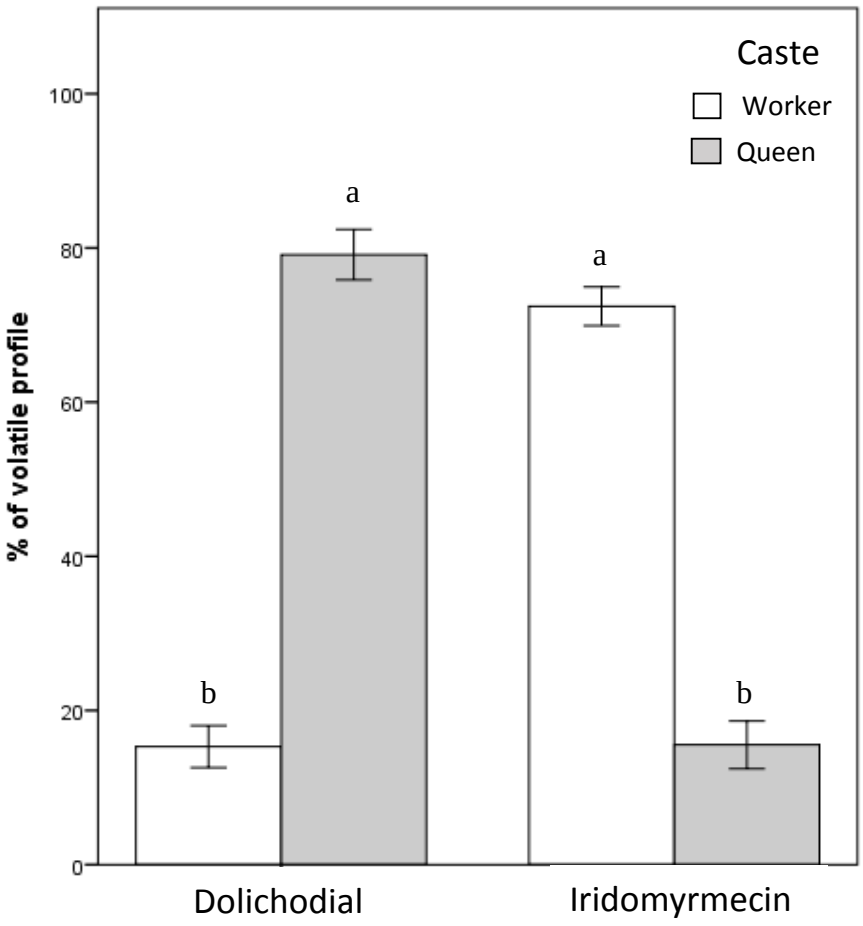

Figure 2.3 Percent (mean ± SE) of the total volatile headspace profile made up of the two dominant pygidial gland products, dolichodial and iridomyrmecin, for queens and workers. Dolichodial was significantly higher in relative abundance in queens (79.1 ± 10.34%) than in workers (15.3 ± 8.6%). Relative abundance of iridomyrmecin was significantly higher in workers (72.4 ± 7.9%) than in queens (15.5 ± 9.7%).

Discussion

Our results show that both the volatile and CHC constituents of *L. humile* differ qualitatively and quantitatively among castes and developmental stages. Our results corroborated previous studies that showed queens of *L. humile* differed from workers (de Biseau et al. 2004; Vasquez et al. 2009). These differences were primarily in the presence of 5-methyl and 5,11-dimethylalkanes, specifically ones with 27, 29, and 31-carbon chains. The presence of a series of long-chain alkenes in queens was particularly striking, because these compounds were not detected at all in workers. Other studies have reported the presence of (*Z*)-9 alkenes in both workers (Martin et al. 2008) and queens (Smith et al. 2013), but our study is the first to report the presence of the isomer (*Z*)-14 in the Formicidae. Both isomers occurred in odd chain-lengths (C_{29}, C_{31}); (*Z*)-9-$C_{30:1}$ was the only identified even-chain alkene.

Queen-specific hydrocarbons have been identified for numerous species from five ant subfamilies, including Ponerinae (Cuvillier-Hot et al. 2004; Heinze et al. 2002; Liebig et al. 2000; Monnin et al. 1998; Smith et al. 2013), Myrmicinae (Eliyahu et al. 2011), Formicinae (Endler et al. 2006; Hannonen et al. 2002; Holman et al. 2010b; Holman 2012), and Dolichoderinae (de Biseau et al. 2004), but functional roles have been established for very few (reviewed in Van Oystaeyen et al. 2014). Experimental evidence from ants in the subfamily Formicinae suggest saturated alkanes are used by workers to detect the presence and fertility of queens, which then suppress their own ovarian development. This evidence, and evidence from other groups within the order Hymenoptera, has led to the hypothesis that these compounds are conserved fertility

signals within the order. However, the degree to which these individual fertility signals suppress worker reproduction relative to the entire complement of queen hydrocarbons was not investigated in those species (Van Oystaeyen et al. 2014). In addition to other queen-specific alkanes that may potentially affect worker response, alkenes that are either queen pheromones or overrepresented in queens have been identified in at least 17 ant species (Cuvillier-Hot 2004; Cuvillier-Hot et al. 2004; Dani 2006; de Biseau et al. 2004; El-Showk et al. 2010; Evison et al 2012; Foitzik et al. 2011; Hannonen et al. 2002; Holman et al. 2010a, 2010b; Holman 2012; Holman et al. 2013; Liebig et al. 2000; Monnin and Peeters 1997; Monnin et al. 1998; Oettler et al. 2008; Peeters et al. 1999; Smith et al. 2012). It is well established in the Hymenoptera, and ants particularly, chemical communication involves multicomponent signals which may modulate or synergize one another (Hölldobler 1995), an extreme example being the alarm response of the African weaver ant *Oecophylla longinoda* (Bradshaw et al. 1979). The chemical and/or environmental context is also important for the perception of chemical signals. Workers of the trap jaw ant *Odontomachus brunneus* (Patton) require other chemicals present in the background profile to accurately assess the presence of the queen (Smith et al. 2015). In *O. brunneus*, workers take on a submissive posture when the contact the queen, which have significantly higher proportions of (*Z*)-9-nonacosene in their chemical profile than workers (Smith et al. 2012). Nestmate workers treated with this compound elicited similar submissive responses when compared with queens. However, queens from foreign colonies and foreign workers treated with the queen signal elicited this response in significantly fewer workers. Additionally, the fertility signal alone did not

inhibit workers from reproducing. These results indicate the importance of chemical context in eliciting behavioral responses from pheromones, and highlight the need to explore these fertility signals in other species if generalizations are to be drawn.

In ants, CHCs are considered to be the main components underlying nestmate recognition and fertility signaling, but they are not the only chemicals found to do so. Eliyahu et al. (2011) and this study reported differences in volatile components of *S. invicta* and *L. humile*, respectively. Fire ant queens produce primer pheromones, chemical signals that have delayed physiological or behavioral effects, that affect caste determination in female larvae, inhibit the production of sexuals, prevent virgin female reproductives from shedding their wings and activating their ovaries, and inhibit egg production in mated queens (Vargo 1998; Vargo and Hulsey 2000). Extracts from the poison gland were initially implicated as the glandular source, and bioassays with poison gland extracts revealed that all of the components of the gland were necessary to restore biological activity. A second glandular source, the postpharyngeal gland (PPG), was later found to elicit the same effects as the poison gland. This suggests that queens may possess multiple mixtures of chemicals and chemical classes that signal her presence to the colony. The finding that Argentine ant queens have a relatively large volume of dolichodial suggests that this compound may be involved in signaling the presence of the queen. It also may be involved in retinue behavior, where workers are attracted to groom and feed queens. Testing this will require separating or synthesizing dolichodial and iridomyrmecin and testing them individually for activity.

In conclusion, this study shows the amazing diversity of chemicals produced by *L. humile* castes and highlights important differences, qualitative and quantitative, between castes and developmental stages. Understanding the chemical differences present at a colony-wide scale will help guide future bioassays to elucidate the functions of these compounds.

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Appendix A

Table S2.1 Retention times (Ret. Time), Kovat's index values (KI), and presence of saturated hydrocarbons in *Linepithema humile* queens (Q), gynes (G), workers (W), males (M), gyne larvae (GL), and worker larvae (WL). Peak numbers identified in the text correspond to values in 'Peak no.' column. Boxes marked with an 'X' indicate presence of a compound within the caste. Retention times and KI index were calculated based on retention time of compounds on a DB-5MS column.

Peak no.	**Compound**	**Ret. Time**	**KI**	**FQ**	**VQ**	**W**	**M**	**QL**	**WL**
	n-C25	22.57	2500					X	X
8	*n*-C27	24.09	2700	X	X	X		X	X
9	13- ;11-MeC27	24.31	2729	X	X	X			
10	7-MeC27	24.38	2741		X				
11	5-MeC27	24.44	2750	X	X	X			
12	3-Me + 5,11-diMeC27	24.60	2773	X	X	X			X
13	*n*-C28	24.79	2800	X	X	X		X	X
14	5-MeC28	25.15	2848	X					
15	4-MeC28	25.20	2855	X					
18	*n*-C29	25.49	2900	X	X	X		X	X
19	13- ;11-MeC29	25.70	2930	X	X	X			X
20	7-MeC29	25.76	2937		X				
22	5-MeC29	25.83	2957	X	X	X			
23	3-Me + 5,11-diMeC29	26.03	2984	X	X	X		X	X
24	*n*-C30	26.15	3000		X	X		X	X
25	3,11- ; 3,7-diMeC29	26.23	3011	X	X				

26	5-MeC30	26.51	3047	X					
27	4-MeC30	26.57	3055	X					
30	*n*-C31	26.89	3100	X	X	X			
31	15- ;13-MeC31	27.12	3126	X	X	X			
33	5-MeC31	27.27	3152	X	X	X			
34	3-Me + 5,15- ;5,11-diMeC31	27.73	3176	X	X	X			
35	3,15- ;3,13-diMeC31	*	3202	X	X	X			
36*	3,23-diMeC31	*	3201	X	X				
37	5,13,17-triMeC31	27.761	3206		X				
38	3,7-diMeC31	27.78	3210	X	X				
41	*n*-C33	27.79	3300	X	X	X			
42	17- ;11-MeC33	28.65	3327	X	X	X			
43	7-MeC33	28.95	3338	X	X				
44	13,15- ; 15,19-diMeC33	29.08	3348		X	X			
46	5-MeC33	29.22	3349	X	X				
47	5,17- ;5,11-diMeC33	29.19	3380	X	X	X			
48	5,13,17-triMeC33	29.53	3410	X	X	X			
49	3,13,17-triMeC33	29.82	3427		X	X			
50	15- ;11- ;9- ;7-MeC35	30.11	3524		X	X			

51	13,15- ;15,19- diMeC35	31.515	3543		X	X			
53	5,11- ;5,15- diMeC35	31.831	3576		X	X			
54	5,13,17- ; 5,15,19- triMeC35	32.349	3605	X		X			
55	5,13,17- ; 5,15,19- triMeC35 + 3,23- diMeC35	32.349	3605		X				
56	3,13,17- triMeC35	32.809	3629	X	X	X			
57	15- ;11- ;9- ;7-MeC37	33.188	3726		X	X			
58	13,15- ;15,19- diMeC37	35.216	3748		X	X			
59	5,11- ;5,15- diMeC37	35.741	3776		X	X			
60	5,13,17- ; 5,15,19- triMeC37	36.394	3805			X			
61	5,13,17triMe + 3,23diMe	36.394	3805		X				
62	3,13,17triMe	37.093	3829		X	X			

Table S2.2 Alkene composition of the various castes and developmental stages of *L. humile*. Double bond position and geometry were confirmed by comparison to epoxide products of synthetic standards. Alkenes marked with (*) indicate alkenes that were present but in extremely low abundance.

	Compound	**FQ**	**VQ**	**W**	**M**	**GL**	**WL**
1	Tetradecene	X	X	X	X	X	X
2	Hexadecene	X	X	X	X	X	X
3	Octadecene	X	X	X	X	X	X
4	Nonadecene	X	X	X	X	X	X
5	Eicosene	X	X	X	X	X	X
16	(*Z*)-14-nonacosene	X					
17	(*Z*)-9-nonacosene	X	*				
21	(*Z*)-9-triacontene	X	*				
28	(*Z*)-14-hentriacontene	X	*				
29	(*Z*)-9-hentriacontene	X	*				
32	Dotriacontene [a]	X	*				
39	Tritriacontene [a]	X	*				
40	Tritriacontene [a]	X	*				
52	Pentatriacontene [a]	X	*				

[a] Indicates putative homologs of (*Z*)-14- and (*Z*)-9-alkenes that could not be confirmed due to low concentrations following epoxidation.

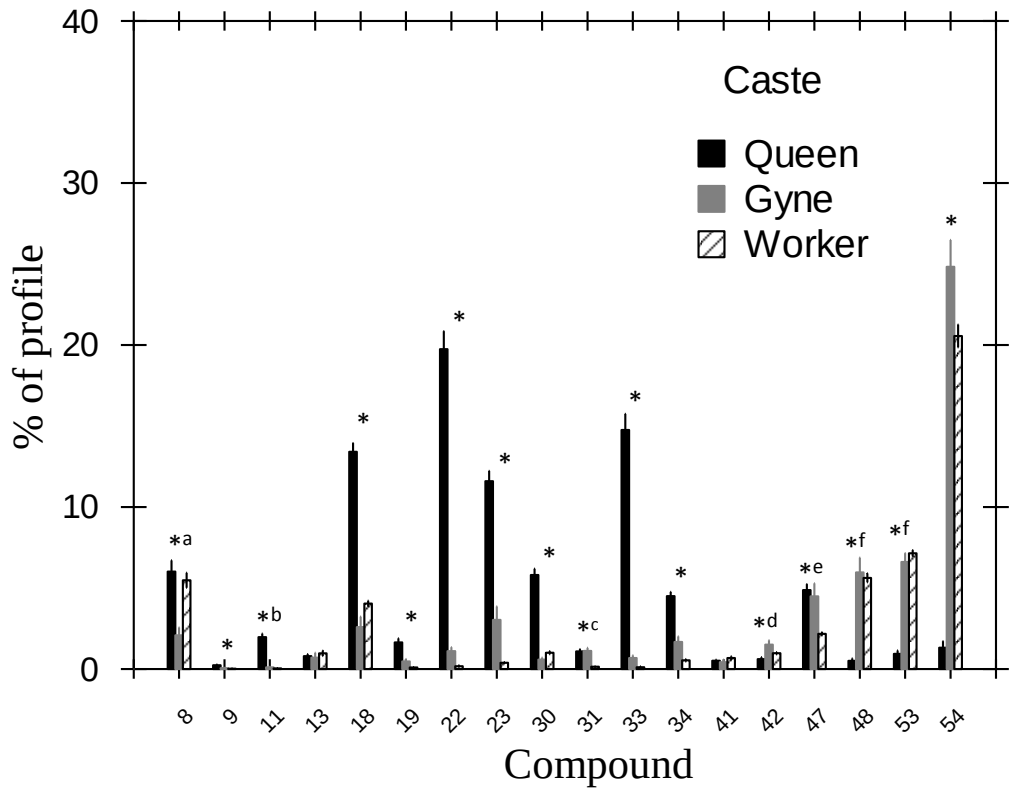

Figure S2.1 Bar chart comparing the abundance of selected alkanes between queens, gynes, and workers ($\alpha = 0.05$, Kruskal-Wallis ANOVA). Numbers on the x-axis correspond to compounds in Supplemental Table 1. Bars marked with '*' indicate significant differences. All castes were significantly different from one another unless specified

a = Queens were not significantly different from gynes ($P = 0.96$)

b = Gynes were not significantly different from workers ($P = 0.08$)

c = Queens were not significantly different from gynes ($P = 0.80$)

d = Gynes were not significantly different from workers ($P = 0.15$)

e = Queens were not significantly different from gynes ($P = 0.49$)

f = Gynes were not significantly different from workers ($P = 0.06$)

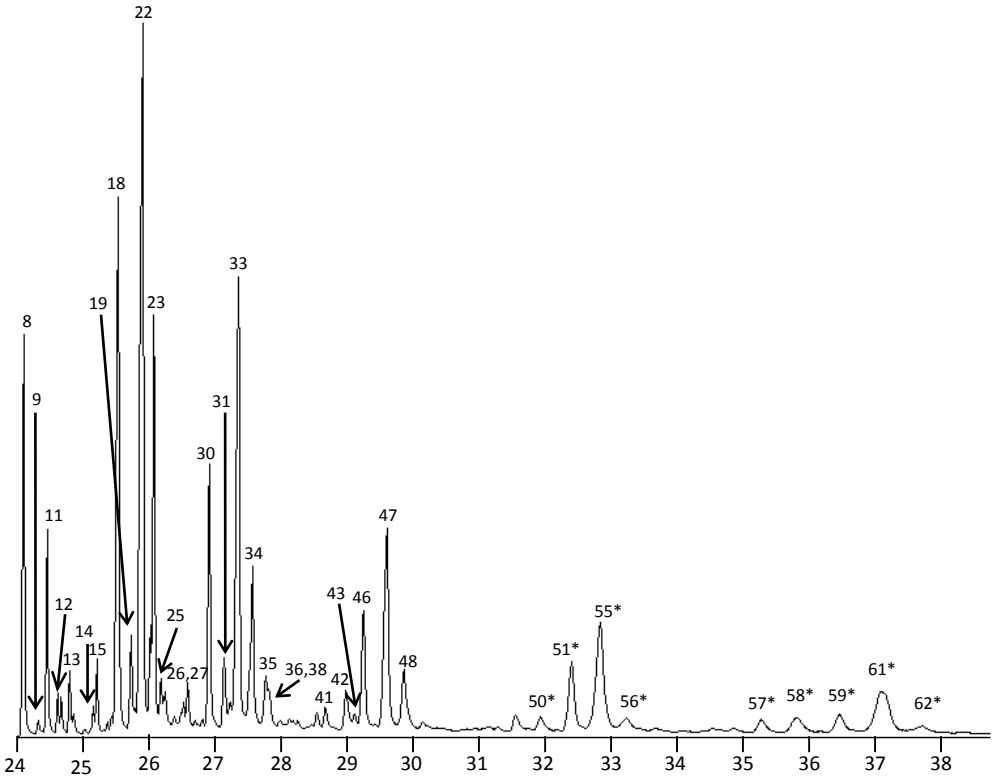

Figure S2.2 Representative chromatogram of *L. humile* queen alkanes. Peaks 11, 18, 19, 22, 23, 30, 33, and 34 were found in all three castes (queens, gynes, and workers) but were significantly higher in abundance in queens. '*' = Compounds were not present in all samples or in sufficient quantities for data analysis. Samples were separated on a DB-5MS column.

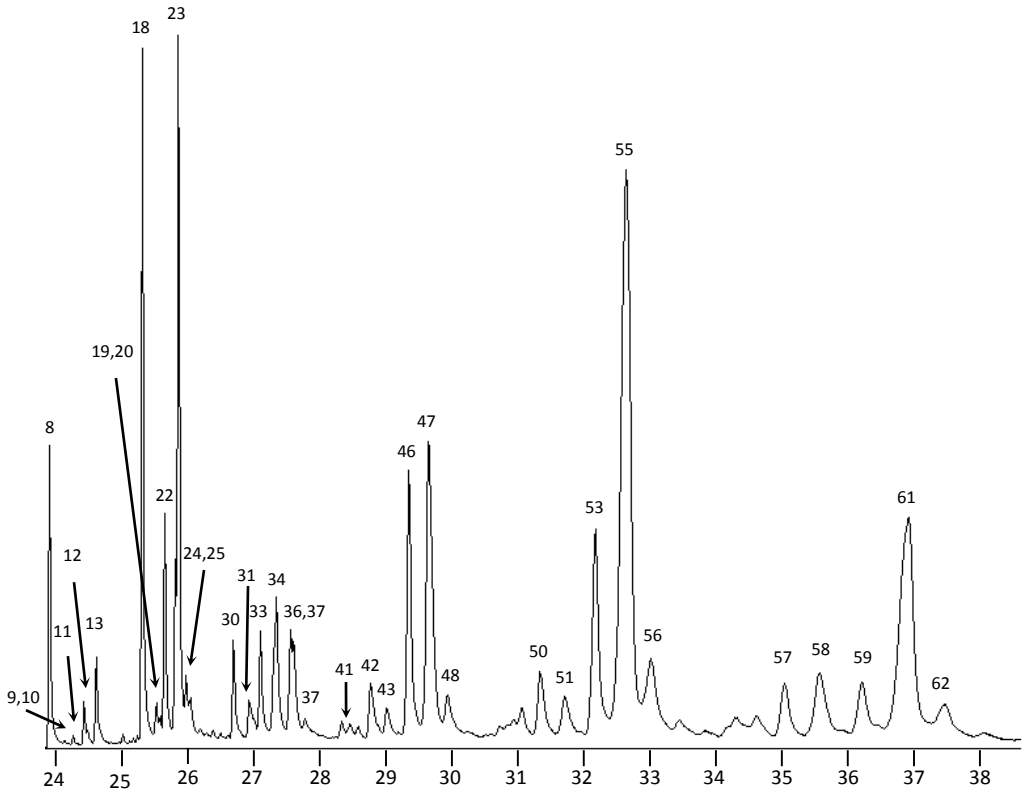

Figure S2.3 Representative chromatogram of gyne alkanes. Peaks 22, 23, 31, 33, and 34 were significantly lower in abundance in gynes than in queens, but were more abundant in gynes than in workers. Peaks 48, 53, and 54 were similar in gynes and workers, and were significanlty higher in abundance in gynes than in queens. Samples were separated on a DB-5MS column.

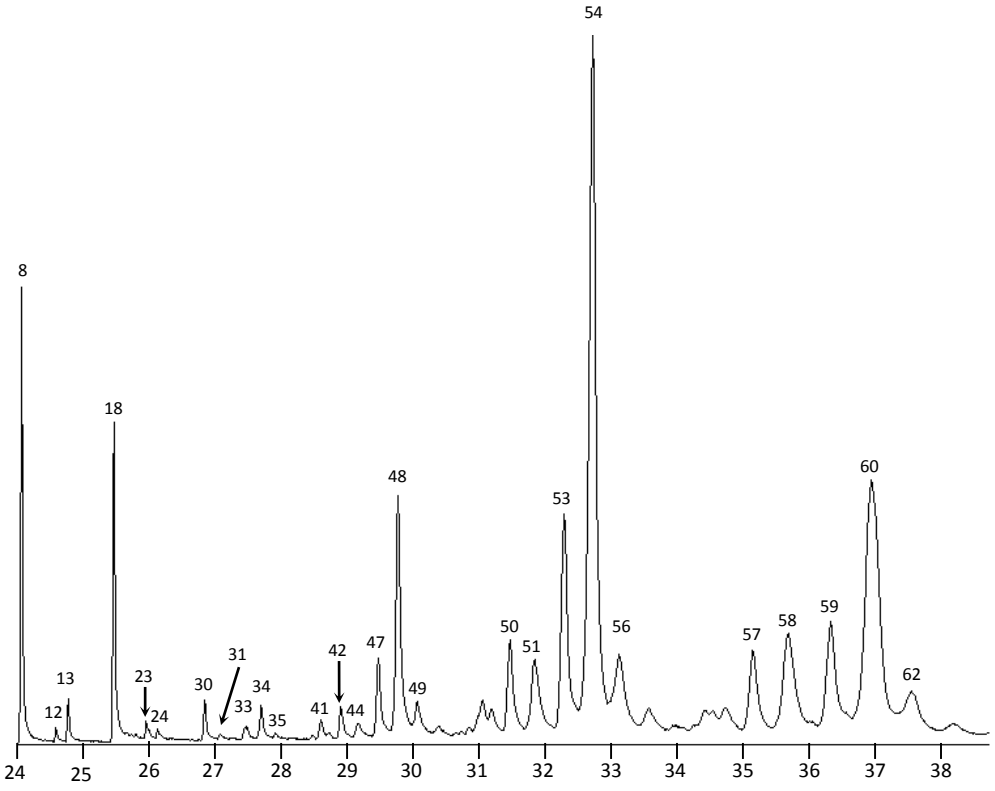

Figure S2.4 Representative chromatogram of worker alkanes. Peak 8 was the only compound that occurred in all three castes (queens, gynes, and workers) that was present at similar abundance in both workers and queens. Samples were separated on a DB-5MS column.

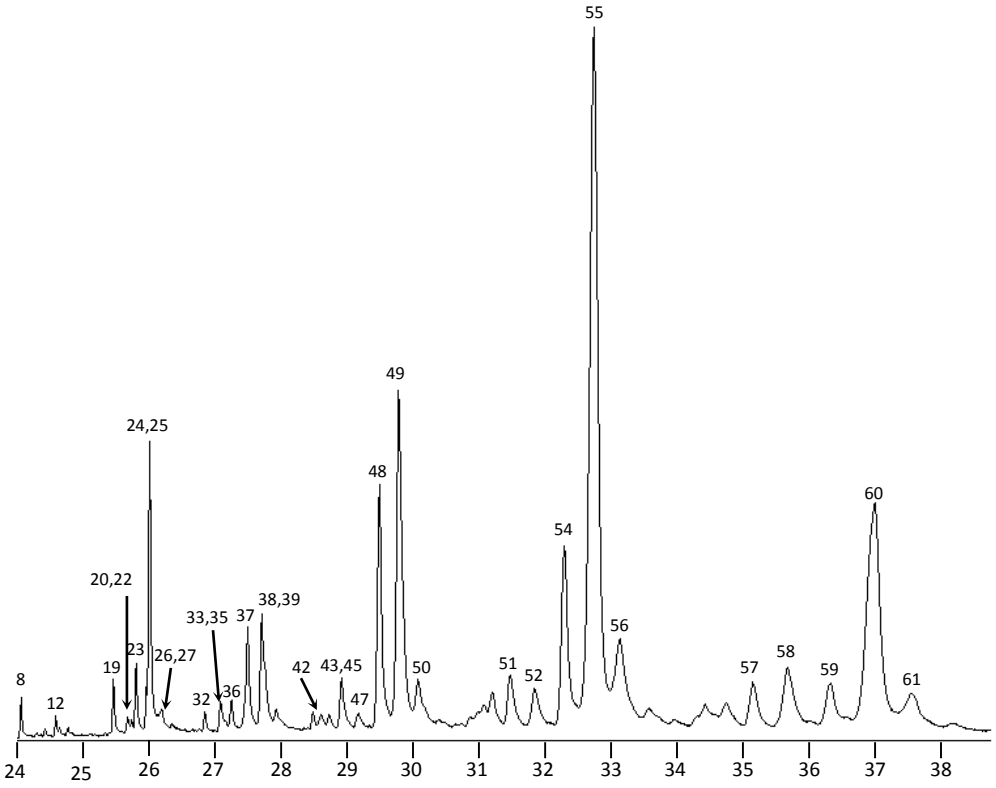

Figure S2.5 Representative chromatogram of male alkanes. Males were characterized by an abundance of peaks 24 and 25, and appeared to have alower abundance of *n*-alkanes. However, GC analysis of male alkanes was highly variable and thus not used for statistical analyses. Samples were separated on a DB-5MS column.

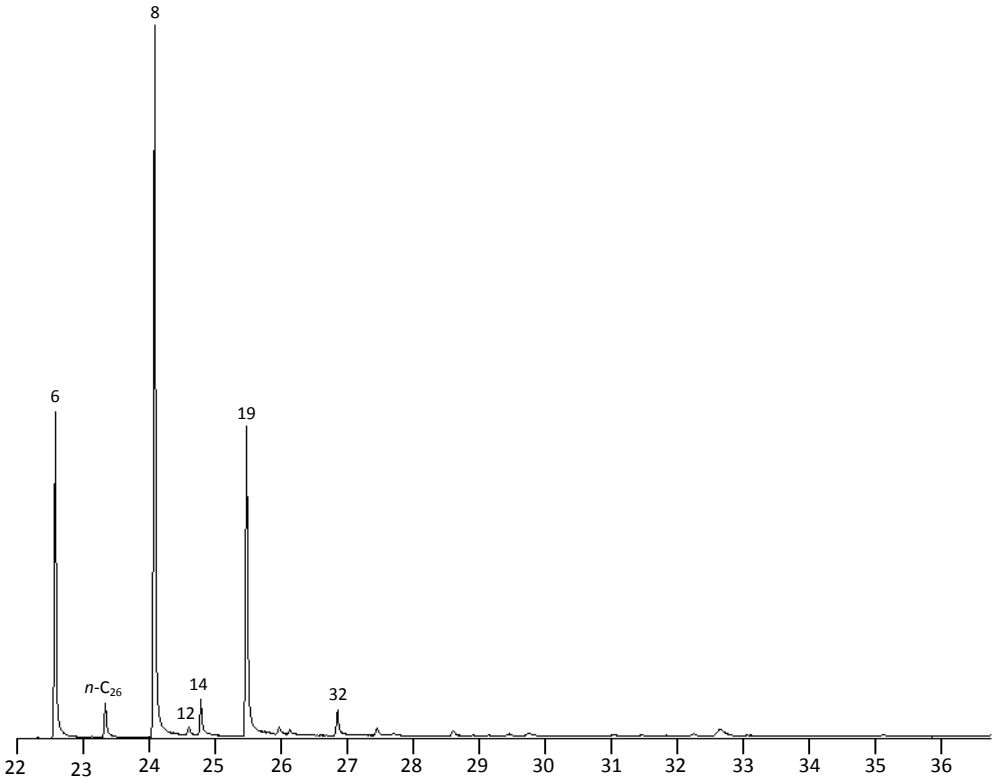

Figure S2.6 Representative chromatogram of alkanes of gyne larvae. The scale is shorter for gyne larvae due to the presence of shorter-chain C_{25} alkanes not present in other samples. *n*-alkanes (*n*-C_{25}, *n*-C_{26}, *n*-C_{27}, *n*-C_{28}, and *n*-C_{29}) comprised the majority of hydrocarbons on the cuticle of gyne larvae. Samples were separated on a DB-5MS column.

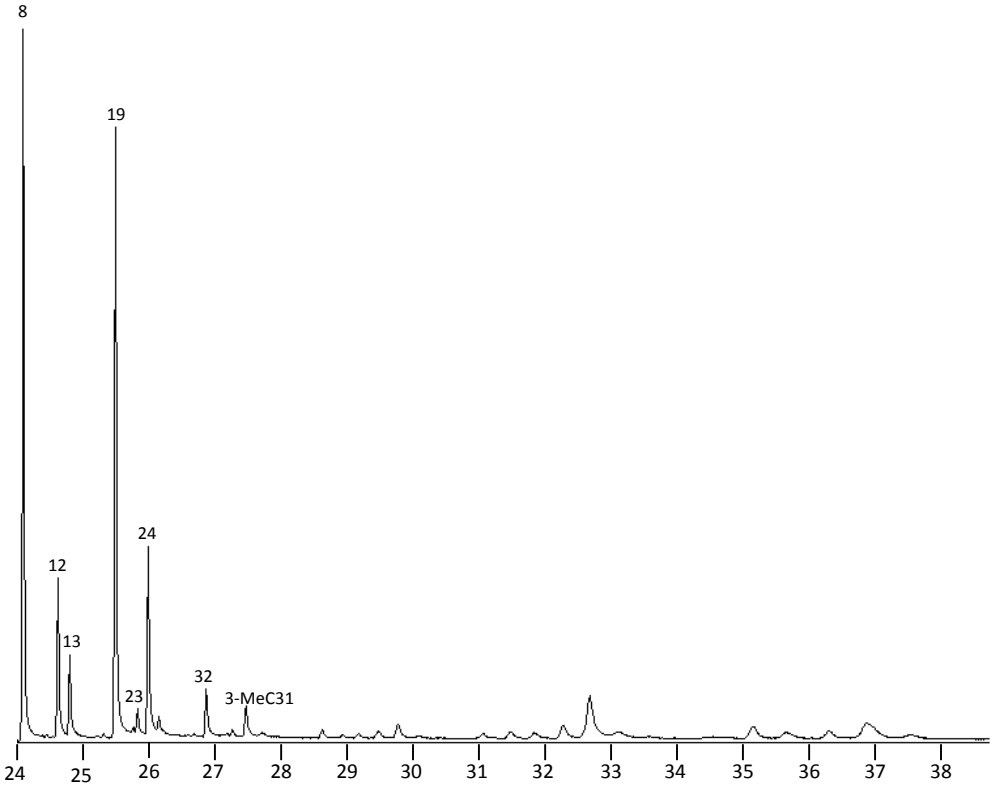

Figure S2.7 Representative chromatogram of worker larvae. Worker larvae generally had higher proportions of 3-Me alkanes (peaks 12 and 23; peak labeled on GC trace) than gyne larvae, although there were not enough samples of gyne larvae to perform statistic analyses. Samples were separated on a DB-5MS column.

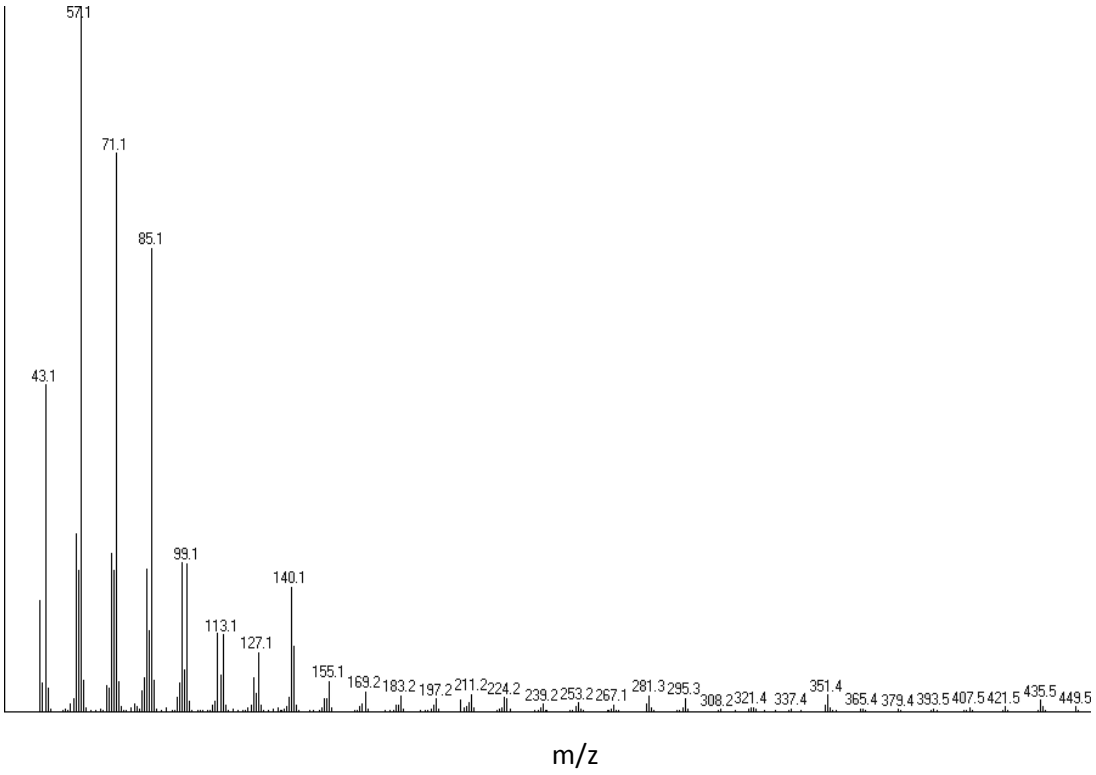

Figure S2.8 Mass spectrum of a putative 3,23-diMeC31 alkane from *L. humile* gynes. Ion fragments at 140, 350, and 435 m/z are indicative of the compound, but the presence of ion fragments from co-eluting 5,13,17-triMeC31 prevents confident identification.

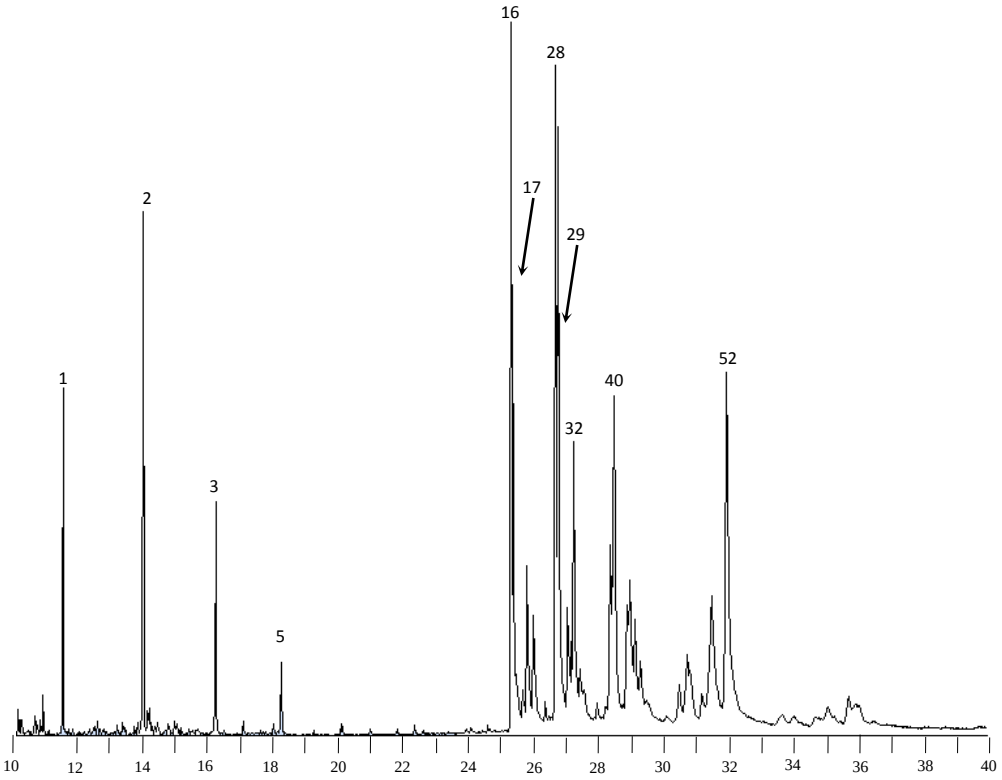

Figure S2.9 Representative chromatogram of queen alkenes. Queens were qualitatively different from all other castes in the presence of long-chain monomethyl alkenes. Samples were separated on a DB-5MS column.

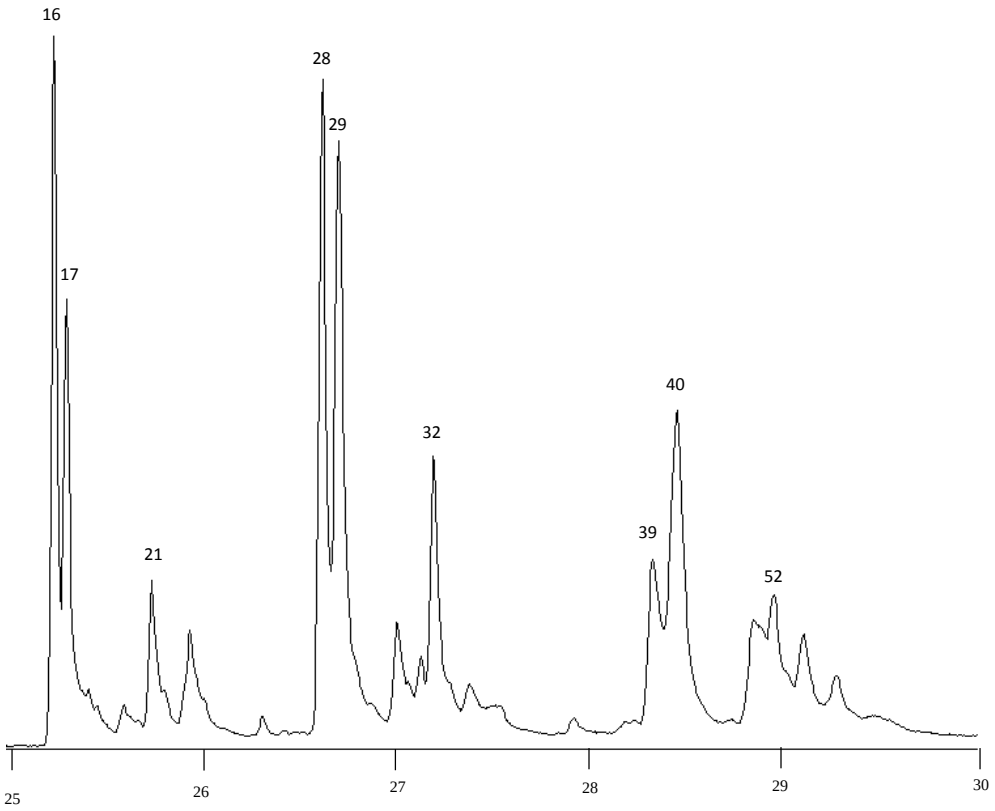

Figure S2.10 Close-up view of long-chain queen alkenes.

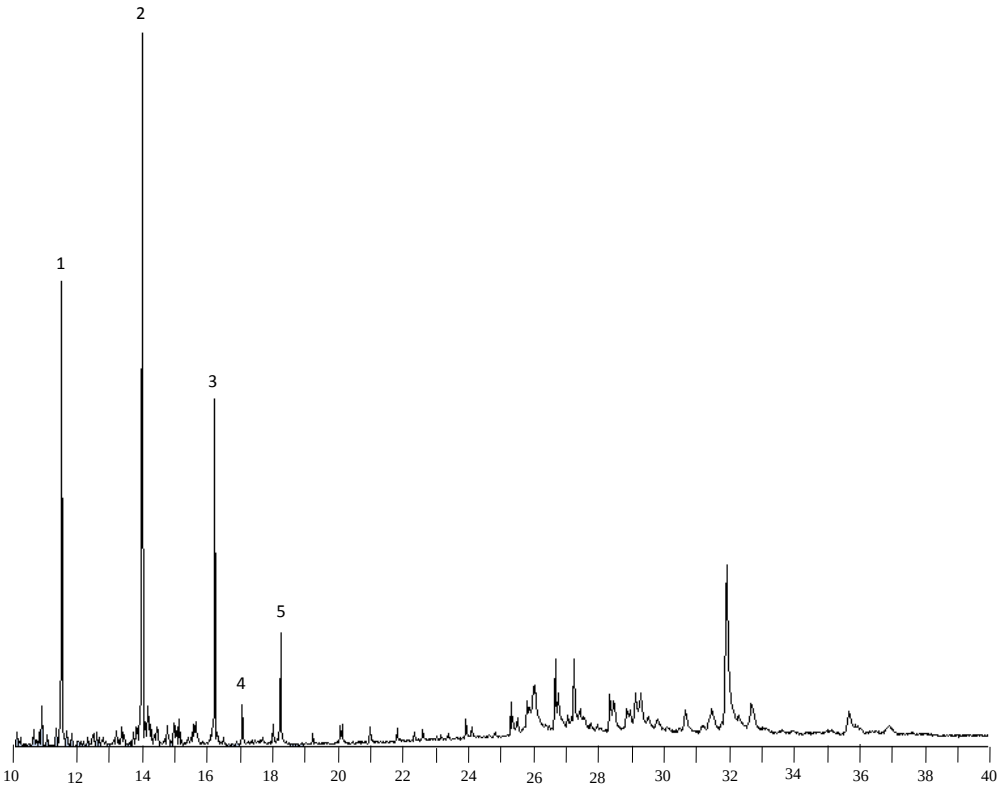

Figure S2.11 Representative chromatogram of gyne alkenes. Gynes appeared to have low concentrations of long-chain alkenes found in queen profiles, but their presence may be because gynes are generally in close proximity to queens inside the nest. Samples were separated using a DB-5MS column.

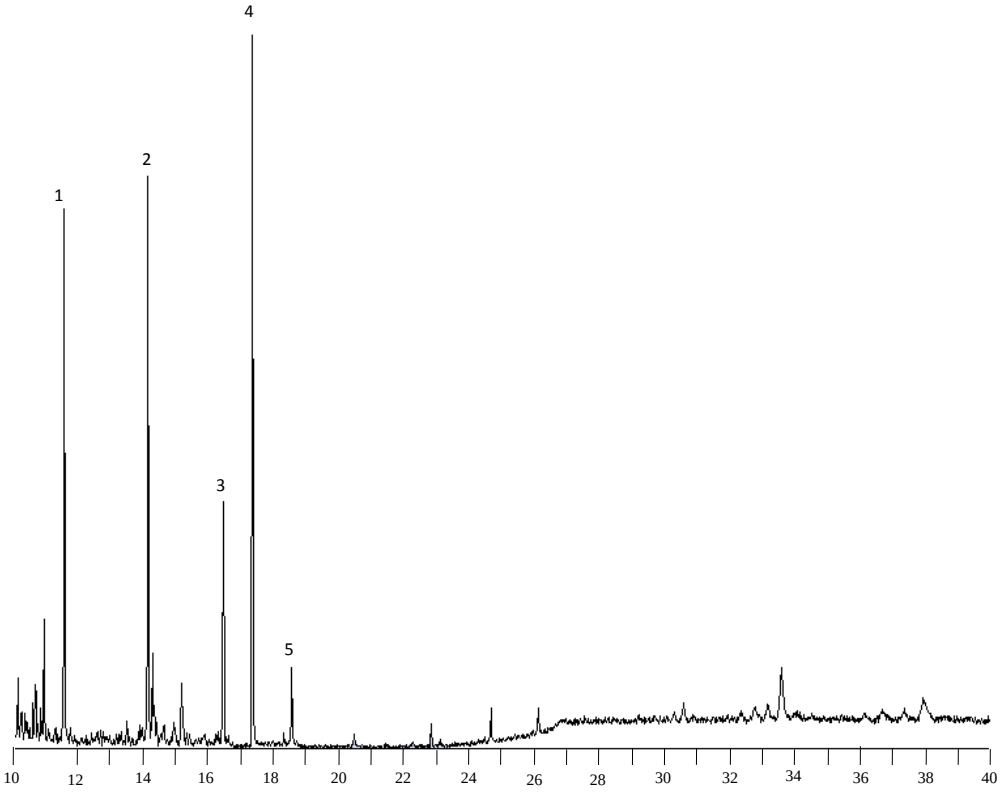

Figure S2.12 Representative chromatogram of worker alkenes. Workers profiles were similar to gyne profiles and did not have any long-chain alkenes associated with queens. Samples were separated using a DB-5MS column.

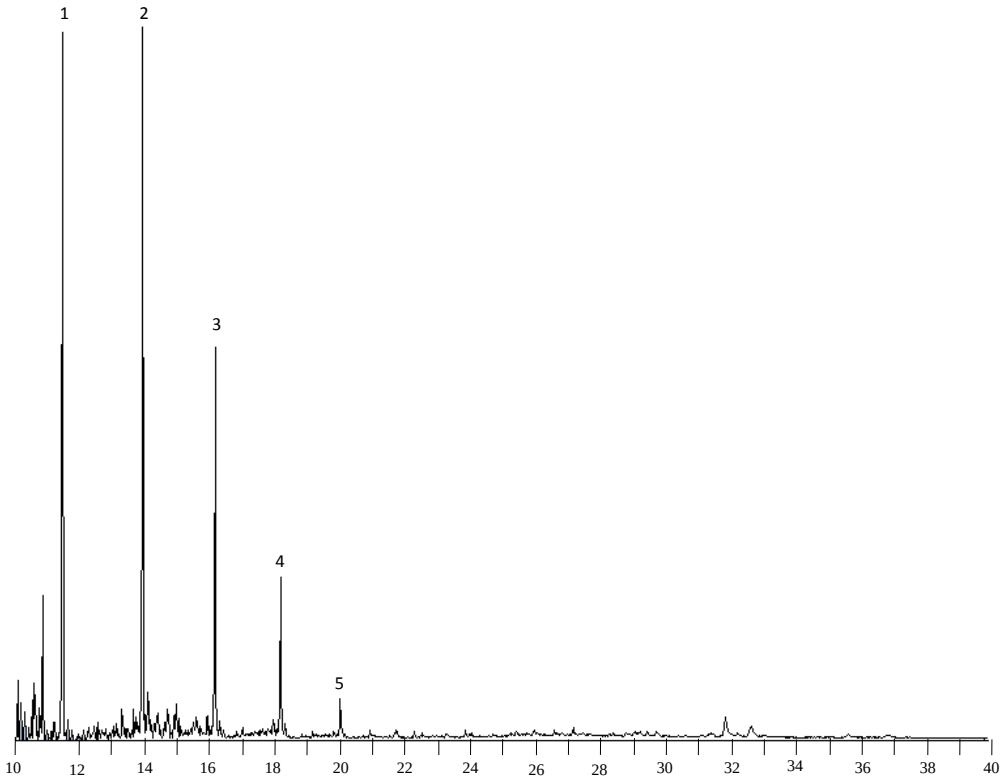

Figure S2.13 Representative chromatogram of male alkenes. Male profiles were similar to worker and gyne alkene profiles. Samples were separated using a DB-5MS.

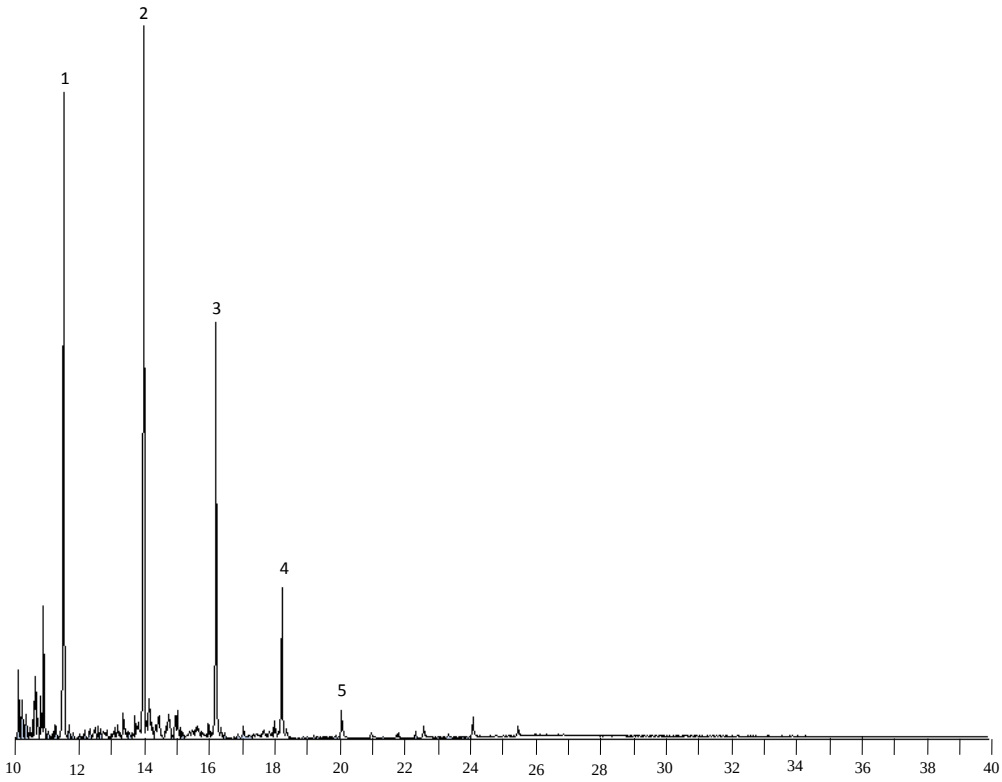

Figure S2.14 Representative chromatogram of alkenes of gyne larvae. Samples were separated using a DB-5MS column.

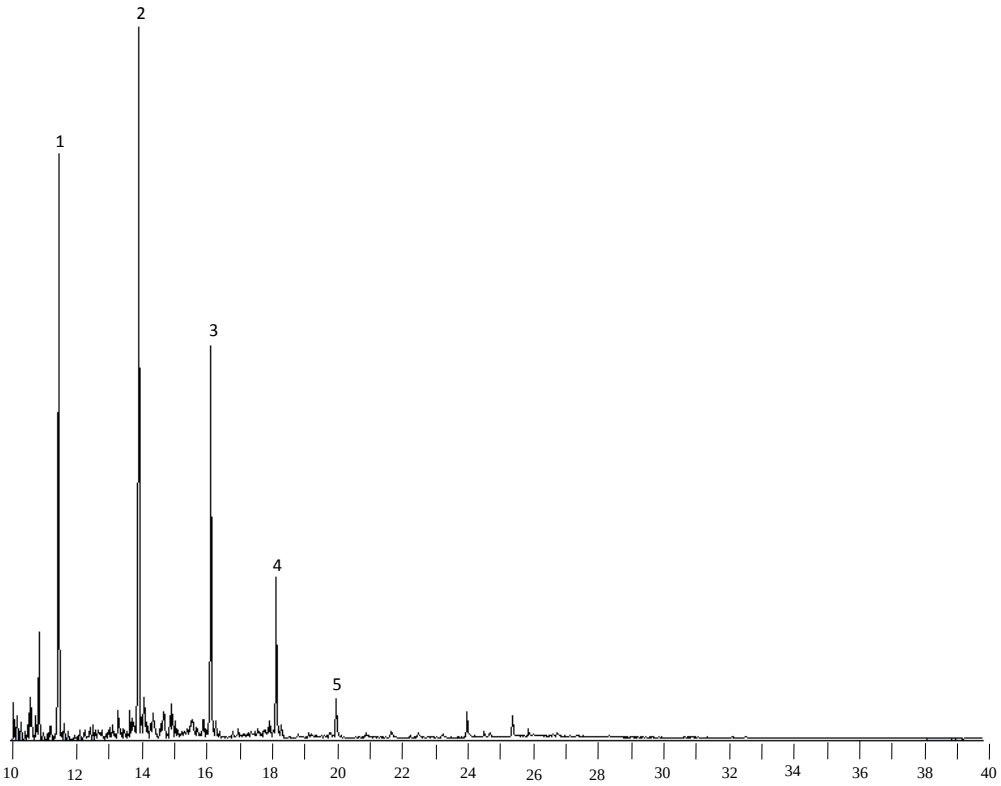

Figure S2.15 Representative chromatogram of alkenes of worker larvae. Samples were separated using a DB-5MS column.

Table S2.3 Between-caste comparisons of volatile constituents from aerations of live ants.

Peak no.	Compound	Queens	Gynes	Males	Workers	Worker larvae
63	2,5-dimethyl-3(3-methylbutyl)pyrazine*	X	X	X	X	X
64	Dolichodial	X			X	
65	*n*-C_{17}	X		X	X	X
66	Iridomyrmecin	X			X	X
67	(*Z*)-9-nonadecene*	X		X	X	X

* The pyrazine was full characterized by Cavill and Houghton (1974a). (*Z*)-9-nonadecene was fully characterized by Cavill and Houghton (1973).

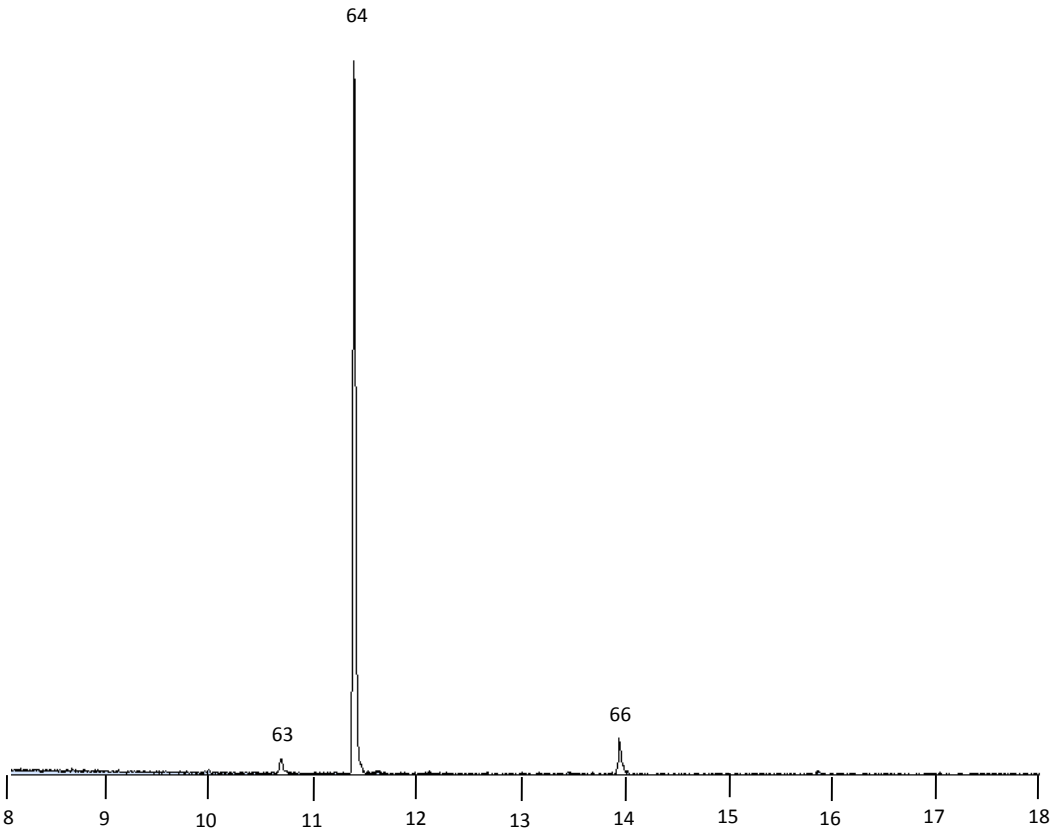

Figure S2.16 GC trace of volatile collections of three live queens. The abundance of dolichodial (peak 64) was significantly higher than the abundance of iridomyrmecin peak 66). Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~ 10 µl and separated with a DB-17 column.

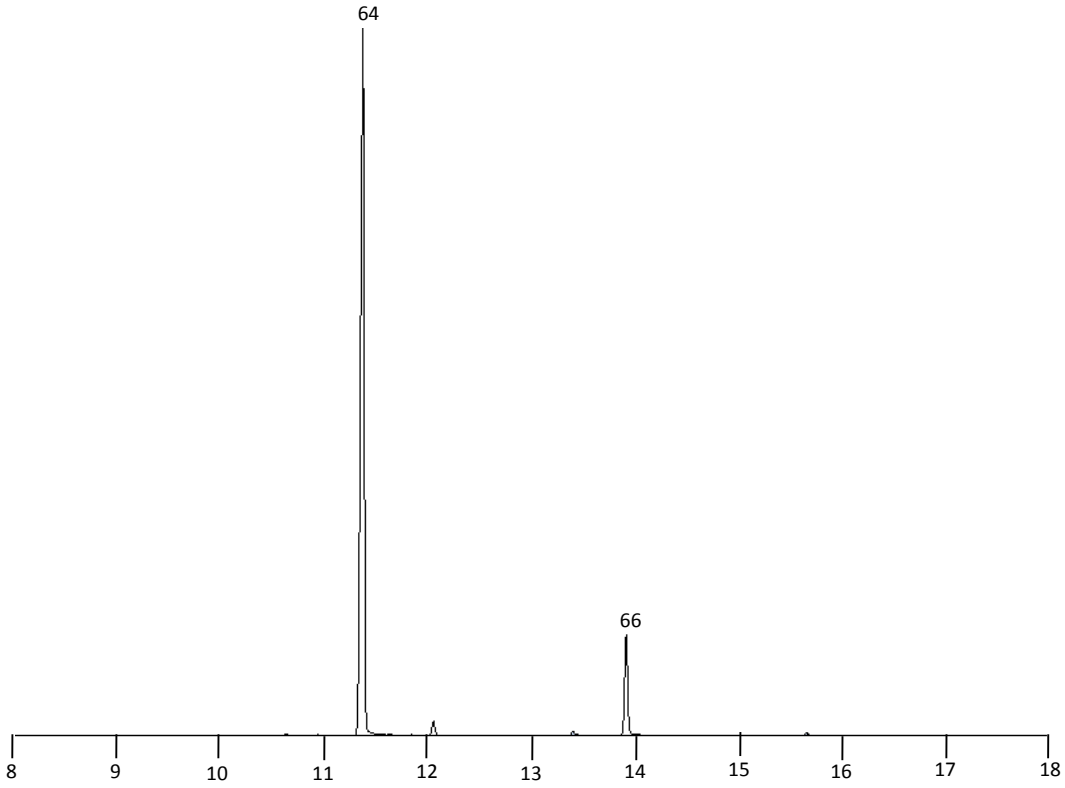

Figure S2.17 GC trace of volatile collections of three crushed queens. Aerations of crushed queens were similar but more concentrated than those of live queens. Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~ 10 µl and separated with a DB-17 column.

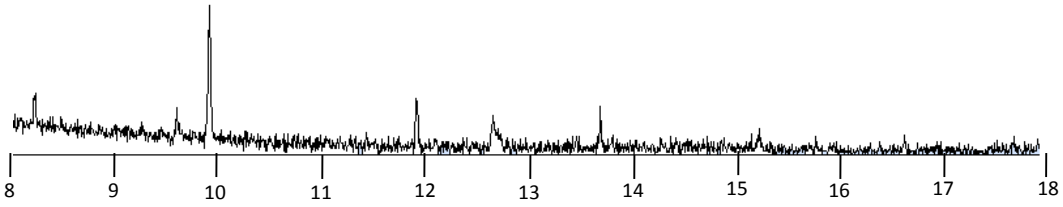

Figure S2.18 GC trace of volatile collections of three crushed queen heads. Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~ 10 µl and separated with a DB-17 column. There were no peaks that corresponded to the pyrazine (~10.7 min), dolichodial (~11.5 min), or iridomyrmecin (~13.9 min). While the pyrazine was detected in aerations of live queens, this may be due to their close proximity to workers and males, which have relatively abundant pyrazine peaks associated with the head capsule.

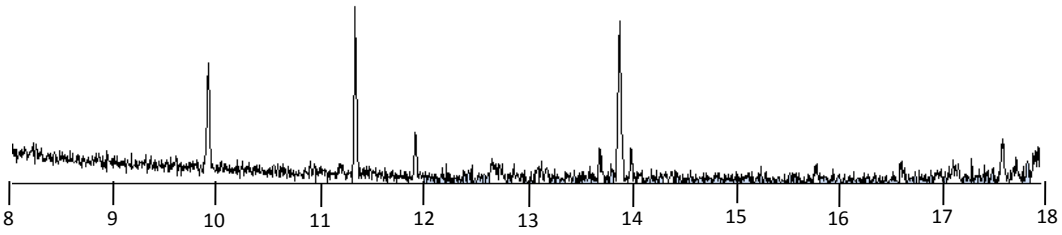

Figure S2.19 GC trace of volatile collections of three crushed queen thoraces. Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~ 10 µl and separated with a DB-17 column. There were no peaks that corresponded to the pyrazine (~10.7 min), dolichodial (~11.5 min), or iridomyrmecin (~13.9 min).

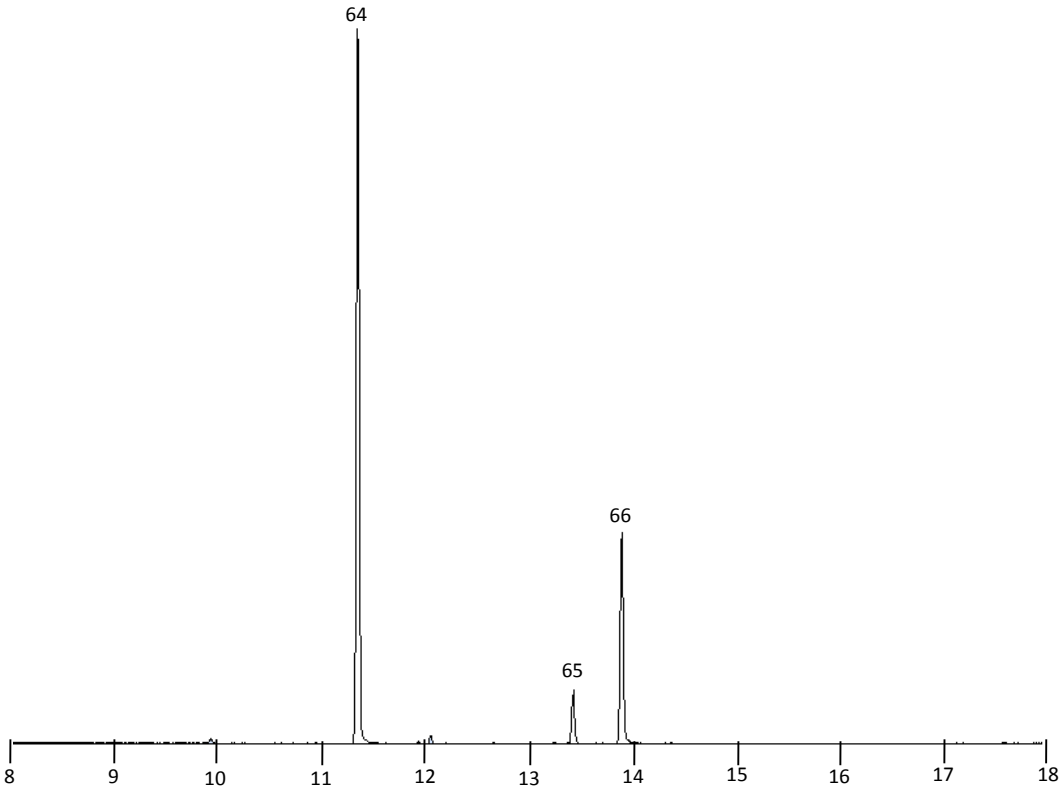

Figure S2.20 GC trace of volatile collections of three crushed queen abdomens. Aerations of gasters resemble those of whole crushed queens, indicating the primary source of queen volatiles is located in glands associated with the abdoment. Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~ 10 µl and separated with a DB-17 column.

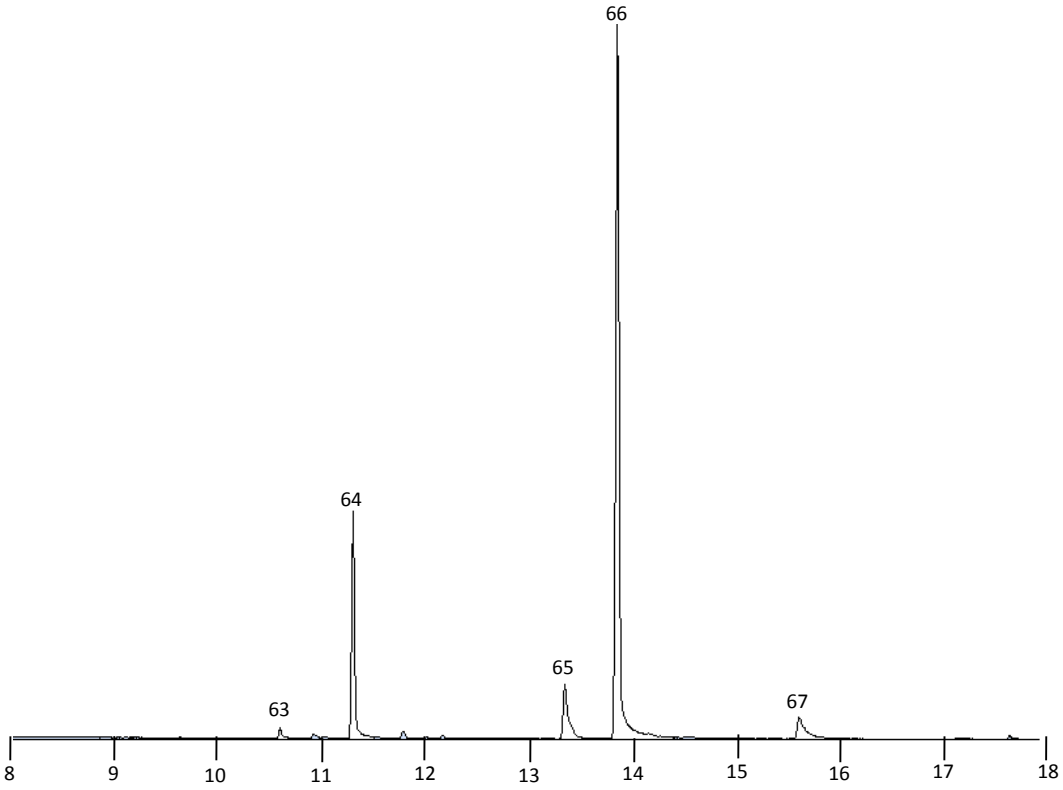

Figure S2.21 GC trace of volatile collections of fifteen live workers. Aerations of workers show an opposite trend to that found in queens; workers have much higher proportions of iridomyrmecin than dolichodial. Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~ 10 µl and separated with a DB-17 column.

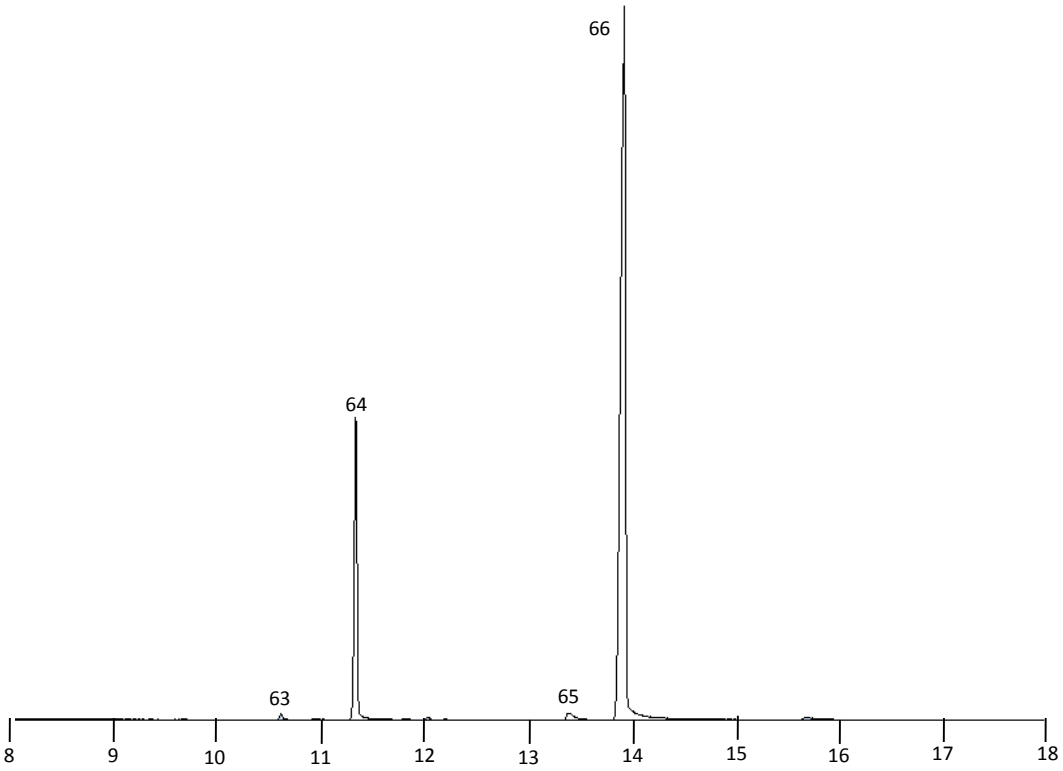

Figure S2.22 GC trace of volatile collections of fifteen crushed workers. The concentrations of dolichodial and iridomyrmecin were predictably higher in crushed workers and overshadow *n*-C_{17} and (*Z*)-9-nonadecene. Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~ 10 µl and separated with a DB-17 column.

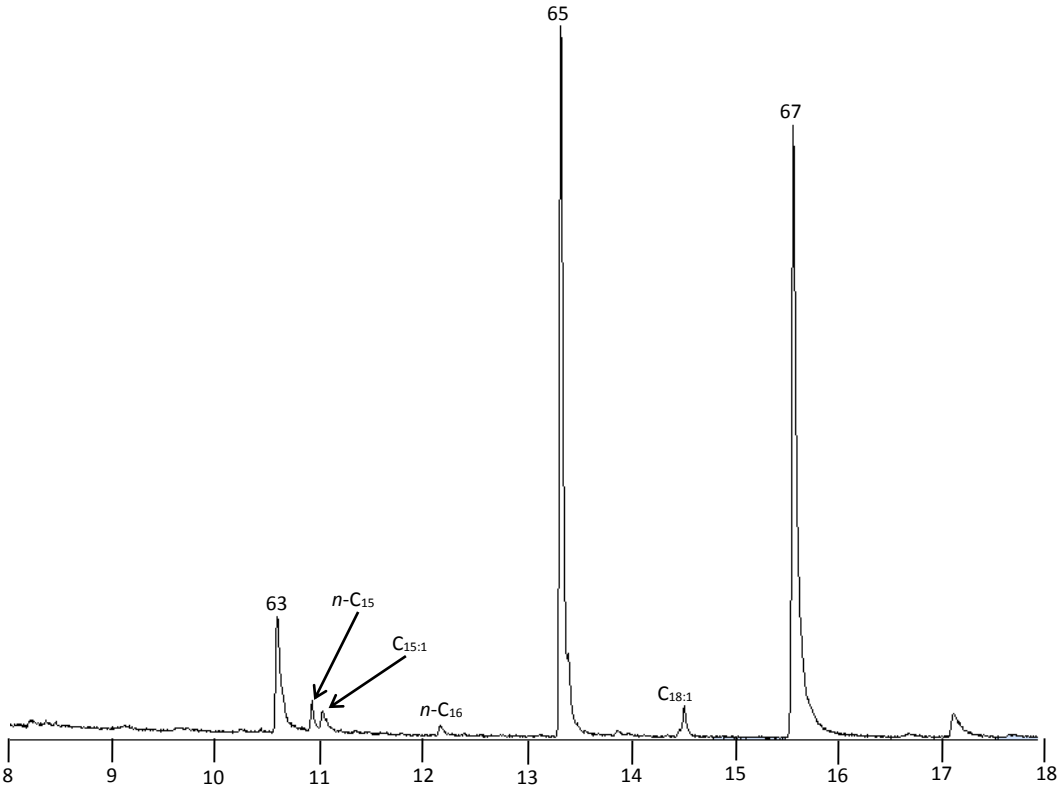

Figure S2.23 GC trace of volatile collections of fifteen crushed worker heads. In addition to the pyrazine, aerations of crushed head capsules were abundant in the aliphatic hydrocarbons *n*-C_{17} and (*Z*)-9-nonadecene. Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~10 µl and separated with a DB-17 column.

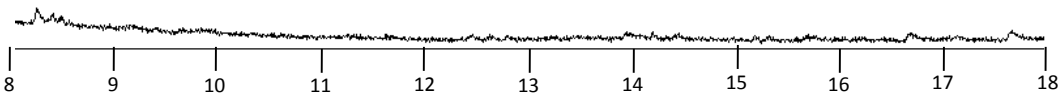

Figure S2.24 GC trace of volatile collections of fifteen crushed worker thoraces. Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~ 10 µl and separated with a DB-17 column. There were no peaks that corresponded to the pyrazine (~10.7 min), dolichodial (~11.5 min), or iridomyrmecin (~13.9 min).

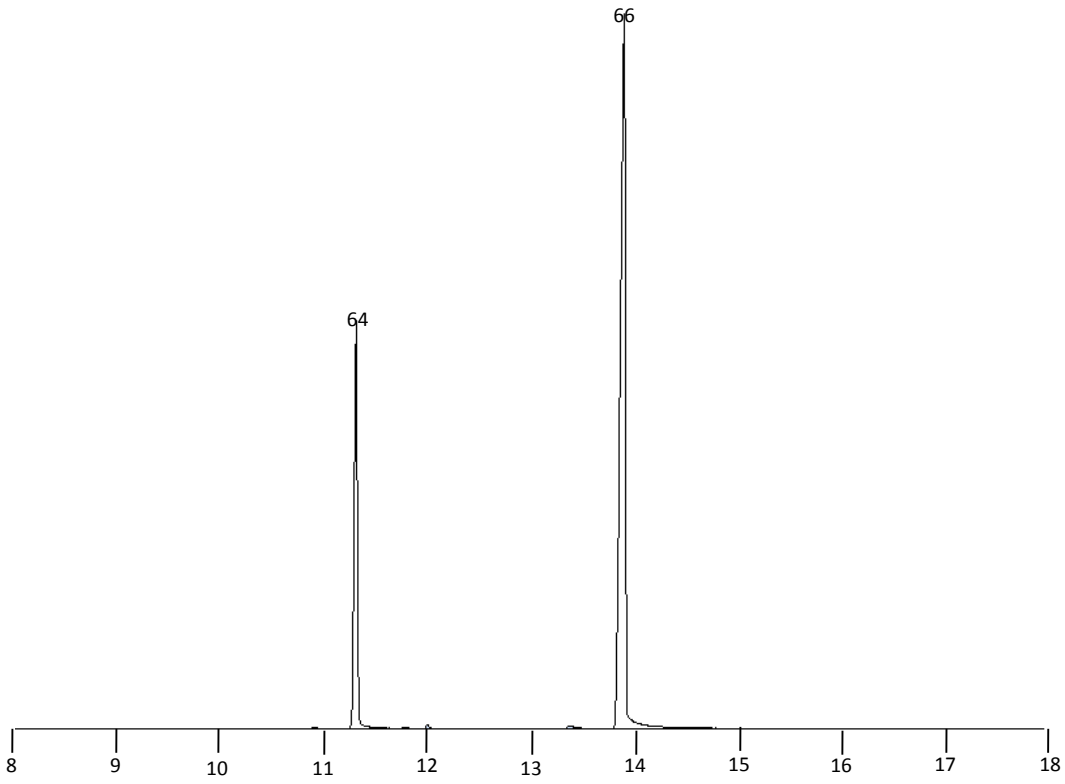

Figure S2.25 GC trace of volatile collections of fifteen crushed worker abdomens. Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~ 10 µl and separated with a DB-17 column.

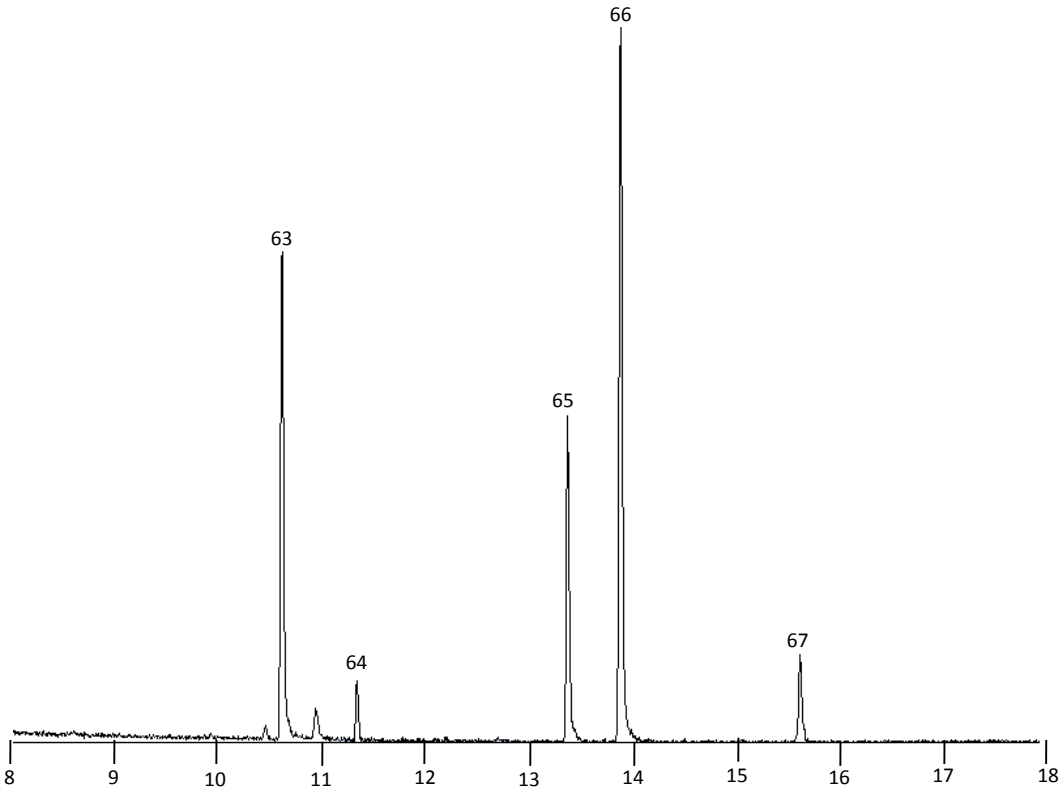

Figure S2.26 GC trace of volatile collections of fifteen live males. Males had noticeably high proportions of the pyrazine (peak 63). Traces of dolichodial and iridomyrmecin were probably contamination resulting from worker ant secretions during the collection process. Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~ 10 µl and separated with a DB-17 column.

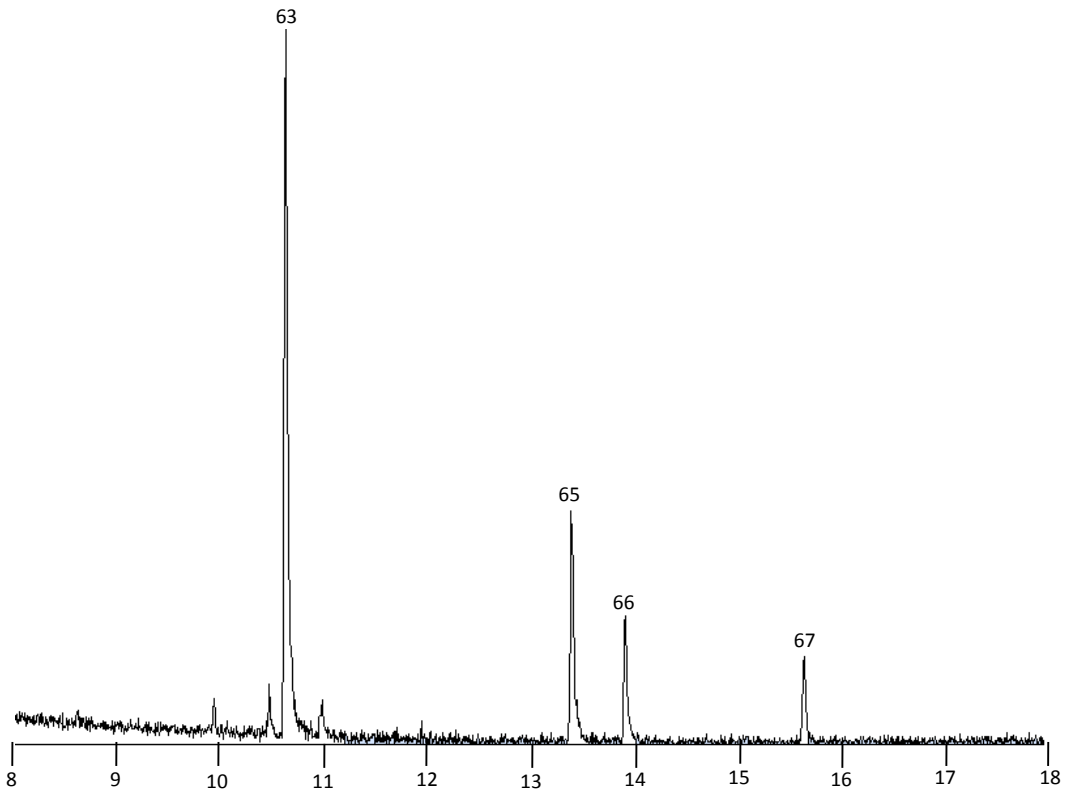

Figure S2.27 GC trace of volatile collections of fifteen crushed males. The pyrazine was the dominant component of crushed males, where dolichodial and iridomyrmecin were the dominant components of crushed queens and workers. Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~ 10 µl and separated with a DB-17 column.

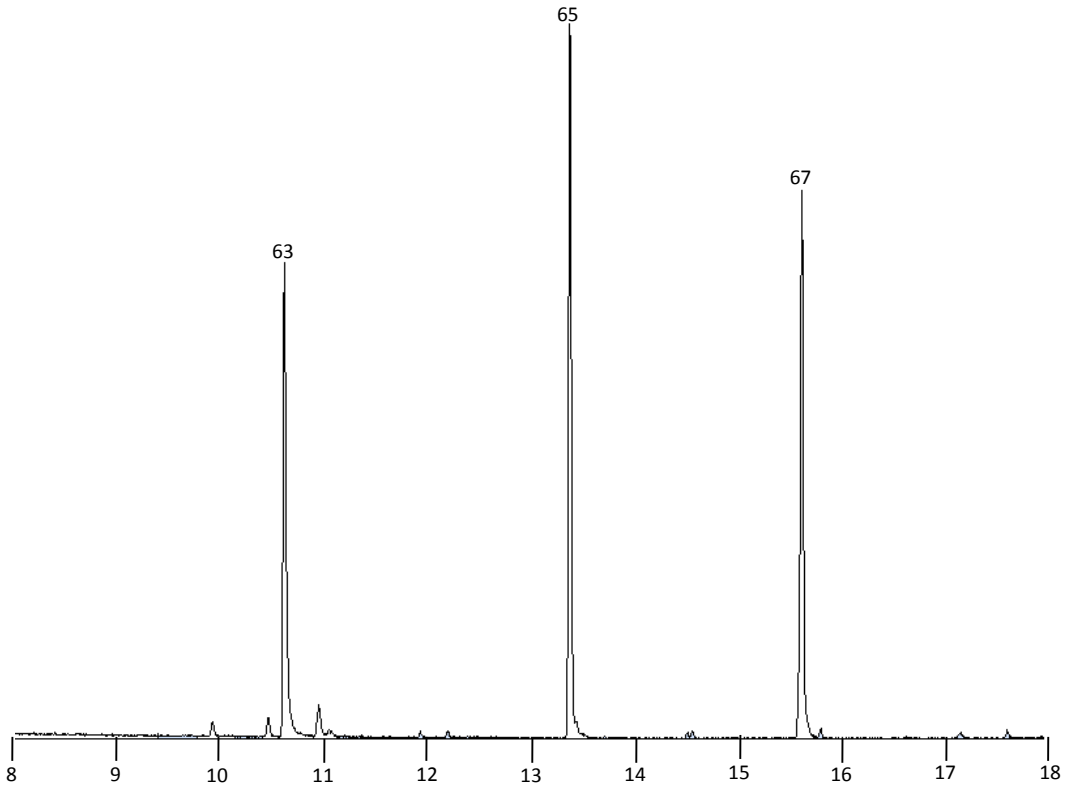

Figure S2.28 GC trace of volatile collections of fifteen crushed male heads. Males head capsules were dominanted by the presence of the pyrazine, *n*-C_{17}, and (*Z*)-9-nonadecene, which was similar to the aerations of crushed worker head capsules. Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~ 10 µl and separated with a DB-17 column.

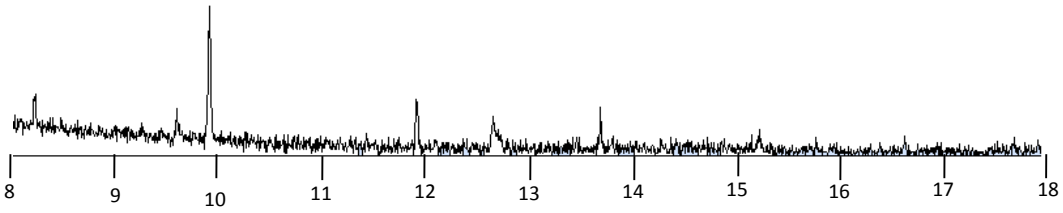

Figure S2.29 GC trace of volatile collections of fifteen crushed worker thoraces. Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~ 10 µl and separated with a DB-17 column. There were no peaks that corresponded to the pyrazine (~10.7 min), dolichodial (~11.5 min), or iridomyrmecin (~13.9 min).

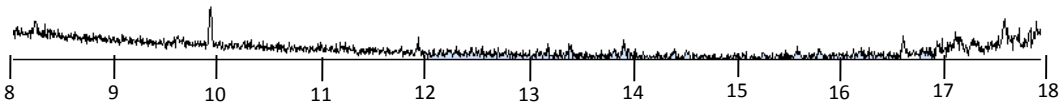

Figure S2.30 GC trace of volatile collections of fifteen crushed worker abdomens. Samples were aerated for 24 h. Volatiles were trapped on activated charcoal, eluted with 1 ml methylene chloride, concentrated to ~ 10 µl and separated with a DB-17 column. There were no peaks that corresponded to the pyrazine (~10.7 min), dolichodial (~11.5 min), or iridomyrmecin (~13.9 min).

Chapter 3: The relative contributions of individual components of a mass recruitment signal in Argentine ants

Introduction

Chemical communication is considered the oldest form of communication, and occurs across a variety of taxa including bacteria, fungi, plants, insects, and vertebrates. In many solitary insects, including moths, beetles, and aphids, intraspecific chemical signals, or pheromones, are used to convey information about the sex of the emitter and are often used before and/or during copulation. In social insects, communication among many individuals is critical in maintaining colony cohesion. In eusocial insects, chemical communication has expanded and diversified to a degree well beyond that observed in solitary insects, and is perhaps at its most complex within the ants (Hymenoptera: Formicidae). Ants coordinate colony-level processes such as mating, defense, sex-allocation of offspring, recruitment, and foraging through a vast array of chemical signals (Hölldobler and Wilson 1990). Of these chemically-mediated behaviors, aggregation and trail-following are among the most complex in social insects (Wilson 1971; Czaczkes et al. 2015).

Trail pheromones vary in their glandular origin and chemical nature, precluding generalizations among ants (Morgan 2009). Pheromones may consist of a single component, as in several *Myrmica* species (Evershed and Morgan 1983; Morgan et al. 1990), or may exist as a multi-component blend, exemplified by the trail pheromone of *Tetramorium meridionale* (Jackson et al. 1990). Venom reservoirs of *T. meridionale*

workers contain ~1 ng of indole and ~100 pg of methylpyrazine, 2,5-dimethylpyrazine, trimethylpyrazine, and 3-ethyl-2,5-dimethylpyrazine. Indole alone shows little activity, but, when combined at 1 ng with 0.1 ng of each of the four pyrazines, shows activity similar to extracts of the venom reservoir (Jackson et al. 1990). Trail pheromones may be produced by a single gland, or come from multiple glandular sources. In the ponerine ant *Pachycondyla analis* (Forel), one compound from the venom gland and one from the pygidial gland are used (Janssen et al. 1995). They may be species-specific (Regnier et al. 1973), or may show cross-reactivity between several species (e.g. eight species of *Myrmica* respond to the venom gland component 3-ethyl-2,5-dimethylpyrazine; Morgan 1984). Trail pheromones are usually deposited on the substrate beneath the ant, though some evidence suggests ants will respond to volatiles emitted into the headspace and orient accordingly (Van Vorhis Key and Baker 1982; Hölldobler et al. 1994).

The trail pheromone of the Argentine ant, *Linepithema humile (*Mayr), has been the subject of research for more than four decades, beginning with the work of Cavill and colleagues in the 1960s and 1970s. Using various analytical and microchemical methods, it was determined that (*Z*)-9-hexadecenal (Z9-16:Ald), produced in the Pavan gland, was a component of an Argentine ant aggregation factor (Cavill et al. 1979). Although Z9-16:Ald was able to induce trail following in *L. humile* workers, it was less effective than the entire lipid fraction in eliciting aggregation by worker ants (Cavill et al. 1979; Cavill et al. 1980). Choe et al. (2012) recently identified two additional components of the Argentine ant trial pheromone, the pygidial gland products dolichodial and iridomyrmecin (herein referred to collectively as iridoids). Using *trans, trans*-dolichodial

obtained from defensive secretions of *Anisomorpha buprestoides* (Stoll) stick insects, and synthetic *cis*, *trans*-iridomyrmecin, they determined that dolichodial and iridomyrmecin, in a ratio of 1:1.87, respectively, were able to elicit trail following by Argentine ant workers.

Despite the apparent role of dolichodial and iridomyrmecin in inducing trail-following by ants, it is not clear what the relative contributions of each are. A single worker contains about 3.8 µg dolichodial and 11.7 µg iridomyrmecin (Choe et al. 2012), which is roughly a 1:4 ratio of dolichodial to iridomyrmecin. However, the ratio of dolichodial to iridomyrmecin in Argentine ant queens is ~7.5:1, which suggests they may have unequal roles in eliciting trail following by workers. These differences may result from different metabolic processes associated with queen and worker phenotypes, or possibly through selection pressure on ancestrally anonymous signals (Hölldobler 1995). Through chemical ritualization - the evolutionary process by which a phenotypic trait is altered to serve more efficiently as a signal (Hölldobler 1995) - dolichodial and iridomyrmecin may have been selected for different signaling functions in queens and workers. *L. humile* queens are primarily egg-layers and seldom venture outside the nest, whereas workers forage year-round (Markin 1970; Rust et al. 2000). These chemical and behavioral differences between queens and workers suggest dolichodial and iridomyrmecin may have different trail-following and recruitment functions.

In addition to the individual functions of the iridoids, the absolute stereochemistry of natural iridomyrmecin has not been resolved. This often overlooked chemical characteristic can have a profound impact on the perception of synthetic pheromones by

insects. Behavioral and physiological responses of insects to different pheromone enantiomers and diastereomers are variable. Enantiomers may act synergistically, as with the aggregation pheromone of the ambrosia beetle *Gnathotrichus sulcatus* sulcatol. Neither the (*R*)- nor (*S*)-enantiomers are active individually, but when combined at a 35:65 mixture of (*R*)- and (*S*)-sulcatol it becomes active (Borden et al. 1976). Alternatively, the wrong enantiomer may inhibit bioactivity. (*R*)-(-)-japonilure, the sex pheromone produced by the female Japanese beetle *Popillia japonica* (Newman), is the active enantiomer, and the presence of (*S*)-(+)-japonilure severely inhibits the activity of (*R*)-(-)-japonilure (Tumlinson et al. 1977). Enantiomeric excess of as little as 1% (*S*)-(+)-japonilure reduces bioactivity of the (*R*)-enantiomer by two-thirds. The effect of chirality on pheromone response has been examined in at least seven ant species (reviewed in Mori 2007). Most species respond to a single enantiomer, while other enantiomers do not inhibit the bioactivity of the active enantiomer (*Myrmica rubra* L., Cammaerts and Mori 1987; *Leptogenys diminuta* Smith, Steghaus-Kovâc et al. 1992; *Crematogaster castanea* Smith, Morgan et al. 2004; *Polyergus breviceps* Emery, Greenberg et al. 2007; *Monomorium pharaonis* L., Kobayashi et al. 1980; *Atta texana* Buckley, Riley and Silverstein 1974). In other species, such as *Myrmica scabrinodis* (Patton), the natural pheromone is a mixture of enantiomers (Mori 2007). The mandibular gland of *M. scabrinodis* workers contains (*R*)- and (*S*)-3-octanol at a ratio of 9:1, and elicits orientation and rapid movement from workers in bioassays (Cammaerts et al. 1985). The (*R*)-isomer alone elicits a partial response from workers, while the (*S*)-isomer is inactive. However, optimal bioactivity was obtained using a 9:1 mixture of synthetic (*R*)- to (*S*)-3-

octanol (Cammaerts and Mori 1987). In Argentine ants the importance of double bond geometry in bioactivity was established for *Z*9-16:Ald (Van Vorhis Key and Baker 1982), but the significance of chirality as it relates to bioactivity for iridomyrmecin and dolichodial has not been investigated.

The aim of this study was to determine the bioactivity of three compounds believed to be involved in Argentine ant mass recruitment, both individually and in combination, and to determine the absolute stereochemistry of iridomyrmecin, which has not yet been confirmed (Choe et al. 2012). First, natural dolichodial and iridomyrmecin (collectively referred to as iridoids) were purified from crude worker extract using a combination of preparative liquid and gas chromatography, followed by selective reduction of the iridomyrmecin fraction to remove trace amounts of dolichodial and/or *Z*9-16:Ald. Trail following by Argentine ant workers was then measured by applying individual purified iridoids or synthetic *Z*9-16:Ald to artificial trails and measuring the time and distance workers spent following trails. A second set of trail-following assays were conducted to determine if combinations of chemicals had modulatory or synergistic roles. While we found no evidence for the involvement of the iridoids in trail following, gas chromatography-electroantennographic detection (GC-EAD) showed worker antennae responded to iridomyrmecin. Finally, the absolute configuration of Argentine ant-derived iridomyrmecin was determined by comparing the retention time and peak shape of natural iridomyrmecin to those of four synthetic stereoisomers of iridomyrmecin and isoiridomyrmecin.

Methods

Insect colonies

Ant colonies were collected from the citrus groves on the University of California, Riverside campus (Riverside, CA). The groves are under biological control and with the exception of a mineral oil and natural pyrethrin treatment to prevent the spread of the Asian citrus psyillid *Diaphorina citri* (Kuwayama), are pesticide free. Ants were excavated from the soil and driven into artificial nests by using heat lamps. Queens were collected by aspirating them from a large plastic container (0.5 m x 0.9 m) lined with talc powder to prevent ants from escaping, and frozen at -20°C in 20 ml scintillation vials until use. Workers were kept in 6 L plastic containers lined with talc to prevent escape, and numerous artificial nests made of 1.6 x 15 cm Pasteur pipets that had been flame-sealed at the narrow end. A foraging arena of the same size was connected by a cardboard bridge to the stock colony. Ants were provided water *ad libitum*, 2 ml 25% w/v sucrose water three times per week, and ~1 g adult American cockroach (*Periplaneta americana* L.) twice per week. Colonies were maintained on a 12: 12 LD cycle at 24°C and 28% relative humidity.

Extraction of iridoids

Approximately 30,000 Argentine ant workers were aspirated from stock colonies, immersed in 20 ml of hexane, and macerated using a solvent-washed glass test tube. Two minutes after maceration, crude extracts were filtered through glass wool in 1 ml aliquots and recombined into two 20-ml glass scintillation vials (10 ml per vial). Filtered extracts

were then applied in 1 ml aliquots to a 3 cm x 0.4 cm bed of silica gel loaded into a glass pipette and eluted sequentially with 4 ml hexane and 4 ml EtOAc. The components of each fraction were identified by coupled gas chromatography-mass spectrometry (GC-MS), using an HP6890 gas chromatograph (Hewlett-Packard, now Agilent Technologies, Santa Clara, CA) equipped with a DB-17MS capillary column (25 m x 0.20 mm, 0.33 µm film thickness; J&W Scientific, Inc., Folsom, CA) coupled to an HP5973 mass selective detector run in full scan mode. The EtOAc fraction contained the iridoids along with other semi-polar constituents. The EtOAc fractions were concentrated by rotary evaporation and recombined for a total volume of 5 ml. Natural *Z*9-16:Ald was not detected in any of the fractions and we were, therefore, required to use synthetic material for subsequent bioassays.

Reverse-phase high performance liquid chromatography (RP-HPLC) purification of iridoids

Following normal phase column chromatography, 250 µl aliquots of the EtOAc fraction were transferred to a 1 ml conical bottom glass auto-sampler vial. The iridoids were separated on an Infinity 1220 HPLC coupled to a 380-Evaporative Light Scattering Detector (Agilent Technologies). The HPLC was equipped with an Eclipse XDB-C18 reverse phase column (5 µm particle size, 4.6 mm i.d. x 250 mm; Agilent Technologies) and a 100 µl sample loop (25 µl injection volume was used). The column oven was set to 20°C and the ELSD was set to nebulize at 40°C, evaporate at 70°C, and the gas flow rate was set to 1.20 SLM. Separated components were collected using an 80:20 fraction

splitter between the HPLC column and the ELSD. Samples were separated using a solvent gradient of 0% EtOAc/100% MeOH for 3 min, during which the iridoids eluted between 2.56-2.90 min, followed by 100% EtOAc/0% MeOH for 7 min to purge the column of less polar compounds. The collected fractions were analyzed by GC-MS to determine purity, then pooled and concentrated by rotary evaporation prior to separation by preparative gas chromatography. The amount of *Z*9-16:Ald present in individual workers is only a few nanograms (Choe et al. 2012). Although GC-MS analysis identified *Z*9-16:Ald in aerations of 5000 macerated worker ants (Fig. 3.1), it was not detectable in any of the RT-HPLC fractions.

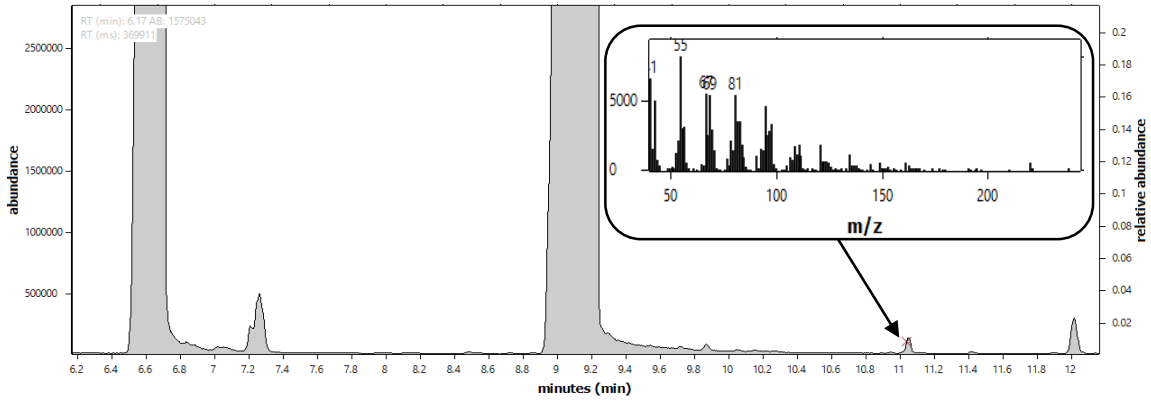

Figure 3.1 GC-MS analysis of volatiles collected from 5000 worker ants macerated in CH_2Cl_2. The respective percent relative abundance of dolichodial, iridomyrmecin, and *Z*9-16:Ald are 39.4, 60.0, and 0.02% of the detectable compounds. Picture inset shows the mass spectrum of *Z*9-16:Ald.

Preparative Gas Chromatography

HPLC-purified samples containing the iridoids and trace amounts of *Z*9-16:Ald were fractionated using an HP-5890 GC equipped with a glass column packed with 10% SP-1000 on 80/100 Supelcoport (2 m x 2 mm i.d.; Supelco Inc., Bellefonte, PA) and with a flame ionization detector (FID). Injector and detector temperatures were 200°C. The oven was programmed from 70°C, increased at a rate of 15°C /min to 200°C with a final hold time of 30 min. The column effluent was split between a 0.1 mm i.d. fused silica capillary to the FID, and a 0.53 mm i.d. fused silica capillary to the collection port, for a split ratio of ~1:28. Compounds were trapped in 15 cm x 2 mm i.d. glass capillary tubes cooled in the middle with dry ice. Trapped volatiles were eluted with ~20 µl hexane into 2 ml glass auto-sampler vials. The total collection time was 35 min, with dolichodial eluting from 21.0-21.8 min, and iridomyrmecin eluting between 27-28 min. Fractions were checked for purity by coupled gas chromatography-mass spectrometry (GC-MS) using an HP6890 gas chromatograph (Hewlett-Packard, now Agilent Technologies) equipped with a DB-17MS capillary column (25 m x 0.20 mm, 0.33 µm film thickness; J&W Scientific, Inc.) coupled to an HP5973 mass selective detector run in full scan mode. Fractions from multiple runs were then combined, evaporated to dryness by rotary evaporation, weighed, and immediately resuspended in 1 ml hexane. Following partial purification by liquid chromatography, the fraction containing iridomyrmecin and dolichodial was concentrated almost to dryness and treated with a few drops of a solution of $NaBH_4$ in ethanol (10

mg/ml) to selectively reduce both dolichodial and *Z*9-16:Ald to the corresponding alcohols, effectively eliminating them. After letting the solution stand at room temperature for 2 h, the mixture was quenched with 1M aqueous HCl and extracted with hexane. The hexane layer was dried by passage through a short column of anhydrous Na_2SO_4. Analysis by GC showed no trace of dolichodial or *Z*9-16:Ald in the resulting extract. Fractions were stored at -20°C in glass vials with Teflon cap-liners until used for bioassays.

Coupled gas chromatography-electroantennography (GC-EAD)

Aliquots of purified natural dolichodial and iridomyrmecin were analyzed by GC-EAD using methods similar to McElfresh and Millar (1999) on an HP-5890 Series II GC in splitless mode, with helium carrier gas. The GC was equipped with a DB-5 column (30 m x 0.32 mm i.d., 0.25 µm film; J&W Scientific Inc.) with a temperature program of 80°C /1 min, 15°C /min to 275°C /10 min. The column effluent was split equally with a press-fit Y-connector (J&W Scientific Inc.) into two branches of 0.25 mm i.d. deactivated fused silica capillaries, with one capillary routed to the GC's FID and the other routed through a heated transfer line (275°C) to a small, septum-sealed hole in the glass sample delivery tube. The delivery tube consisted of a 1 cm i.d. glass tube, with the distal end terminating in a hose nipple and the proximal end being slightly flattened and flared to accommodate the insect antennal preparation. Air flow rate through the sample delivery tube was 400 ml/min, with the air being humidified by passage over a pad of water-saturated glass wool on the bottom of the tube.

The antenna of a worker ant was removed by first decapitating workers, separating the head into 2 equal section, and finally removing excess cuticle and tissue until the intact antenna plus a small portion of the basal tissue remained. The terminal end of the antenna was cut off with a razor blade and the antenna was suspended between two glass capillary electrodes filled with insect physiological saline (modified Locke's saline, 7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, 0.20 g $NaHCO_3$ in 1 L distilled water) fitted over gold wires, with the wires running down the centers of the electrodes to within 2 mm of the suspended antenna. The preparation was moved as close as possible to the end of the delivery tube. Antennal responses were amplified with a custom-built multistage amplifier and the amplified signal was recorded on a pair of HP 2294 recording integrators.

Trail-following bioassays

Trail following by workers was examined by tracing a 5 cm circle with a number 2 pencil on a sheet of 16.5 cm x 21.6 cm construction paper, and marking the circle at 1 cm intervals to give both time and distance data (Fig. 3.2). Circles were treated with

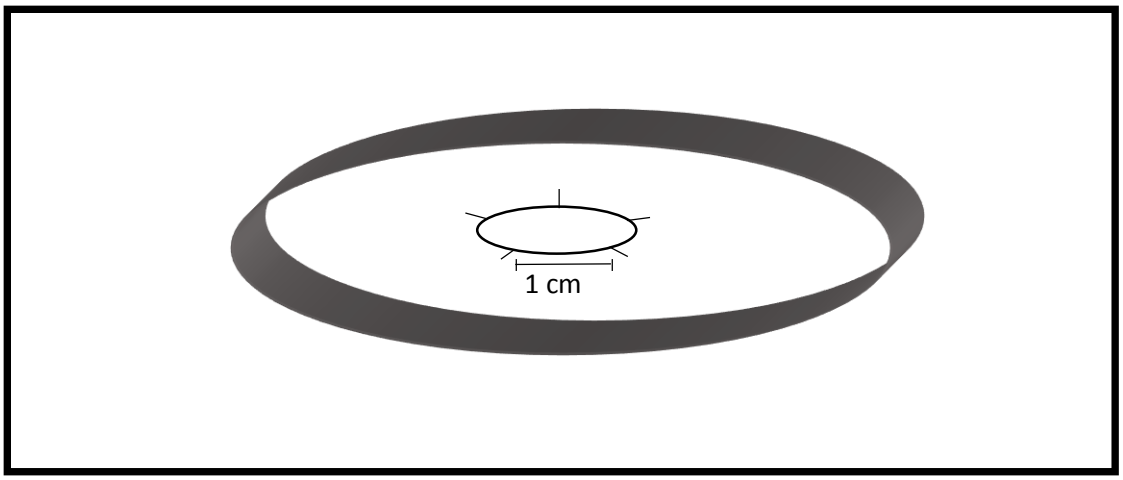

Figure 3.2 Trail following arena, consisting of a 15 cm o.d. x 1.5 cm depth glass petri dish cover, and a 16.5 cm x 21.6 in sheet of paper with a 5 cm circular trail marked at 1 cm intervals. The sides of the petri dish cover were coated with Fluon to prevent ants from escaping the assay arena.

EtOAc (solvent control), natural dolichodial (0.0067, 0.067, 0.67 ng/µl), natural iridomyrmecin (0.028, 0.28, 2.8, 28 ng/µl), or synthetic *Z*9-16:Ald (0.0002, 0.002, 0.02, 0.2, 2.0 ng/µl), at doses of 1 µl/cm to determine which concentrations were physiologically relevant. Solutions were applied using a 5 µl micropipette. A 2 min interval between extract application and the introduction of ants permitted evaporation of the carrier solvent. Worker ants from stock colonies were guided onto a glass micropipette and placed at the center of the treated circle, and immediately covered with a 15 cm x 1.5 cm glass petri dish lid. During preliminary bioassays ants often climbed the petri dish, so the sides of the petri dish were coated with Fluon to prevent them from escaping the assay arena. The total amount of time in seconds that each worker ant spent

trail-following on the treated circle was recorded for 180 s periods. The number of 1 cm intervals completed by ants was recorded over the same 180 s period. Trail following was defined as continuous movement along the treated area after completing a full 1 cm interval, as well as remaining within 5 mm of the treated area. Ants that simply arrested on the treated circle were not considered to be trailing, and this behavior was rarely observed. Each treated circle was used only once to ensure results were not confounded by the deposition of pheromones by the previous ant. The counter surface on which the arena was set and petri dish cover were cleaned with water between assays to prevent contamination of the assay arena. All bioassays were conducted at ambient room temperature (~24°C) and light conditions, between 0800 and 1600 hours. Results from preliminary bioassays guided subsequent tests using combinations of putative trail pheromone components.

Synergism between compounds

To investigate possible synergistic effects of combinations of extracts samples were recombined into two-component blends and a blend that included all three extracts. Sample concentrations were based on previously published results (Choe et al. 2012) and on results described in Chapter 2. The ratio of dolichodial:iridomyrmecin used for experiments (1:4) was determined by comparing the ratios found in aerations of live workers (~2:8, Chapter 2) and solvent extracts of workers (~4:6, Fig. 3.1), and taking the average of the two. For *Z*9-16:Ald, the lowest bioactive concentration from bioassays with individual compounds was used (0.002 ng/cm, Fig. 3.3a). Synergism was defined as

a significant increase in the time or distance workers traveled on treated circles compared to treatments using individual compounds only.

Absolute configuration of iridomyrmecin

To determine the absolute configuration of natural iridomyrmecin, peak shape and retention time of the natural extract were compared to four synthetic stereoisomers. (+)-iridomyrmecin, (-)-iridomyrmecin, (+)-isoiridomyrmecin, and (-)-isoiridomyrmecin were synthesized as described by Fischman et al. (2013). Synthetic compounds were diluted with hexane to concentrations similar to those of bioactive natural iridomyrmecin. Retention times of synthetic iridomyrmecin enantiomers and natural iridomyrmecin were compared using chiral GC. An HP-5890 GC equipped with a Cyclodex B column (20 m x 0.25 mm i.d. x 0.25 µm film; J&W Scientific Inc.) was programmed from 50°C for 1 min, 3°C per min to 200°C, with a 4 min hold time. The injection and detector temperatures were 150°C and 220°C respectively. Samples were diluted to concentrations of 25 ng/µl prior to injection, and samples were injected in splitless mode.

Statistics

All analyses were performed using IBM SPSS Statistics version 22 (IBM Corp., Chicago, IL). Time and distance data were square root transformed in order to meet the assumption of normality using the Shapiro-Wilk test ($\alpha = 0.05$). Trail-following rate data were normally distributed and were analyzed by one-way ANOVA. Transformed time and distance data were heteroschedastic (Levene Statistic < 0.001 for time and distance), and

were thus compared using the Games and Howell post-hoc test for pairwise comparisons. Pairwise comparisons of rate data were performed using Tukey LSD (Levene Statistic = 0.53).

Results

Bioactivity of individual compounds

Of the three compounds tested, only *Z*9-16:Ald (0.02 and 0.002 ng/cm) induced trail-following by Argentine ant workers. Worker ants spent significantly more time following circles treated with *Z*9-16:Ald than on circles treated with dolichodial, iridomyrmecin, or solvent ($F = 120.3$, $df = 437$, $P < 0.01$; Fig. 3.3a-c). Ants exhibited marked trail following on circles treated with 0.02 and 0.002 ng/cm *Z*9-16:Ald (mean 65.9 and 49.4 s, respectively) compared with solvent controls ($P < 0.01$). Neither dolichodial (0.0067, 0.067, 0.67 ng/µl) nor iridomyrmecin (0.028, 0.28, 2.8, 28 ng/µl) elicited trail following by workers and were not significantly different from solvent controls. Workers travelled significantly further on trails treated with *Z*9-16:Ald than on solvent or iridoid-treated trails ($F = 100.1$, $df = 437$, $P < 0.01$). Workers followed circles treated with *Z*9-16:Ald at doses of 0.02 and 0.002 ng/cm, and covered an average distance of 45.9 cm and 27.3 cm, respectively. Distance traveled on trails treated with dolichodial (0.0067, 0.067, 0.67 ng/µl) and iridomyrmecin (0.028, 0.28, 2.8, 28 ng/µl) and non-active *Z*9-16:Ald (0.0002, 0.2 and 2.0 ng/µl) doses were similar to solvent controls; ants in these treatment groups did not exhibit any obvious trail-following.

Synergism between compounds

We compared time, distance, and rate data of bioactive extracts to all possible combinations of extracts, and found no evidence for synergism. Combinations of extracts did not result in increased time spent on trails, increased distance traveled, or increased rate of trail-following compared to active doses of *Z*9-16:Ald (0.02 and 0.002 ng/cm; Fig. 3.3d).

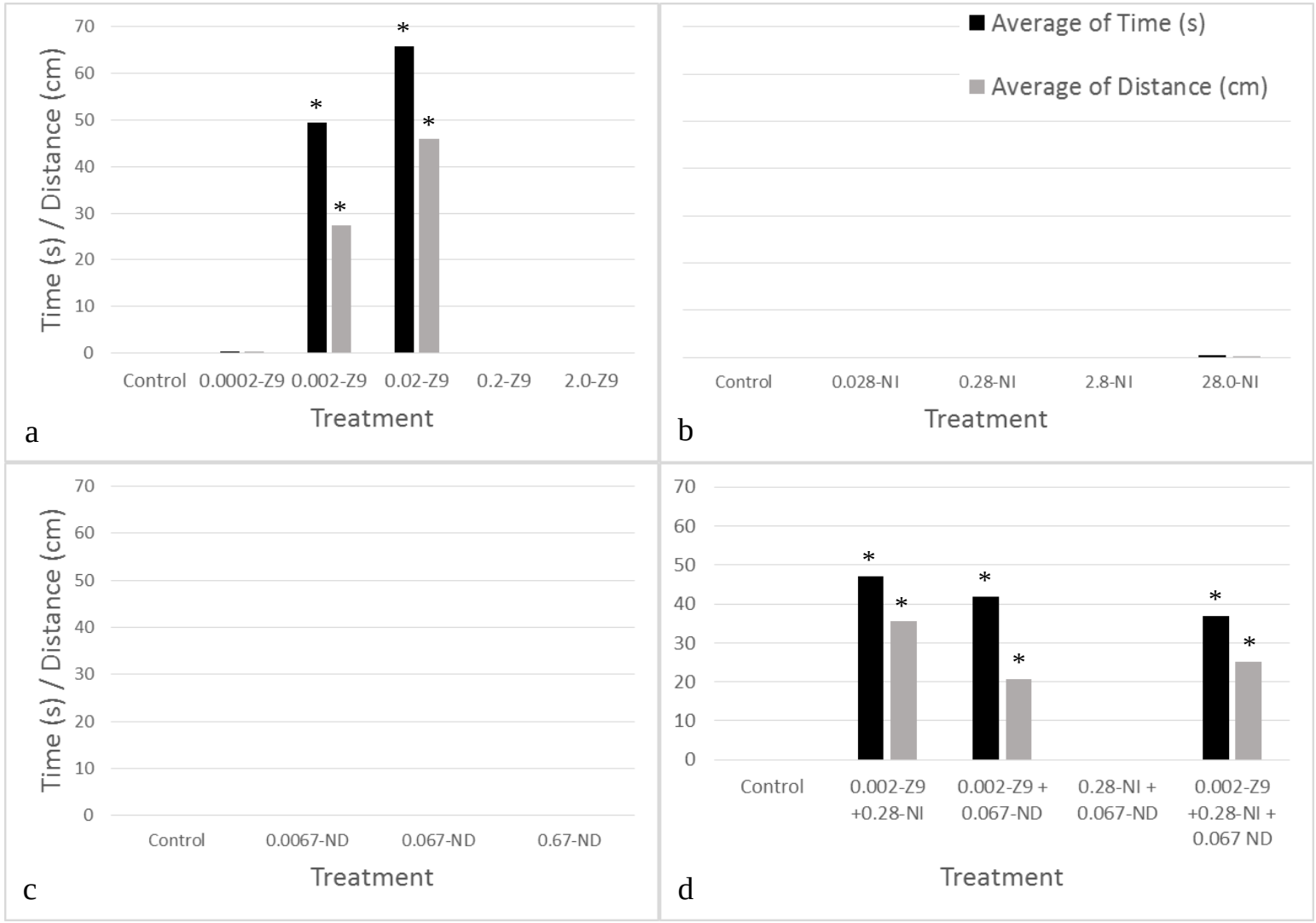
Time (s) / Distance (cm)
70
60
50
40
30
20
10
0
Control
0.0002-Z9
0.002-Z9
0.02-Z9
0.2-Z9
2.0-Z9
Treatment
a
Average of Time (s)
Average of Distance (cm)
Control
0.028-NI
0.28-NI
2.8-NI
28.0-NI
Treatment
b
Time (s) / Distance (cm)
Control
0.0067-ND
0.067-ND
0.67-ND
Treatment
c
Control
0.002-Z9 +0.28-NI
0.002-Z9 + 0.067-ND
0.28-NI + 0.067-ND
0.002-Z9 +0.28-NI + 0.067 ND
Treatment
d

Figure 3.3 Mean (± SE) trail-following time (in seconds) and distance (in cm) of individual ants on 5 cm trails marked with trail pheromone constituents (n = 20 for all groups). Values on the x-axis indicate doses in ng/cm in 1 µl solvent. The y-axis applies to both time and distance values. (a) Preparative GC-purified natural iridomyrmecin (NI); (b) Preparative GC-purified natural dolichodial (ND); and (c) synthetic *Z*9-16:Ald (Z-9); (d) Blends of trail pheromone components. Both individual compounds and blends of compounds were significantly more effective at eliciting trail following compared to controls. (*) indicates significant difference in time or distance compared with 5 cm circles treated with solvent onl

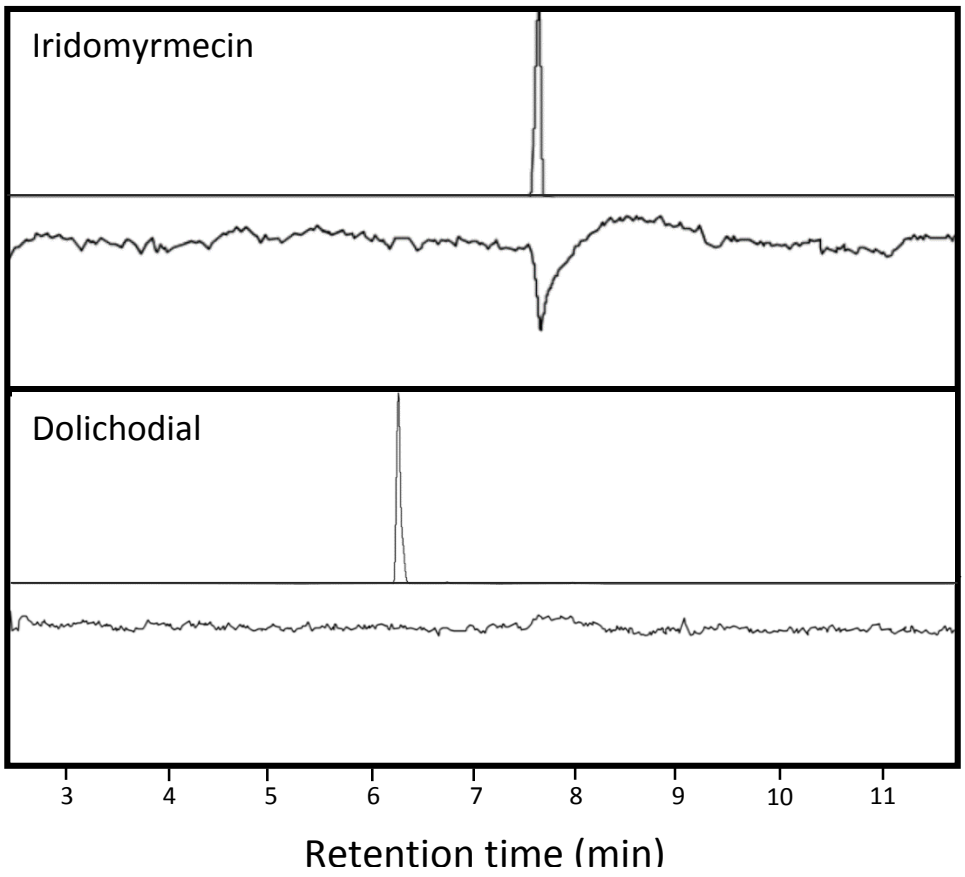

Figure 3.4 Coupled GC-EAD traces for an *L. humile* worker antenna responding to 2 µg natural iridomyrmecin and natural dolichodial, respectively. Top trace = gas chromatograph detector response (FID); bottom trace = antennal response.

Worker antennal responses to iridoids

Although the natural iridoids had no obvious effect on worker trailing time and distance, GC-EAD analysis revealed worker antenna responded to iridomyrmecin. Antennae of workers responded to iridomyrmecin at an injection dose of 2 µg in 16 different antennae

out of a total of 33. This dose is approximately one order of magnitude greater than the amount found in an individual ant (Choe et al. 2012).

Absolute configuration of iridomyrmecin

The composition of natural iridomyrmecin was a mixture of 94.5% (+)-iridomyrmecin, 1.9% (+)-isoiridomyrmecin, and 3.6% (-)-isoiridomyrmecin. Synthetic (+)-iridomyrmecin was 84.8% pure, with small amounts of (-)-iridomyrmecin and (-)-isoiridomyrmecin (1.9% and 13.3%, respectively). The presence of trace amounts of (-)-isoiridomyrmecin is likely the result of epimerization of (+)-iridomyrmecin. However, there is no mechanism by which the diastereomeric (+)-isoiridomyrmecin could arise because there is no (-)-iridomyrmecin present that could be epimerized. Nor is there another mechanism by which (+)-isoiridomyrmecin could be generated by isomerization because it would require changing two chiral centers, one of which is not epimerizable. Together this suggests that the small amount of (+)-isoiridomyrmecin in the natural iridomyrmecin fraction is an additional component of the blend and not the result of epimerization or isomerization of (+)-iridomyrmecin.

Table 3.1 Relative abundance of iridomyrmecin isomers in natural and synthetic samples. Sample retention time in minutes is indicated in parentheses next to row headers. Column headers are abbreviated. Abundance was calculated as percent of total chiral GC peak area. Compounds are ordered based on retention time with a Cyclodex-B column programmed at 50°C for 1 min, 3°C per min to 200°C, with a 4 min hold time.

Sample iridoid	(-)-irido	(+)-irido	(+)-isoirido	(-)-isoirido
Natural iridomyrmecin	-	94.5	1.9	3.6
(-)-iridomyrmecin (39.43)	74.1	11.4	13.0	1.5
(+)-iridomyrmecin (39.71)	1.9	84.8	-	13.3
(+)-isoiridomyrmecin (40.43)	3.9	0.6	84.1	11.4
(-)-isoiridomyrmecin (40.62)	-	5.6	1.2	93.2

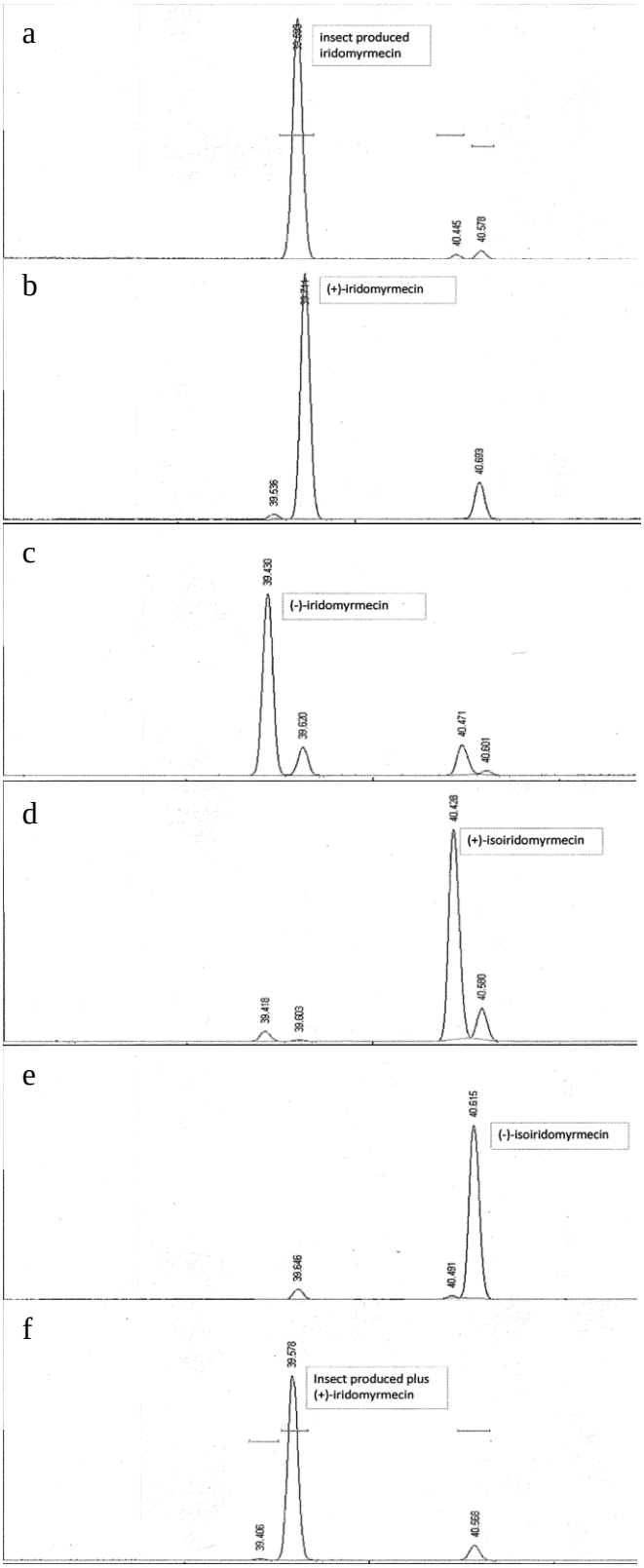

Fig. 3.5 Retention times and peak shapes of natural iridomyrmecin (a) and synthetic iridoids (b-e) on a Cyclodex-B column.
a) Natural iridomyrmecin,
b) (+)-iridomyrmecin,
c) (-)-iridomyrmecin,
d) (+)-isoiridomyrmecin,
e) (-)-isoiridomyrmecin, and
f) co-injection of natural iridomyrmecin and (+)-iridomyrmecin.

Discussion

Choe et al. (2012) was the first study of Argentine ant trailing behavior to examine the chemical nature of naturally laid trails. They found that recruitment trails of Argentine ants contained dolichodial and iridomyrmecin, and that a combination of both at a 1:1.87 ratio attracted workers and induced trail-following behavior. In this study neither natural dolichodial nor natural iridomyrmecin, alone or as a 1:4 mixture, induced trail-following behavior in workers under experimental conditions. One reason for these conflicting results may be the source of the iridoids used in experiments. In the previous study availability of purified natural dolichodial and iridomyrmecin was a limiting factor. Instead, the authors obtained synthetic *cis,trans*-iridomyrmecin, (+)-iridomyrmecin in this study, and *trans,trans*-dolichodial extracted from *Anisomorpha buprestoides* stick insects (Dossey et al. 2008), and it is possible that trace constituents may have influenced worker ant behavior. Although the dolichodial and iridomyrmecin standards used by the authors were 94% and 99% pure, respectively, ants may have responded to compounds that were present below the detection limit of the analytical equipment. This is exactly what occurred during a preliminary screening of iridoids for this study, and initially led us to believe iridomyrmecin was involved in trailing behavior as well. Only after the failure of synthetic (+)-iridomyrmecin to induce trail-following was the possibility raised that the natural iridomyrmecin fraction might be contaminated. GC-MS analysis with selective ion monitoring (SIM) for ion fragments m/z 55 and 81 showed that the trace peaks found in the natural samples had the same ion ratios as the *Z*9-16:Ald standard. The presence of trace amounts of *Z*9-16:Ald was confirmed following the loss of extract

bioactivity after the sample was reduced with $NaBH_4$. Therefore, the activity observed in the standards used by Choe et al. (2012) may have been the result of contamination by *Z*9-16:Ald, or possibly other trace components that still unidentified.

The major iridoid isolated from Argentine ant workers was (+)-iridomyrmecin. The fraction containing natural iridomyrmecin also had minor amounts of (-)-iridomyrmecin and (+)-isoiridomyrmecin. The presence of (-)-isoiridomyrmecin in the natural fraction was expected, as (+)-iridomyrmecin can epimerize at the C4 position under acid, base, or high temperature conditions (Stökl et al. 2012). Thus, any sample containing (+)-iridomyrmecin or (-)-isoiridomyrmecin will appear contaminated with the other when analyzed by GC because the temperature of the injection port is hot enough to cause partial epimerizatin at C4. However, (+)-iridomyrmecin and (-)-isoiridomyrmecin cannot react to form their antipodes, (-)-iridomyrmecin or (+)-isoiridomyrmecin, respectively. Therefore, the small amount of (+)-isoiridomyrmecin identified in the natural fraction is likely biosynthesized by the ants and not an artifact of the storage and analysis of the sample.

Neither dolichodial, iridomyrmecin, nor a combination of the two induced trailing behavior. Furthermore, no modulatory affect was observed when combinations of two or all three components were tested. Even so, we cannot exclude the possibility that our experimental design was insufficient to identify subtle behavioral responses by workers to these compounds. It is possible that the trail following response on which the bioassay was based requires pheromone components be presented a different chemical context than the setup used. Although trail pheromones have the obvious function of recruiting

workers to resources, evidence suggests worker response to trail pheromone components depends both on the environmental context in which they are perceived and the quantity in which they occur (Czaczkes et al. 2015 and references therein). Such a context-dependent response has been observed in Argentines ant workers foraging in an unfamiliar area. Laboratory colonies of Argentine ant workers explore new territory by "sweeping" – chemically marking – an unmarked area (Deneubourg et al. 1990). Workers randomly explore the area closest to the nest and work their way out. As workers advance into the unmarked area, a trail forms between the nest entrance and the frontier, indicating ants are being recruited to explore this area. Trails only begin to form when a sufficient amount of pheromone is deposited on the substrate. Because exploratory trails are laid continually, and ants typically avoid exploring the same area twice, the relatively low concentrations used in our bioassay (those that did not induce trail-following) may actually deter ants from following the trail. Once a certain threshold has been reached (i.e. 0.002 ng/cm *Z*9-16:Ald in this study), treated circles may be perceived as part of a trunk trail.

Iridoids are known to be involved in communicatory functions outside of trailing and recruitment. Dolichodial and iridomyrmecin play an important role in inhibiting necrophoresis in workers (Choe et al. 2009). Iridoids produced by Argentine ants, and other dolichoderine ants for that matter, are believed to serve defensive, alarm, and antibiotic roles (Cavill et al. 1976; Morgan 2008; Wilson and Pavan 1959). Argentine ant queens produce dolichodial in much higher abundance than iridomyrmecin. In other eusocial hymenoptera volatile emmissions from queens often elicit a retinue response

from workers, causing workers to groom and feed the queen. In the red imported fire ant *Solenopsis invicta* (Buren) experimental evidence suggests that volatile compounds produced in the poison sac are recognized as queen-specific by worker ants (Vargo 1998), and it is possible dolichodial may have a similar function in Argentine ant colonies. Other possible roles for dolichodial include signaling queen quality and/or quantity, as well as her presence. Alternatively, dolichodial could be a form of background noise that only elicits a response when it is absent. Choe et al. (2009) demonstrated that following an ant's death, the gradual dissipation of dolichodial and iridomyrmecin signals its' death to other ants and the body is then moved to a refuse pile. In queens, decreased levels of dolichodial might signal reduced fecundity and lead to execution. The absence of dolichodial may also signal to workers that there is no longer a functional queen and prompt them to begin rearing new sexual offspring to replace her. These hypotheses will be tested in the future pending the development of appropriate bioassays.

The results of this study definitively show that dolichodial and iridomyrmecin have little, if any, effect on trail-following by Argentine ant workers. Although it had no obvious role in inducing trail-following in workers, worker antennae responded to iridomyrmecin, highlighting the possibility of other communicatory functions. The absolute stereochemistry of Argentine ant-derived iridomyrmecin was identified as (+)-iridomyrmecin, although a suitable behavioral bioassay still needs to be developed to determine its range of function.

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Chapter 4: A complex reproductive signal in the unicolonial Argentine ant *Linepithema humile* (Mayr)

Introduction

Chemical communication is the predominant signaling modality within social insect colonies. Fertility signals, which identify the presence of a queen as well as her fecundity, are fundamental to maintaining colony structure. In ants (Hymenoptera: Formicidae) the presence of the queen affects behavior (Smith et al. 2013, 2015), ovarian development (Holman et al. 2010; Van Oystaeyen et al. 2014), chemical profile (Liebig et al. 2000, Monnin 2006; Moore and Liebig 2010), gland secretion (Eliyahu et al. 2011), and gene expression (LeBoeuf et al. 2013; Yan et al. 2014) of other colony members. Although a glandular source of queen fertility signals has been identified in the red imported fire ant *Solenopsis invicta* (Buren) (Vargo and Hulsey 2000), the vast majority of experimental evidence implicates cuticular hydrocarbons (CHCs) as the source of social insect queen fertility signals (Van Oystaeyen et al. 2014).

CHC profiles have been correlated with dominance and fertility in the ant subfamilies Ponerinae (*Hypoponera opacior* Forel, Foitzik et al. 2011; *Harpegnathos saltator* Jerdon, Liebig et al. 2000), Myrmeciinae (*Myrmecia gulosa* F., Dietemann et al. 2003), Myrmecinae (*Aphaenogaster cockerelli* Andre, Smith et al. 2008; *Crematogaster smithii* Creighton, Oettler et al. 2008), Formicinae (*Formica fusca* L., El-Showk et al. 2010), and Dolichoderinae (*Linepithema humile* Mayr; de Bisseau et al. 2004). CHCs associated with reproductive status have been identified in over 40 ant species and

include *n*-alkanes, methyl-branched alkanes, and alkenes (Van Oystaeyen et al. 2014). CHCs may differ between workers and reproductives in the relative proportions of chemicals within the profile (Monnin et al. 1998), or in the presence/absence of certain components (de Biseau et al. 2004). These chemical differences likely serve as honest signals of queen fertility, and are often correlated with the reproductive state and health of the queen (Liebig et al. 2000; Holman et al. 2010, 2013). Fertility signals prompt workers to forego their own direct fitness (worker egg-laying) in favor of supporting a highly fecund reproductive caste, thereby increasing their inclusive fitness (Keller and Nonacs 1993; Peso et al. 2014). By removing queens from experimental colonies, and thus removing the fertility signal, workers will respond to a change in the social environment by activating their ovaries or by rearing new queens from existing brood, depending on the biology of the species. However, the precise chemical components responsible for eliciting stereotyped behavior in colony members, and the context which they are detected, remains poorly understood.

Currently, there are two lines of experimental evidence that address the perception of fertility signals by workers. The first line of evidence suggests that workers will respond to individual fertility signals presented alone and without the remainder of the chemical background. In three formicine species, *Lasius niger* (L.), *L. flavus* (Fabr.), and *Cataglyphis iberica* (Emery), presentation of individual queen-specific CHCs decreased worker ovarian activation. In *L. niger* and *L. flavus*, 3-methylhentriacontane (3-MeC_{31}) was also reported to signal queen health (Holman et al. 2010) and to suppress ovarian activity of functional queens (Holman et al. 2012). However, a recent study examining

the fertility signal of *Odontomachus brunneus* (Patton) queens suggests that individual compounds are insufficient for conveying queen presence. When *O. brunneus* workers encounter a queen, they assume a stereotyped submissive posture. This response can be replicated by applying the fertility signal, (*Z*)-9-nonacosene (*Z*9:C_{29}), to nestmate workers, which have similar chemical profiles except for the relative abundance of *Z*9:C_{29}. This response was not observed when workers were presented with non-nestmate queens, or when the fertility signal was applied to non-nestmate workers. Additionally, presentation of the fertility signal in the absence of the background chemical profile did not inhibit worker reproduction. These results suggest that fertility signals are perceived correctly by workers only when presented in the appropriate chemical context.

While it appears that chemical context is important for fertility signaling in at least one ant species, the nature of this chemical context has not been investigated. Chemical context may also depend on methyl-branch position and carbon chain length, or on the presence of appropriate classes of CHCs (e.g. alkanes and/or alkenes). In the Argentine ant *Linepithema humile*, evidence suggests that homologous CHCs, that is, CHCs with the same methyl-branching patterns or sites of unsaturation but different carbon chain lengths, encode the same information and are essentially chemical "synonyms" in the context of nestmate recognition (van Wilgenburg et al. 2010, 2012). Chemical class has also been shown to affect nestmate recognition in *L. humile* workers, and both saturated hydrocarbons (SHCs) and unsaturated hydrocarbons (UHCs) were required to elicit species recognition responses from workers under field conditions (Greene and Gordon 2007).

In *L. humile*, pheromones are thought to regulate queen production in both field and laboratory colonies. Laboratory colonies in which queens have been removed will rear new queens from early-instar larvae, but the daily addition of queen corpses inhibits new queen production (Vargo and Passera 1991). Extracting queen corpses with pentane prior to placement in colonies does not inhibit queen production, suggesting a queen pheromone used by workers to identify the reproductive caste is removed with organic solvents (Vargo and Passera 1991). The chemical nature of this signal was initially examined by de Biseau et al. (2004), and a more in-depth identification was recently conducted by our group (Chapter 2). Argentine ant queen SHCs differ from workers in their relative abundance of 5-methylalkanes and 5, 11-dimethylalkanes, which occur as a homologous series at carbon chain lengths of 27, 29, and 31. Queens also possess a unique homologous series of UHCs, primarily composed of (*Z*)-14- and (*Z*)-9-monounsaturated HCs with chain lengths of 29, 31, 33, and 35 carbons. Here, we provide the first experimental evidence that queen extracts are recognized by *L. humile* workers and that treatment of laboratory colonies in which queens have been removed with SHCs and UHCs is sufficient to prevent workers from rearing new queens. The second part of this study explored the chemical context necessary for proper recognition of the fertility signal. By separating queen SHCs and UHCs into subsets based on carbon chain length, extracts in which individual subsets were removed could be used to determine if the entirety of the profile was necessary to be properly recognized by workers. We also tested the hypothesis that homologous compounds were in fact chemical "synonyms" that convey the same information by creating extracts made up of only the most abundant

SHCs and UHCs. It should be noted that throughout this paper the terms "inhibit", "prevent", and "suppress" are used to describe the effect queen pheromones have on worker reproduction and brood rearing. The meaning of these descriptors is not to suggest that queen pheromones are in any way controlling workers at a cost to their own fitness. Rather, when queen pheromones are added to colonies in which queens have been removed, workers behave as though a queen is present, which prevents, inhibits, or suppresses a different set of behavioral or physiological processes that would otherwise occur.

Methods

Stock colonies

Ant colonies were collected from the citrus groves on the University of California, Riverside campus (Riverside, CA). The groves are under biological control and, with the exception of a mineral oil and natural pyrethrin treatment to prevent the spread of the Asian citrus psyillid *Diaphorina citri* (Kuwayama), were pesticide free. Ants were excavated from the soil and transferred to a wooden box (1 m x 0.6 m x 0.1 m) lined with Fluon to prevent them from escaping. Ants were driven from the soil into artificial nests by placing heat lamps with 60 W lightbulbs above the soil. Artificial nests were composed of plaster discs (100 mm x 15 mm) placed on top of one another that had been immersed in water. Artificial nests were provisioned with 25% sucrose water (wt/wt) to encourage colonies to move out of the soil. Queens were collected by aspirating them from large mixed groups of workers and queens held in a large plastic container (0.6 m x

0.9 m) lined with talc powder to prevent them from escaping. Queens were frozen at -20°C in 20 ml scintillation vials until use. Workers were kept in plastic containers lined with talc to prevent escape, and numerous artificial nests made of 1.6 x 15 cm Pasteur pipets that had been flame-sealed at the narrow end. A foraging arena of the same size was connected by bridge to the stock colony. Ants were provided water *ad libitum*, 2 ml 25% w/v sucrose water three times per week, and ~1 g adult American cockroach (*Periplaneta americana* L.) twice per week. Colonies were maintained on a 12: 12 LD cycle at 24°C and 28% relative humidity.

Experiment 1

Linepithema humile queens (ca. 1500) were collected between 1 October 2012 and 15 December 2012. Queens were extracted by immersion in 5 ml *n*-hexane for 2 min, followed by 5 1-ml *n*-hexane washes, resulting in a total of 10 ml of crude extract. The crude extract was concentrated under reduced pressure, reconstituted in 500 µl, and loaded onto liquid chromatography columns prepared from Pasteur pipettes filled with 300-500 mg of silica gel impregnated with silver nitrate (10% wt/wt; +230 mesh, Aldrich Chemical Co., Milwaukee, WI). Columns were sequentially eluted with 4 ml hexanes (saturated hydrocarbons, or SHCs), 4 ml 10% cyclohexene in hexane (unsaturated hydrocarbons, or UHCs), and 4 ml diethyl ether (polar compounds). The components of each fraction were identified by coupled gas chromatography-mass spectrometry (GC-MS), using an HP6890 gas chromatograph (Hewlett-Packard, now Agilent Technologies, Santa Clara, CA) equipped with a DB-17MS capillary column (25 m x 0.20 mm, 0.33 µm

film thickness; J&W Scientific, Inc., Folsom, CA) coupled to an HP5973 mass selective detector run in full scan mode. Queen equivalence (qe) was established by comparing GC-MS peak areas of the fractions to peak areas of a crude hexane extract of 5 queens concentrated to 5 µl, of which 1 µl was injected (1 qe/µl). Fractions were used singly or in 1:1 v/v ratios when combined to establish 6 treatment groups (Fig. 4.1): 1) solvent control, 2) queen-right control, 3) saturated hydrocarbons (SHCs) + unsaturated hydrocarbons (UHCs) + polar compounds (recombined extract), 4) SHCs + UHCs, 5) SHCs, and 6) UHCs. Experimental colonies were established from overwintered ants collected in January 2013. In the laboratory, colony fragments in which queens have been removed are 3 to 8 times more likely to produce queens compared with colonies collected in the summer or fall (Vargo and Passera 1992). Experimental colony fragments were kept in 6 L plastic containers under the same conditions as stock colonies (see above) and composed of 2 queens, 500 workers, and brood of various developmental stages. Queens were allowed to lay eggs for 4 weeks, after which queens were removed. Colonies were provided with 2 artificial nests composed of 1.6 x 15 cm Pasteur pipets that were flame-sealed at the narrow end, so that ants had only one point of access to nests. Artificial nests were placed ~5 cm apart and perpendicular to each other inside the plastic boxes. One of the two nests was covered with a 5 cm x 15 cm piece of cloth to shield ants from light and stimulate the ants to relocate into the covered nests. Following the removal of queens from experimental colonies, extracts were applied at a dose of 2 qe/d to the interior surface of the pipettes' large diameter opening (nest entrance). The pipettes were rotated as the solvent dried to ensure the inside of the pipette was completely covered,

and were returned to the colony after ~5 min to allow the solvent to evaporate. The cloth cover that previously covered the occupied nest was placed over the treated nest. The colony boxes were placed under 65 W light bulbs to stimulate ants to relocate into the treated nest. This was repeated every 24 h and colonies were monitored daily for 12 weeks for the presence of queen pupae.

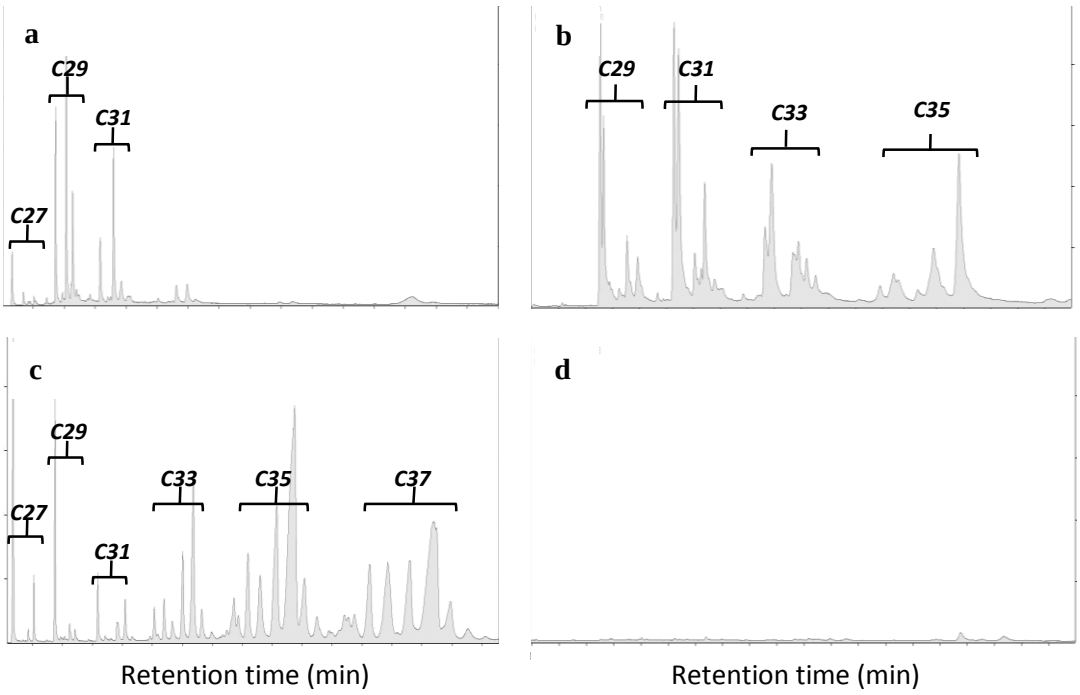

Figure 4.1 Representative chromatograms of queen (top) and worker (bottom) cuticular hydrocarbons. **a**) Queen alkanes, **b**) queen alkenes, **c**) worker alkanes, and **d**) worker alkenes. Queens have significantly higher proportions of 5-methylalkanes and 5,11-dimethylalkanes and have a series of (*Z*)-14- and (*Z*)-9-monounsaturated alkenes. Brackets indicate subsets of CHCs that differ in methyl branch position **a, c**) or double bond location **b**) but have the same carbon chain length.

Experiment 2

Queens were collected between 6 May 2013 and 30 June 2013. Queen SHCs and UHCs were isolated from ca. 2500 queens as described in experiment 1 (see above). Fractions were evaporated to dryness and taken up in 2 ml hexanes. Aliquots of each fraction were added to a 1 ml conical auto-sampler vial, evaporated to dryness, and reconstituted in 200 µl ethyl acetate. The resulting solution was fractionated by preparative RP-HPLC as described by Bello et al. (2015) using an Agilent 1220 Infinity HPLC coupled to a 380-Evaoprative light scattering detector (ELSD) (Agilent Technologies, Santa Clara, CA), fitted with an Eclipse XDB-C18 reverse phase column (5 µm particle size, 4.6 mm i.d. x 250 mm; Agilent Technologies) and a 100 µL sample loop (25 µL injection volume was used). The column oven was set to 50°C and the ELSD was set to nebulize at 40°C, evaporate at 70°C, and the volumetric flow rate of the gas was set to 1.20 standard liters per minute (SLM). Each separated component was collected using an 80:20 fraction splitter between the HPLC column and ELSD. The collected fractions were analyzed by GC-MS to determine purity, then pooled and concentrated *in vacuo* prior to establishing queen equivalence (Figs. 4.2, 4.3).

As in experiment 1, the effectiveness of a treatment group was determined by the inhibition of queen production in colony fragments in which queens had been removed. Treatments were: 1) hexanes only (+ control); 2) two mated queens (- control); 3) total CHC extract (TE); 4) TE – C_{29} alkanes; 5) TE – C_{31} alkanes; 6) TE – C_{29} alkenes; 7) TE – C_{31} alkenes; 8) TE – C_{33} alkenes; 9) TE – C_{36} alkenes; 10) C_{29} alkanes + C_{29} alkenes; 11) C_{31} alkanes + C_{31} alkenes; and 12) all C_{29}-C_{31} alkanes and alkenes. Groups 3 – 10 were

designed to test if all queen-specific CHCs are necessary for proper recognition of queens, or if removal of certain subsets of the total CHC blend would prevent workers from correctly perceiving the extract. Treatment groups 11 – 12 tested the hypothesis that homologous CHCs convey the same information about queens to workers, thus acting as chemical synonyms. Colonies were established from ants collected in June 2013. Extracts were applied to glass Pasteur pipettes, flame sealed at the tip, at a dose of 2 qe/d. One artificial nest was covered with a piece of cloth to shield ants from light and stimulate the ants to relocate into covered nests. Following removal of queens, extracts were applied to the nest entrance of the unoccupied nest. The cloth cover was moved over the treated artificial nest and colonies were placed under 65 W light bulbs, prompting workers to move the brood and themselves into the treated nest. This was repeated every 24 h and colonies were monitored daily for 12 weeks for the presence of queen pupae.

Experimental colonies were maintained under the same conditions as described for experiment 1, with the exception of diet. In addition to insect protein (*P. americana* L.), colonies were given 0.1 ml of a nutrient-rich diet three time per week. The diet consisted of 5 g protein and vitamin-rich powder (Optimum Nutrition Inc. Aurora, IL), 2 g royal jelly (eBeeHoney, Ashland, OH), and 35 ml water. This diet was administered to ensure queens and brood had access to a highly nutritious food source. Nutrition is linked with queen-worker caste determination in many eusocial Hymenoptera, and because queen production is not as efficient in non-overwintered laboratory colonies, maintaining well-fed colonies would potentially make the assay more robust. Queens were kept in colonies

where they laid eggs for 4 weeks before being removed to stimulate the production of new queens.

Statistics

All analyses were performed using Systat 2013. Queen production was highly non-normal as determined by the Shapiro-Wilk test and required the use of non-parametric tests. Queen production in both Experiments 1 and 2 were compared between treatment groups using Kruskal-Wallis ANOVA with Conover-Inman post-hoc test ($\alpha = 0.05$).

Results

Experiment 1

There was a dramatic difference among treatments in the production of queens in laboratory colonies in which queens were removed ($H = 32.72$, $P < 0.01$). With the exception of a single colony in the TE treatment that produced four queens, no queens were produced in the queen control, TE, or SHC + UHC treatment groups (Fig. 4.2). In contrast, hydrocarbon classes tested singly were ineffective at preventing queen production in orphaned colonies. The fact that the recombination of all three chemical classes were no more effective than the SHCs + UHCs treatment suggests that polar compounds are not part of the queen signal. These results are the first in the Argentine ant to provide evidence that CHC extracts of mature queens are perceived by workers, which respond by not rearing queens in laboratory colonies in which queens have been removed.

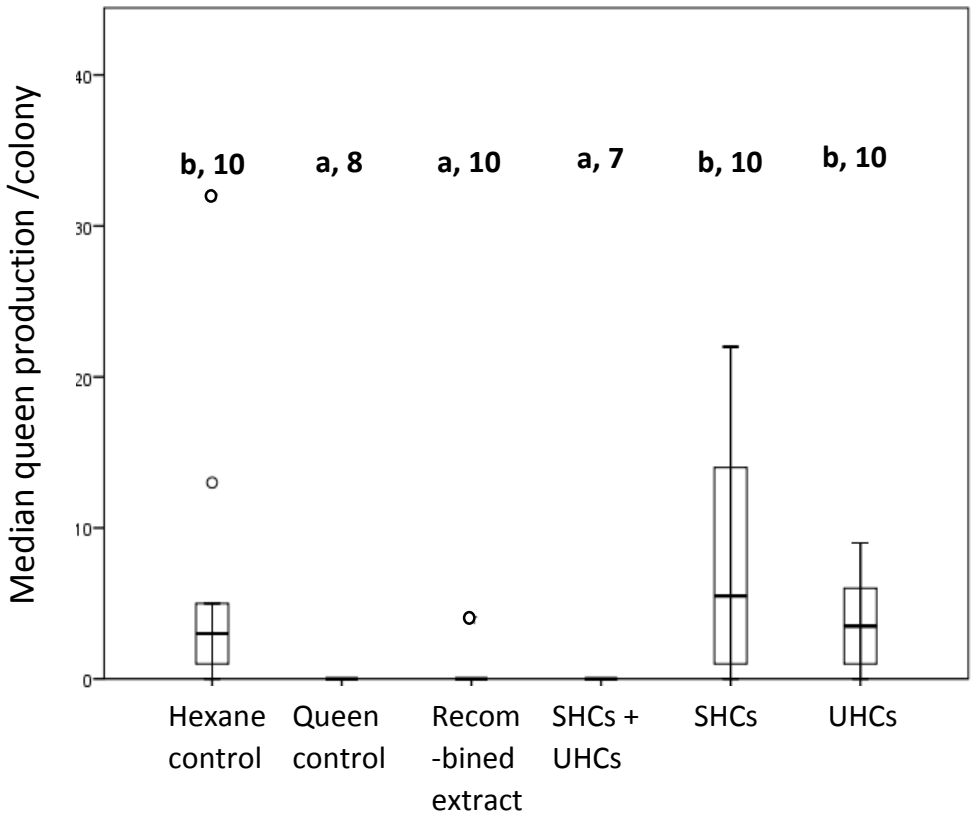

Figure 4.2 Boxplot indicating the effect of queen extracts on median queen production in laboratory colonies of *L. humile* in which queens have been removed. Circles represent outliers. Sample size is indicated. Letters indicate significant differences of pairwise comparison with Conover-Inman post hoc test ($P < 0.05$).

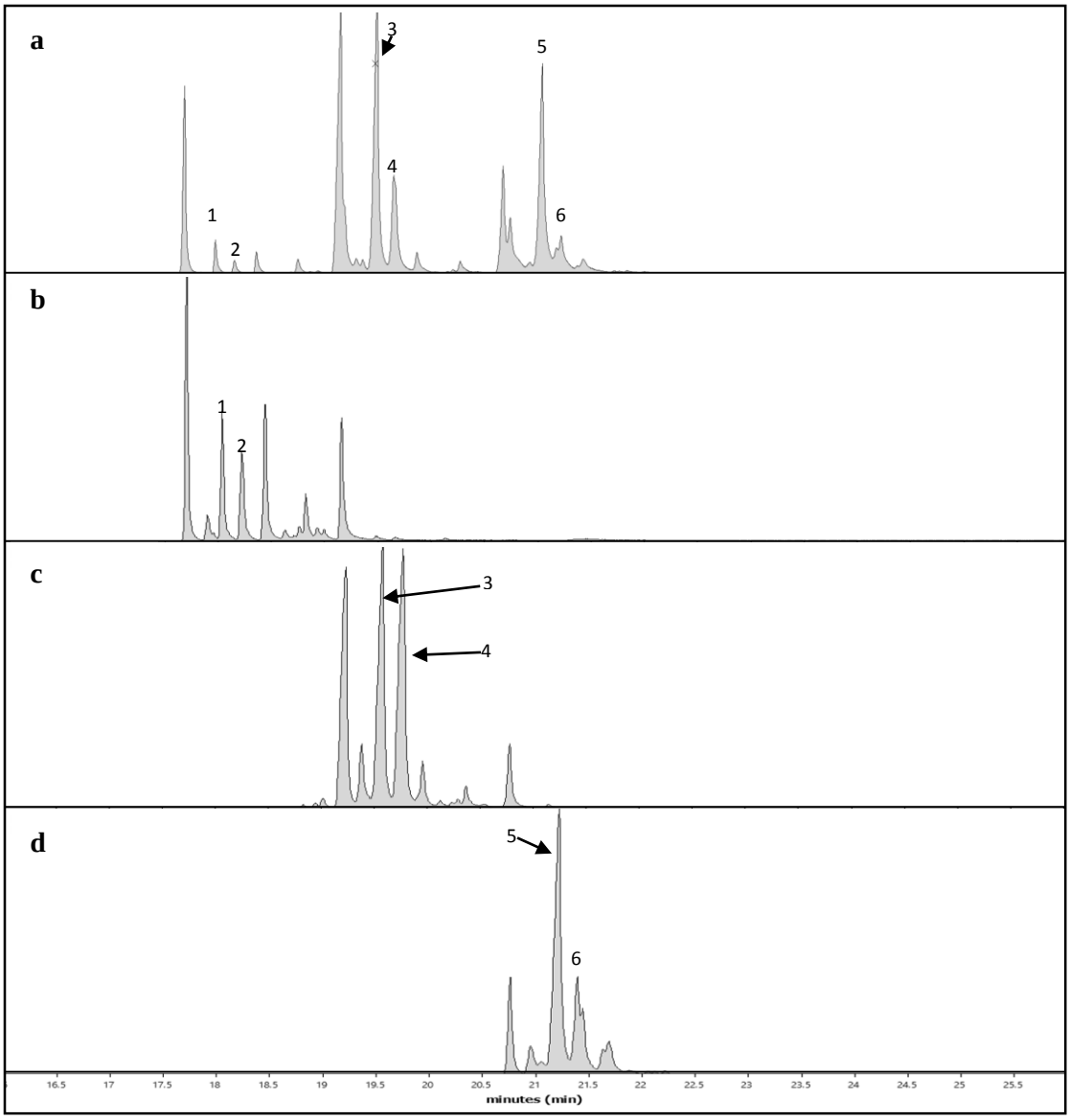

Figure 4.3 Representative GC traces of subsets of queen alkanes following separation using RP-HPLC. **a**) Total queen alkanes, **b**) C_{27} alkanes, **c**) C_{29} alkanes, **d**) C_{31} alkanes. Peaks that are numbered are queen-specific methylalkanes. 1: 5-methylheptacosane, 2: 5,11 dimethylheptacosane, 3: 5-methylnonacosane, 4: 5,11-dimethylheptacosane, 5: 5-methylhentriacontane, 6: 5,11-dimethylhentriacontane.

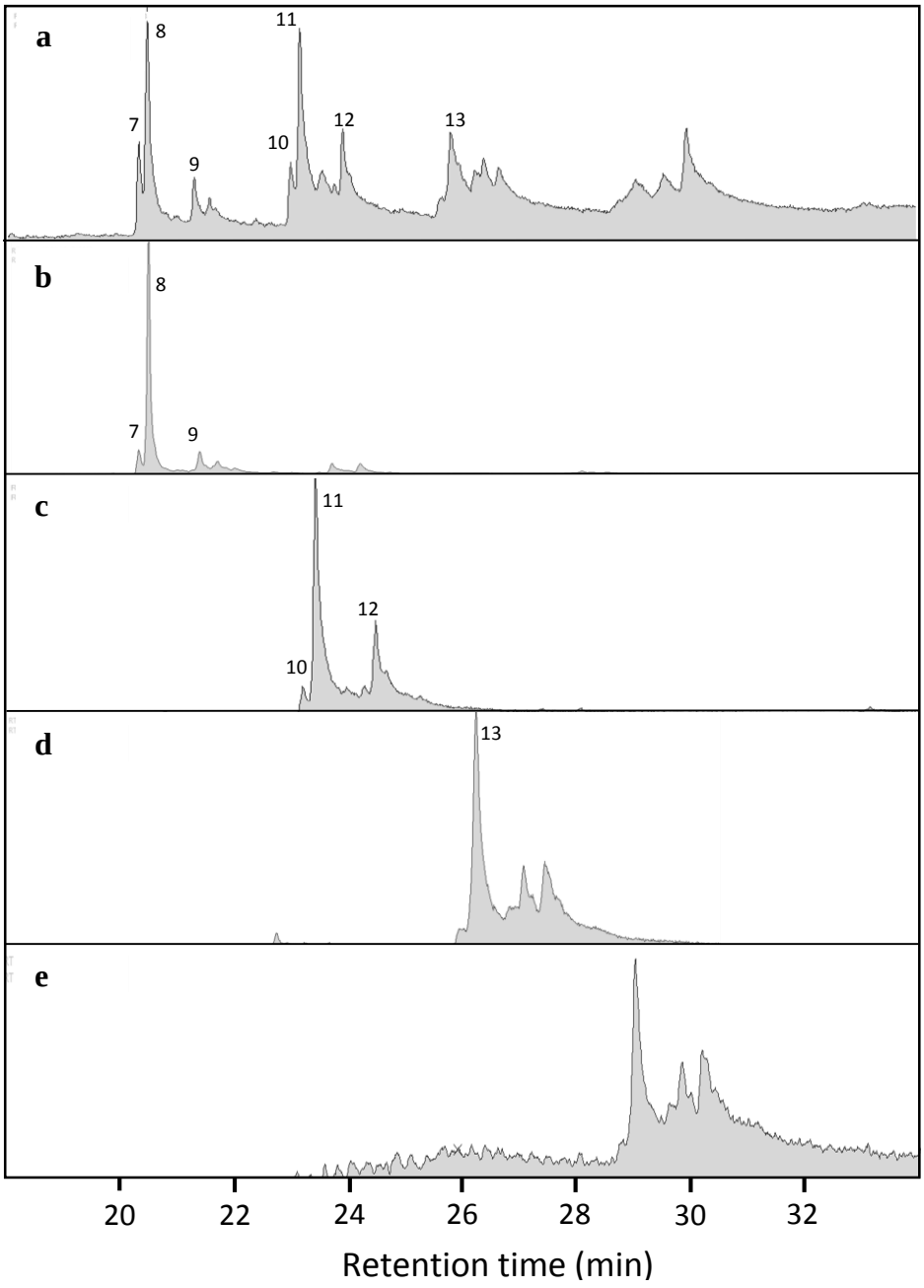

Figure 4.4 Representative GC traces RP-HPLC-separated **a**) recombined queen alkenes, **b**) C_{29} alkenes, **c**) C_{31} alkenes, **d**) C_{33} alkenes, and **e**) C_{35} alkenes. Peak numbers indicate queen-specific monounsaturated compounds. 7: (*Z*)-14-nonacosene, 8: (*Z*)-9-nonacosene, 9: (*Z*)-9-triacontene, 10: (*Z*)-14-hentriacontene, 11: Δ^9-hentriacontene, 12: Δ^9-dotriacontene, 13: Δ^{11}-tritriacontene.

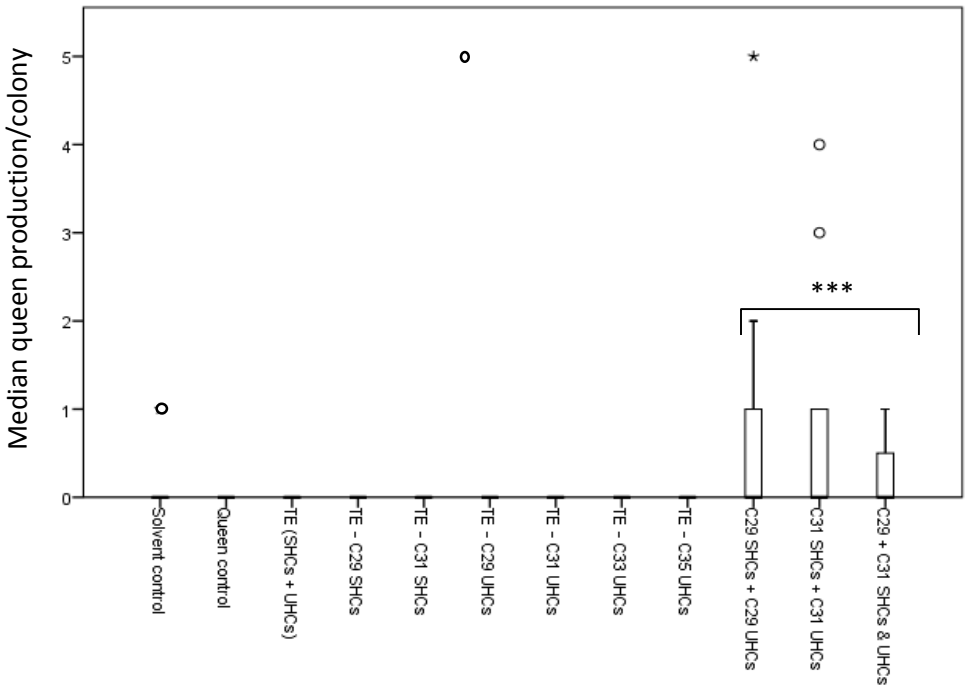

Figure 4.5 Boxplot indicating the effect of fractions of queen extracts on median queen production per colony in laboratory colonies of *L. humile* in which queens have been removed. $n = 10$ for all treatment groups. '***' Indicates statistical significance in treatment groups compared to solvent controls (Kruskal-Wallis ANOVA, $P < 0.01$). Circles indicate outliers.

Experiment 2

The use of RP-HPLC to separate queen SHCs and UHCs into subsets based on carbon chain length was successful (Figs 4.2, 4.3). Extracts of C_{27} and C_{29} SHCs also contained C_{29} and C_{31} *n*-alkanes, respectively. This was due to the inability of the chromatographic methods used to achieve complete baseline separation. Because these same *n*-alkanes are

present in worker chemical profiles at similar relative proportions (Chapter 2), this was not considered to be a confounding factor in measuring the bioactivity of these fractions.

Removing single subsets of SHCs or UHCs (treatment groups 3-10) had no impact on queen production in laboratory colonies in which queens have been removed (Fig. 4.5). Queen-production in treatment groups 3-10 was not significantly different from colonies containing live queens, or from colonies exposed to recombined SHCs and UHCs ($H = 0.0$, $df = 9$, $P > 0.1$). In contrast, colonies exposed to C_{29} SHCs + UHCs, C_{31} SHCs + UHCs, or C_{29} and C_{31} SHCs + UHCs (treatment groups 11-13) produced significantly more queens than queen-right colonies and colonies treated with recombined extract ($H = 161.2$, $df = 4$, $P < 0.01$). Workers were not inhibited and readily produced new queens in these treatment groups suggesting that even natural extracts containing the most abundant compounds are not sufficient to signal queen presence to workers. However, we cannot draw any definitive conclusions from this data due to the lack of queen production in solvent control colonies. These colonies should have readily produced new queens in the absence of a fertile queen, or queen signals, yet only one colony produced a single queen.

Discussion

These experiments are the first to provide evidence that queen extracts are sufficient to inhibit workers from rearing new queens in colony fragments of Argentine ants in which queens have been removed. Both SHCs and UHCs appear to act as components of a primer pheromone which inhibits queen production in laboratory colonies in which

queens have been removed. This result is not surprising given the marked caste-specificity of these compounds. Queens have an entire series of long-chain (*Z*)-14 and (*Z*)-9 alkenes, with chain lengths between 29 and 35 carbons, and methylalkanes that are either not present in workers or are significantly more abundant in queens (Chapter 2). These queen-specific compounds account for over 50% of queen CHCs, but only make up ~6% of worker CHCs. The finding that multiple classes of compounds were required to elicit responses differs from results found in other ant species.

Individual racemic synthetic pheromones that represent a subset of the observed differences in CHCs have been shown to effectively inhibiting worker ant reproduction in the proximate sense (see Peso et al. 2014 for an explanation of proximate versus ultimate pheromone control). In the Iberian ant *C. iberica*, queens have characteristic differences in two linear alkanes, four monomethylalkanes, and two dimethylalkanes (Van Oystaeyen et al. 2014). Of these, *n*-C_{27}, *n*-C_{29}, and 3-MeC_{29} were found to decrease the odds of workers developing their ovaries compared with solvent controls. Queen pheromones appear to be conserved in the genus *Lasius*; workers and queens show consistent differences in both their SHCs and UHCs. Of these compounds, only 3-MeC_{31} was assayed and found to function as a queen pheromone that influenced worker reproduction, queen reproduction, and worker behavior (Holman et al. 2010, 2012, 2013; Van Oystaeyen et al. 2014). In these studies the efficacy of putative synthetic pheromones was compared with queenless colonies treated with solvent alone. In these species, it remains to be tested whether the perception of individual fertility signals by workers is similar to worker perception of the full complement of queen chemicals.

In *L. humile*, both SHCs and UHCs were required to restore pheromone activity, indicating that these compound classes are not redundant signals. That removal of a single subset of SHCs or UHCs was still able to prevent workers from rearing queens in queenless colonies would seem to argue at least some redundancy in the signal exists. However, this is purely speculation due to the failure of the control colonies to produce sufficient numbers of queens. If all compounds in the queen chemical profile were necessary in order for workers to respond, all treatment colonies except for those treated with all queen CHCs should have produced queens. However, the inability of combinations of SHCs and UHCs with 29 and 31 carbon backbones (~40% of total queen CHCs) to prevent workers from rearing queens suggests this redundancy is minimal.

Several properties of these complex queen mixtures make studying the various components difficult. Co-eluting di- and tri-methylalkanes are difficult to identify by their spectra due to the large number of fragments (Carlson et al. 1998). The convoluted spectra are further complicated by the possibility of overlapping fragments. Stereochemistry adds another layer of complexity to this puzzle. Monomethyl alkanes can exist as (*R*)-, (*S*)-, or may be a mixture of the two (racemic). With each additional branch the number of possible stereoisomers increases, and few studies have investigated the importance of stereochemistry in contact pheromone response. In the parasitic wasp *Lariophagus distinguidus* (Forster), male response to glass dummies coated in different stereoisomers of a contact sex pheromone are similar to response to racemic mixtures (Kühbandner et al. 2012). Recently, Bello et al. (2015) measured the optical rotations of methylakanes isolated from 20 insect species and found that, in all cases measured,

insects produced the (*R*)-configuration. Response of eusocial insects to stereospecific volatile pheromones has been studied far more frequently. Males of the little fire ant *Wasmannia auropunctata* (Roger) are preferentially attracted to the (*S*)-enantiomer of a pyrazine pheromone (Yu et al. 2014). Workers of *Myrmica scabrinodis* (Nyl.) are more attracted to a mixture of (*R*)-3-octanol and (*S*)-3-octanol [(*R*)-:(*S*) = 9:1] than to pure (*R*)-3-octanol or to racemic 3-octanol. The (*S*)-enantiomer alone was inactive (Cammaerts and Mori 1987). Trail-following in the ponerine ant *Leptogenys diminuta* is observed in response only to (3*R*, 4*S*)-methyl-3-heptanol; none of the remaining three stereoisomers were found to elicit a behavioral response (Steghaus-Kovâc et al. 1992). The queen sex pheromone of *Polyergus breviceps* is composed of a 1:6 ratio of (*R*)-3-ethyl-4-methylpentan-1-ol and methyl 6-methylsalicylate. (*S*)-3-ethyl-4-methylpentan-1-ol was neither inhibitory nor attractive to males (Greenberg et al. 2007). Alternatively, all stereoisomer of the pheromone component 2-methyl-1-butanol were active in the termite *Reticulitermes speratus* (Kolbe) (Yamamoto et al. 2012).

The difficulties in identifying and synthesizing all components of a mixture, and the lack of data concerning stereospecificity, present a rather unwieldy number of variables. Applying the preparative methodologies described in Bello et al. (2015) and this study to other social insect systems will enable the systematic testing of putative queen pheromones, as well as nestmate recognition signals, and hopefully aid in elucidating the role of the various compounds in these pheromone mixtures. It could be that certain parts of the queen's profile influence ovarian activation or ovarian regression, while different subsets of compounds regulate other queen-specific behaviors. It could

also be that all of the compounds associated with queens communicate the same signal in some ants. For example, both linear alkanes and alkenes may communicate the same information, thus serving as redundant signals of queen fertility. Our data suggest this is not the case in Argentine ants, where the queen signal appears require both SHCs and UHCs to affect worker rearing behavior. In fact, the Argentine ant queen signal may be multicomponent and quite complex. More research needs to be done to determine which components of the profile are active, how they interact to influence worker behavior and/or physiology, and in what context they must be presented in order to replicate full biological activity.

While the queen pheromone of *L. humile* appears to be more complex than most other identified ant pheromones, it is similar in complexity to another ant species with an obligately sterile worker caste, *S. invicta*. The components of this pheromone are found in both the poison sac and the postpharyngeal gland (PPG) of mated queens, and the pheromone has wide-ranging effects; queen pheromones inhibit virgin queen from shedding their wings, inhibit virgin and mated queen ovarian activation, inhibit female caste determination, and they may influence worker aggression (Alonso and Vander Meer 2002; Fletcher and Blum 1983; Vargo 1998; Vargo and Fletcher 1986; Vargo and Hulsey 2000). The poison sac contains an abundance of alkaloids and less-abundant neutral components, while the PPG contains an abundance of hydrocarbons that in other ant species is similar in composition to CHCs (Bagnères and Morgan 1991; Soroker et al. 1995). In a bioassay to determine which portions of the poison sac were responsible for inhibiting ovarian activation in virgin queens, the components of the poison sac were

separated into a basic fraction, which contained the alkaloids, and a neutral fraction. The basic constituents alone were not sufficient to inhibit ovarian activation in virgin queens. Neutral components were moderately effective at inhibiting ovarian activation, and full inhibition was restored when both fractions were combined (Vargo 1998). Further complicating this system was the finding that queens whose poison glands were removed still produced an active queen pheromone in the PPG (Vargo and Hulsey 2000). Queen-specific differences in fire ant venom alkaloids and UHCs have since been found (Eliyahu et al. 2011) and suggest a multi-component, multi-class queen pheromone may exist for *S. invicta*.

Our results clearly demonstrate that both SHCs and UHCs are required to for *L. humile* queen pheromone activity. However, the failure of control colonies to produce new queens in Experiment 2 prevents us from drawing of concrete conclusions concerning the importance of homologous CHCs and their possible role as chemical "synonyms". As with any research project in the field of chemical ecology, the most difficult aspect is developing a sensitive and reliable bioassay (Vargo 1998). Model systems in which worker perception of queens can be identified immediately (Smith et al. 2015) or within a few days (Vargo 1998) would be ideal for using the methods described here. Additionally, studies attempting to identify CHCs involved in nestmate recognition signals, which usually involves an immediate detection of aggression levels, would benefit from using this approach.

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Chapter 5: Effect of diet on JH, fecundity, and weight in queens of a highly polygynous ant

Introduction

Eusocial insects are among the most successful terrestrial arthropods, especially eusocial Hymenoptera, which include ants, honey bees, and some wasps. Eusociality is in part characterized by a reproductive division of labor, where egg-laying is delegated to one or a few individuals while a sterile (facultative or obligate) worker caste perform tasks such as nest-building/maintenance, foraging, and defense of the colony (Wilson 1971; Oster and Wilson 1978). Despite the central role of this division of labor within the colony, the mechanisms controlling developmental fate and caste differentiation are poorly understood with the exception of a few well-studied species (Kamakura 2010; Libbrecht et al. 2013).

Until recently it was thought that in all social insect species the brood were fully totipotent, capable of developing as either a worker or reproductive until reaching an irreversible stage in their maturation. Developmental fate was shaped by cues from the physical and social environment of the brood (Schwander et al. 2008; Smith et al. 2008; Schwander et al. 2010; Libbrecht et al. 2013). This form of developmental regulation is known as environmental caste determination (ECD) (Schwander et al. 2008). Alternatively, some species exhibit genetic caste determination (GCD) where an individuals' genotype predicts its caste (Schwander et al. 2008; Libbrecht et al. 2011). An example of GCD occurs in many populations of harvester ants in the southern US, where

differentiated genetic lineages occur from hybridization between *Pogonomyrmex rugosus* (Emery) and *P. barbatus* (Smith) (Helms Cahan and Keller 2003; Anderson et al. 2006). Within these societies, queens mate with multiple males from both lineages; interlineage offspring develop into workers, while intralineage offspring develop into queens (Helms Cahan et al. 2002; Julain et al. 2002; Volny and Gordon 2002; Helms Cahan and Keller 2003). Most social insect caste determination systems lie on a continuum between strict ECD and GCD. In the Argentine ant *Linepithema humile* (Mayr), the maternal and paternal genetic background influence whether offspring develop into queens or workers, although environment is still the primary factor dictating offspring developmental fate (Libbrecht et al. 2011).

In order to influence developmental changes, environmental cues must be transduced into physiological responses. One well-studied exogenous factor is diet. Nutrition has been repeatedly shown to influence the endocrine system of insects, thereby affecting a wide range of behavioral, developmental and physiological processes (Rembold 1987; Rachinsky et al. 1990; Hartfelder and Engels 1998; Kucharski et al. 2008; Kamakura 2010; Mutti et al. 2011).

In highly eusocial insects, which have discrete castes of reproductives (queens) and non-reproductives (workers) (Oster and Wilson 1978; Hölldobler and Wilson 2009), both caste differentiation and reproductive capacity are nutritionally sensitive, but the responses can vary widely across and even within species. For example, in the ant *Leptothorax (= Temnothorax) longispinosus* (Roger), protein supplementation was initially shown to increase the production of males but not queens (Backus and Herbers

1992). A similar experiment with the same species found an increase in queen production when colonies were supplemented with protein (Herbers and Banschbach 1998). In *Myrmica brevispinosa* (Wheeler), colonies receiving carbohydrate-supplemented diets produced more queens and a more female-biased sex ratio compared to colonies receiving protein or no supplementation (Bono and Herbers 2003). Food supplementation was also shown to increase the production of male and female sexual forms in *Formica podzolica* (Francoeur) (Deslippe and Savolainen 1995), *Aphaenogaster rudis* (Enzmann) (Morales and Heithaus 1998), and *Linepithema humile* (Mayr) (Aron et al. 2001).

It is widely believed dietary supplementation affects the endocrine system, which, in turn, influences caste differentiation (Smith et al. 2008; Bono and Herbers 2003; Kamakura 2010). In honey bees (*Apis mellifera* L.), queens and workers can be reared from the same eggs, but the amount of royal jelly developing larvae are fed by workers determines the caste (Kamakura 2010). Diet modulates activity of the epidermal growth factor receptor (EGFR), insulin/insulin-like signaling (IIS), and target of rapamycin (TOR) signaling pathways. These pathways regulate the production of juvenile hormone (JH) and other regulatory factors, which in turn mediate gene expression to determine adult honey bee phenotype (Yan et al. 2014).

Although studies linking diet to hormonal changes and caste differentiation in ants have not been conducted, hormones alone have been shown to affect caste differentiation and reproductive capacity. In the queenless ponerine ants *Diacamma sp*. and *Streblognathos peetersi* (Robertson), JH levels were lowest in reproductively active

workers, while subordinate workers had significantly higher JH titers (Sommer et al. 1993, Brent and Dolezal 2009). In these ants, ecdysteroids were associated with reproductive activity. In contrast, queens of the red imported fire ant *Solenopsis invicta* (Buren) appeared to have retained JH as the gonadotropic hormone (Brent and Vargo 2003). In addition to its gonadotropic role in fire ant queens, the manipulation of JH, either by topical application or feeding JH analogues (JHAs) to colonies, stimulated queen production in several ants species, including *Harpegnathos saltator* (Jerdon) (Penick et al. 2012), *Pheidole pallidula* (Nylander) (Suzzoni et al. 1980; de Menten et al. 2005), and *Pogonomyrmex rugosus* (Emery) (Libbrecht et al. 2013). Although these studies indicate the importance of hormones on developmental fate, with the exception of recent studies of *A. mellifera* and *P. rugosus*, they have failed to show how environmental factors impacted hormone titer and how this subsequently affected caste differentiation.

To address the mechanistic gap between diet and development, we expanded upon earlier work with *L. humile*. In this species, laboratory colonies in which queens have been removed produce queens throughout the year, although they are more likely to do so following an overwintering period (Vargo and Passera 1992). Diet also appears to play a role in *L. humile* the production of sexuals; queenless laboratory colonies provisioned with protein rear significantly more queens than did queenless colonies fed only sucrose water. Further enhancement of dietary protein did not lead to an increase queen production, although that may have been due to the limited accessibility of the mealworm diet provided (Aron et al. 2001). By providing an enriched diet in more accessible form,

such as concentrated protein suspension, it may be possible to enhance food intake to a level at which the reproductive output of a queen and the developmental fate of her offspring are affected. Given the presumptive mediatory role of JH in egg production and caste development, we hypothesize that such dietary enrichment would increase queen JH levels, resulting in enhanced fecundity and a greater proportion of offspring that develop into queens. We tested these predictions using laboratory colonies provisioned with one of four diets, then examining queens for relative differences in body mass, JH content, and fecundity. Offspring produced by these queens were monitored for queen production.

Methods

Effect of diet on queen JH titer and queen production

Laboratory colonies of *L. humile* were collected from a citrus orchard under biological control located on the University of California, Riverside campus in March 2014. Ants were separated from the soil and placed in large plastic boxes lined with Fluon (Asahi Glass Co., Tokyo, Japan) to prevent escape. Queens were aspirated individually and placed in laboratory colonies containing 1000 workers and various stages of brood. In this way, 84 colonies, each consisting of 20 queens and 1000 workers, were established and maintained at 28ºC, 55% RH, with a 12:12 [L:D] h photoperiod. To determine the effects of supplementation, 21 replicate colonies (84 total) were fed one of four test diets:

1) Nutrient Poor (sugar water only)

2) Standard (sugar water + cockroaches)

3) Nutrient Rich (artificial diet)

4) Highly Enriched (artificial diet + cockroaches)

Sugar water (25% *w:v*) and a protein- and vitamin-rich artificial diet (25% *w:w*; Pro Complex Gainer, Optimum Nutrition Inc., Aurora, IL) was provided three times each week in 1 ml aliquots. Roughly 5 g of fresh cockroach (*Periplaneta americana* L.) parts were provided twice each week.

Initially each colony fragment had 20 egg-laying queens, as verified by direct observation. After 4 weeks the queens were removed to measure dietary effects on circulating juvenile hormone, a known gonadotrope in many social hymenoptera (Robinson and Vargo 1997). When queens are removed from laboratory colonies, workers rear new queens from existing female larvae (Passera et al. 1987). This occurs presumably by workers altering the diet of developing female larvae in response to the absence of a queen pheromone (Keller et al. 1989; Vargo 1998). The colonies in which queens were removed were maintained for another 16 weeks (or until all brood had pupated) under identical conditions and surveyed daily to quantify the production of new queens. Colonies that had fewer than 20 queens at the time of orphaning were not used for JH quantification but were still monitored for the production of new queens.

Effect of diet on queen weight and fecundity

A single colony from each treatment group was removed and used to determine average queen mass and fecundity which was measured as the number of eggs produced over a five-day period. Queens were removed from source colonies, anesthetized using CO_2, and their weights were recorded to the nearest microgram with a Sartorius M2P balance

(Sartorius AG, Goettingen, Germany). Queen mass reflects the accumulated endogenous resources necessary to support sustained oogenesis. Each queen from a particular diet group was then moved to a colony fragment with 500 workers, an artificial nest made up of a 1.6 x 12.7 cm Pasteur pipette covered with aluminum foil, and held at 28ºC, 55% RH, 12:12 [L:D] h. Colonies were maintained on their original diet and after five days the number of eggs visible inside the artificial nest was counted under a microscope. Twenty colonies were each provisioned with one of the four diets described above.

Juvenile hormone content

The whole body content of JH was measured using gas chromatography-mass spectrometry (following Brent and Dolezal 2009; Dolezal et al. 2009, 2012; Penick et al. 2012). JH form was confirmed by first running test samples in SCAN mode for known signatures of JH 0, JH I, JH II, JH III and JH III ethyl; JH III was confirmed as the primary endogenous form in this species. Subsequent samples were analyzed using the MS SIM mode, monitoring at m/z 76 and 225 to ensure specificity for the d3-methoxyhydrin derivative of JHIII. For each sample, 20 queens, 100 µl 2% NaCl, 100 µl acetonitrile and 200 pg farnesol (internal standard; Sigma-Aldrich, St. Louis, MO) were homogenized three times in 1 ml 50% acetonitrile. Samples were then extracted three times with 2.5 ml hexane. The extracts were combined and concentrated in a Centrivap concentrator (Labconco, Kansas City, MO). The extract was re-suspended in hexane and eluted through an Al_2O_3 (activated with 6% water) column with hexane, 10% ethyl ether–hexane and 30% ethyl ether–hexane. The 30% ethyl ether–hexane fraction was dried in

the concentrator. The residue was re-suspended with 75- µl methyl d alcohol, then 75 µl of 5% trifluoroacetic acid (Sigma-Aldrich Corp., St. Louis, MO) in methyl d alcohol was added. The sample was derivatized in an oven at 60 °C for 20 min. Derivitization was stopped with the addition of 500 µl hexane and the sample was dried in the concentrator. The residue was dissolved in 600 µl hexane and eluted through a second Al_2O_3 column with hexane, 30% ethyl ether–hexane to remove any non-derivatized compounds, and ethyl acetate–hexane (1:1) to obtain d3-methoxyhydrins. The ethyl acetate–hexane fraction was dried in the concentrator, the residue re-suspended with 200 µl hexane and dried down to less than 3 µl under ultra-high purity nitrogen. Analysis was conducted on a 7890A series gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a 30 m × 0.25 mm Zebron ZB-WAX column (Phenomenex, Torrance, CA) and coupled to a 5975C series inert mass selective detector with helium as the carrier gas. Total abundance was quantified against a standard curve of derivatized JH III.

Statistics

For comparison of JH content, queen mass, and queen fecundity, normaility was tested using a Shapiro-Wilk test, followed by one-way ANOVA with Holm-Sidak pairwise comparisons. The production of new queens was analyzed using non-parametric Kruskal-Wallis ANOVA. The relationship between queen weight and fecundity was analyzed using Pearson's correlation (SPSS version 22, IBM Corp., Chicago, IL).

Results

Diet supplementation had a significant impact on queen juvenile hormone levels ($F = 4.94$, $df = 3$, $P < 0.01$; Fig. 5.1). Queens fed a standard diet of sugar water and cockroaches had significantly lower whole-body JH levels (1.85 ± 0.19 pg/queen) than did ants fed an enriched or highly-enriched diet (2.73 ± 0.65 and 2.60 ± 0.81 pg/queen, respectively; $P < 0.01$). Mean JH titers of queens fed only 25% sugar water were not significantly different from other treatment groups (2.24 ± 0.15 pg/queen). Production of new queens in orphaned colonies was also affected by diet ($H = 63.02$, $df = 3$; $P < 0.01$). Only colonies fed a standard diet produced queens.

Diet significantly affected queen mass ($F = 12.79$, $df = 3$, $P < 0.01$; Fig. 5.2). Queens fed a nutrient-poor diet weighed significantly less (2.53 ± 0.08 mg) than did queens fed a standard diet (2.97 ± 0.12 mg; $P < 0.01$), an enriched diet (3.02 ± 0.08; $P < 0.01$), or a highly-enriched queens fed a nutrient-poor diet (2.32 ± 0.69; $P < 0.01$). Queens fed a standard diet were also significantly more fecund than nutrient-poor diet (3.32 ± 0.08 mg; $P < 0.01$). Queen fecundity was also affected ($F = 23.92$, $df = 3$, $P < 0.01$; Fig. 5.3). Queens fed an enriched diet had the highest mean fecundity (57.75 ± 5.92 eggs/5 d; $P < 0.01$). Queens receiving a highly-enriched diet were significantly less fecund than those fed enriched diets (36.25 ± 5.73; $P < 0.01$), but significantly more fecund than queens (36.83 ± 4.11; $P < 0.01$). Diet had a significant impact on the relationship between queen mass and fecundity. Queens fed a standard diet had a significant positive linear correlation between weight and fecundity (Pearson's

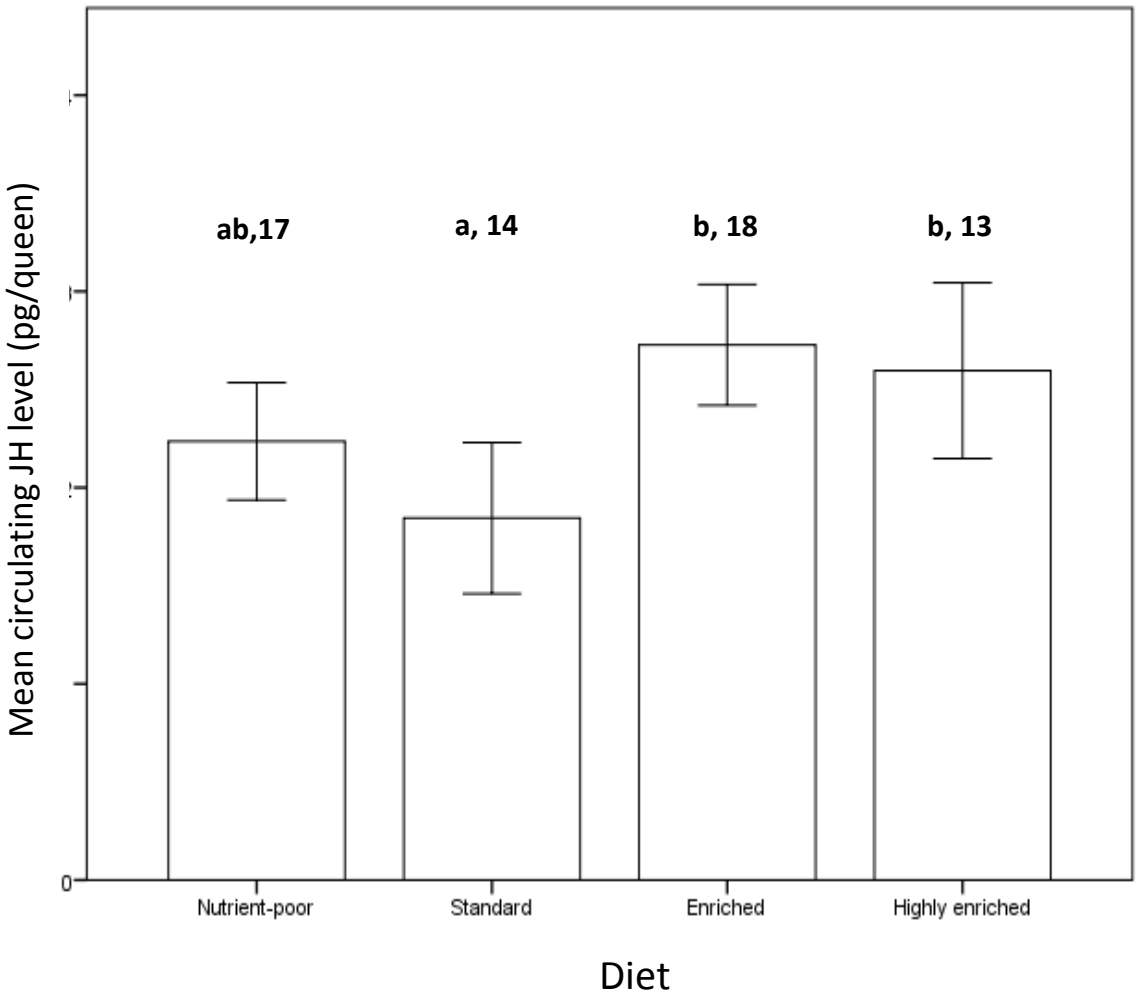

Figure 5.1 The effect of colony diet on the mean (±SE) JH titer of queens (F = 4.94, df = 3, $P < 0.01$), with queens on a standard diet having significantly lower titers (Holm-Sidak test, $P < 0.01$). Sample size is indicated. Differences between groups assigned different letters are statistically significant (Holm-Sidak test, $P < 0.01$).

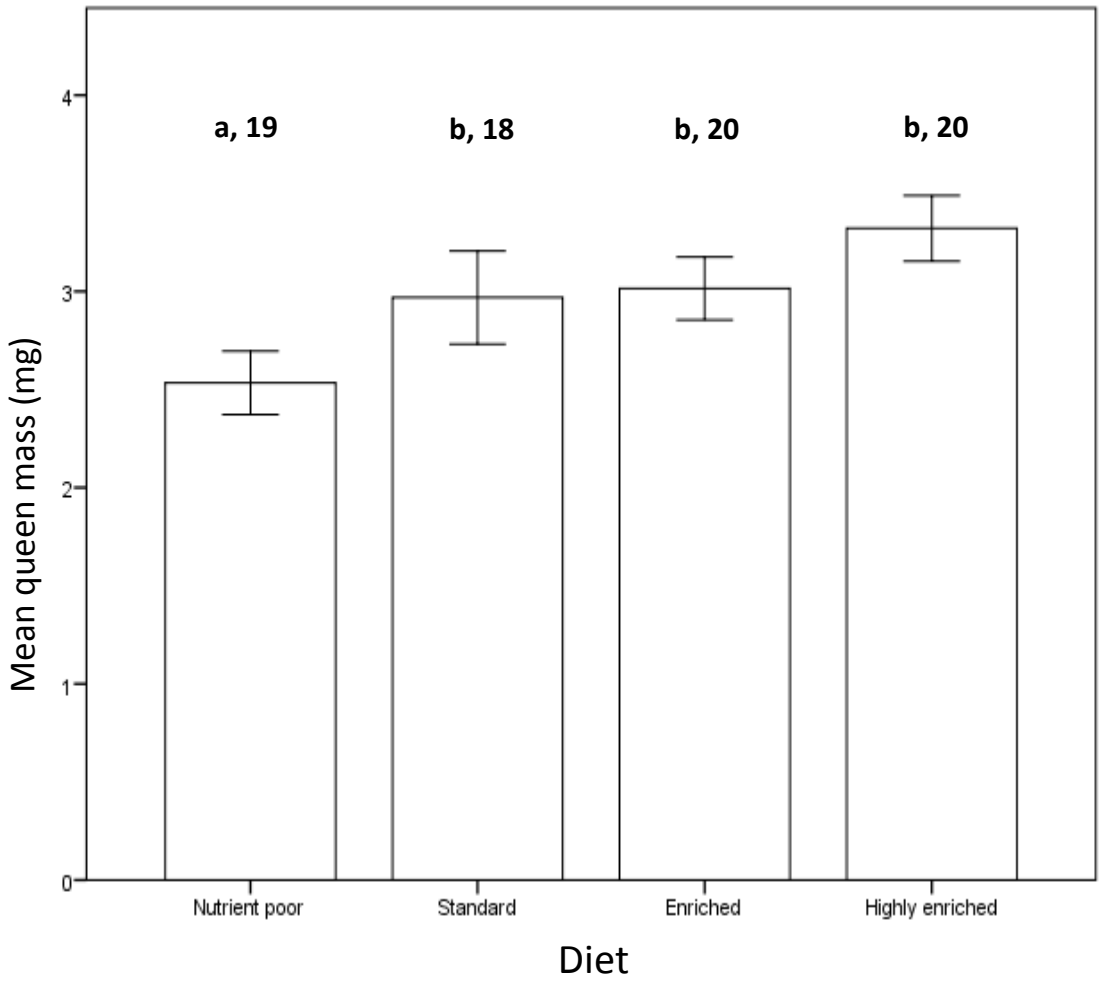

Figure 5.2 Body mass (mean ± SE) was reduced in queens fed a nutrient poor diet compared those on other diets (F = 12.79, df = 3, $P < 0.01$; Holm-Sidak test, $P < 0.01$). Sample sizes are indicated.

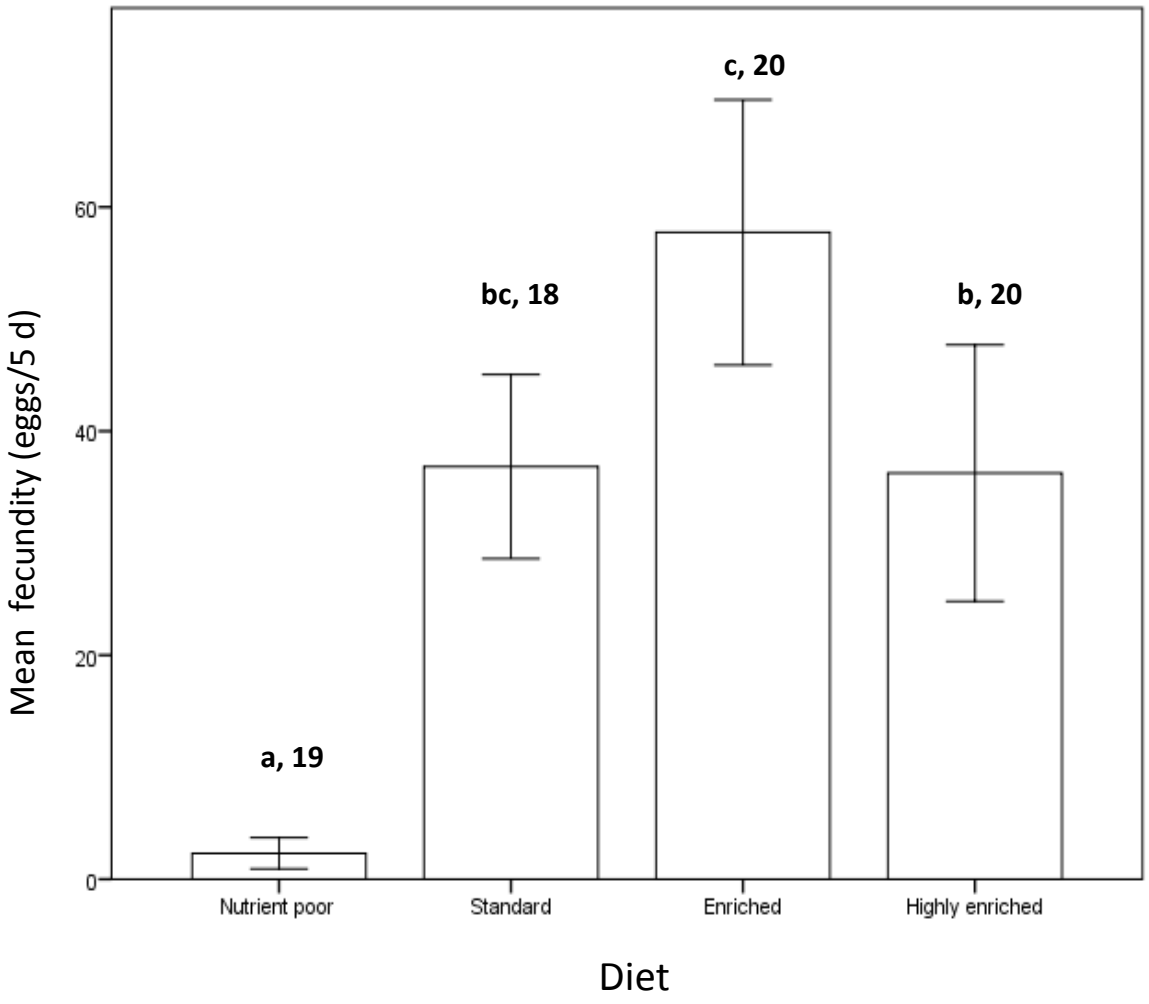

Figure 5.3 Diet affected mean (± SE) fecundity (F = 23.92, df = 3, $P < 0.01$) with queens on a nutrient poor diet being significantly less fecund (Holm-Sidak test, $P < 0.01$). Sample sizes are indicated.

correlation; $r = 0.25$, $P < 0.05$) while a nutrient-rich diet produced a significantly negative linear correlation ($r = -0.34$, $P < 0.01$). Queens fed a nutrient poor diet had low levels of fecundity across all weights and showed no discernable linear trend ($r = 0.01$, $P = 0.67$). Queens fed a highly enriched diet showed a negative trend in the association between weight and fecundity, although this trend was not significant ($r = -0.01$, $P = 0.13$).

Discussion

The results of this study suggest that diet affects both maternal JH levels and the developmental fate of Argentine ant larvae. Enriched diets led to significant increases in circulating JH levels in queens. If the impact of diet is expressed in the developing brood, then it appears elevated JH levels have a significant negative impact on queen production in laboratory colonies in which queens have been removed. One drawback from our experimental design is that we cannot separate maternal effects of diet on caste fate from direct effects on larvae because colonies were continuously provisioned with the same diet throughout. However, changing the diet after removing queens from the colonies would not address the confounding factor of direct or indirect effects. Because the exact nutritional requirements for queen/worker determination are unknown, switching diets could potentially affect larval development and/or worker behavior in an unpredictable way.

Social hymenoptera have evolved a variety of mechanisms for influencing the developmental fate of offspring. In some species, direct effects of environment on larvae

are believed to lead to developmental changes in some larvae that result in queen production. In the honey bee *A. mellifera*, queen/worker determination is almost entirely dependent on larval nutrition regulated by workers, although minor genetic effects have been suggested. In the absence of a queen, workers will alter the proportion of royal jelly, a highly-nutritious diet produced in the hypopharyngeal glands of workers, fed to particular larvae. Royal jelly acts through an epidermal growth factor receptor (Egfr)-mediated pathyway and increases juvenile hormone titer, resulting in a developmental switch to a queen phenotype (Kamakura 2010). In paper wasps (genus *Polistes*) an increase in the quantity of food provided to developing females increases their probability of becoming a future reproductive foundress (Hunt and Amdam 2005). In other species, maternal factors such as nutrition, age, and overwintering have been shown to affect caste determination. Harvester ant queens (genus *Pogonomyrmex*) develop only from eggs produced by overwintered queens (Schwander et al. 2008). In *P. rugosus*, elevated maternal JH levels brought about through overwintering resulted in increased queen production. Only colonies headed by overwintered queens produced queens. The effect of overwintering was similar to the effect of artificially increasing queen hormone levels using the JH analogue methoprene, suggesting that overwintering induces a quantitative increase in endogenous JH levels, and is associated with queen production (Libbrecht et al. 2013). Additionally, queen-destined eggs differ from worker-destined eggs in their ecdysteroid content, suggesting that queens can influence caste determination by translating environmental conditions to subsequent generations. A similar effect has been observed in the ant *Pheidole pallidula* (Nyl.), in which topical applications of methoprene

increased the production of queens (Passera and Suzzoni 1979); queen-destined eggs were characterized by low ecdysteroid levels compared with worker-destined eggs (Suzzoni et al. 1980). Due to the small size of queen larvae, as well as the low number produced by colonies in this experiment, we were unable to investigate hormone content of worker and queen larvae.

In *L. humile*, caste determination has long been thought to be regulated by direct effects on developing larvae rather than maternal effects that affect queen/worker determination. Although queens are only produced during the spring in field populations (Markin 1970; Passera et al. 1987), they may be reared from 1st instar larvae at any time of the year following the loss of a functional queen. The probability of larvae developing as queens is greatly increased depending on the overwintering status of the larvae (Vargo and Passera 1992), as well as the availability of protein (Aron et al. 2001). Our results corroborate the findings of Aron et al. (2001) that protein supplementation increases queen production, although our results indicate there is a threshold above which dietary supplementation may have a negative impact. Our results also suggest a potential effect of maternal physiological state on caste determination, albeit a speculative one. Because of the small size of *L. humile* workers and brood, it was necessary to sample queens in order to determine the effect of diet on hormone levels. That queen JH levels were negatively correlated with queen production suggests that maternal effects could also play a role in caste determination, although future studies would require a separate feeding regime for offspring produced by queens that differ in their dietary intake in order to separate the effects of larval feeding from maternal dietary effects.

Diet may also have affected other pathways that play a role in developmental fate, including insulin signaling, vitellogenesis, and ecdysteroid content. Increases in transcription of genes involved in insulin/insulin-like signaling (IIS) occurred in ant queens that were exposed to cold temperatures and fed methoprene, and these treatments were linked with increased queen production (Passera and Suzzoni 1979; Libbrecht et al. 2013). However, the roles of ecdysteroids and JH in vitellogenesis and ovarian activation vary considerably between ant subfamilies. Reproductive individuals in the subfamily Ponerinae have relatively high ecdysteroid levels compared with foraging workers, while JH titer is much lower compared with foragers (Penick et al. 2012), while in the Myrmicine species *Solenopsis invicta*, JH appears to be the primary gonadotrope (Brent and Vargo 2003). In our study higher JH titer was associated with increased egg production, which suggests it may retain this function in Dolichoderine ants, although confirmation would require artificially manipulating queen hormone levels. Such artificial manipulations have been performed on larger ant species by topically applying JHAs to the gasters of queens (*Streblognathos peetersi* Robertson, Brent et al. 2006; *Harpegnathos saltator* Jerdon, Penick et al. 2012; *S. invicta*, Vargo and Laurel 1994). Several preliminary studies were conducted to determine if this would be feasible with *L. humile* queens, but their small size was prohibitive. Topical applications of 1 µl of 0.1 µg/µl methoprene in acetone resulted in very high queen mortality. Queens often died immediately following treatment using 5 µl micropipettes, while others were executed by workers after returning them to experimental colonies (unpublished data). Future

experiments should explore a less traumatic method of administering JHAs (i.e. mixing compounds with food, Libbrecht et al. 2013).

In many species, the weight of a reproductive female is considered a reliable indicator of fecundity (Vargo and Fletcher 1989; Vander Meer et al. 1992; Vargo 1992). Our results indicate that diet can significantly alter this relationship. Queens that received enriched diets were the heaviest but were no more fecund than queens that received standard diets. However, among queens that were fed a standard diet there was a positive linear correlation between weight and fecundity, whereas negative correlations were observed between weight and fecundity in queens fed enriched diets. In the pome fly *Drosophila melanogaster* (Meigen), overfeeding developing flies with either high sugar or high protein diets diminishes insulin signaling, resulting in a fly with a metabolic disorder similar to type 2 diabetes (Morris et al. 2012). Additionally, in extreme cases of excessive protein consumption, flies showed decreased levels of storage molecules and reduced fertility. In the case of *L. humile* queens that received enriched or highly enriched diets, the excessive sugar and protein intake could have disrupted metabolic processes similar to *D. melanogaster*, which could explain the reduced fecundity observed in the heaviest queens in these treatment groups.

In conclusion, our study is the first to identify how dietary supplementation affects hormone levels in *L. humile* queens, and by inference, on hormone levels of developing larvae. Interestingly, increased levels of JH were negatively correlated with queen production, which is opposite of what has been found in most social insect species. Although enriched diets resulted in increased weight and fecundity in queens, linear

regression analyses revealed a negative relationship between weight and fecundity in enriched diets, suggesting that increased intake in these groups above a certain threshold results in a deleterious effect on egg-laying. We provide circumstantial evidence that maternal effects may also play a role in caste determination, although future studies will be needed to determine if maternal hormone levels affect caste determination.

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Chapter 6: Concluding Remarks

Communication between individuals is critical for the success of social insect colonies. Communication is necessary to delineate group membership, coordinate activities, and identify castes or individuals; without communication, there would be no society. Communication may be via chemical, visual, acoustical, tactile, or substrate-borne signals. Chemical communication is the most ancient and wide-spread form of communication in animals (Candolin 2003), and chemical signals are often used to rapidly and efficiently disseminate information about traits such as sex, health status, dominance, and task specificity, as well as during foraging and recruitment, and in nestmate recognition (Richard and Hunt 2013). One of the biggest challenges in studying chemical communication is to understand the precise signals and signal patterns involved. This begins with proper identification of chemicals or chemical blends that potentially transmit information, as well as the development of robust bioassays that can accurately elucidate the function of chemical signals.

The most widespread and best-studied examples of chemical communication in social insects are the volatile and semivolatile glandular compounds associated with recruitment and foraging, and the nonvolatile cuticular hydrocarbons (CHCs) involved in the recognition of species, populations, colonies, castes, and individuals (Richard and Hunt 2013). The broader goal of this research was to accurately identify and purify ant-produced chemicals associated with foraging and recruitment and caste recognition, so that these compounds could be systematically tested for possible functional roles as

signals for the Argentine ant *Linepithema humile* (Mayr). In chapter 2, I identified volatile and nonvolatile compounds associated with each of the adult castes of the Argentine ant using previously described methods to separate CHCs into saturated and unsaturated fractions using $AgNO_3$-impregnated silica gel (Greene and Gordon 2007; Bello et al. 2015). By separating CHCs of each caste into their respective saturated and unsaturated fractions, I was able to identify clear caste-specific patterns based on qualitative and quantitative differences. The results of chapter 2, as they apply to differences between queens and workers, present a more accurate description of queen-specific CHCs, most notably the identification of homologous 5,11-dimethylalkanes and (*Z*)-14 and (*Z*)-9-monounsaturated alkenes. The first step in identifying putative queen pheromones is to determine which compounds are queen-specific, or present in larger relative amounts in extracts of queens than in other castes. In most ants, there are multiple quantitative and/or qualitative differences between queen and worker CHC profiles (Van Oystaeyen et al. 2014). These differences are often associated with ovarian activation, and range from quantitative shifts in particular CHCs to dramatic qualitative shifts including the production of new classes of CHCs (de Biseau et al. 2004).

In addition to CHC differences between queens and workers, I found that the two castes differ in the relative proportions of dolichodial and iridomyrmecin, which are part of a group of monoterpene derivatives known as iridoids (Morgan 2008). These two compounds were previously identified from dichloromethane extracts of macerated workers (Cavill and Houghton 1974; Cavill et al. 1976). Dolichodial and iridomyrmecin are produced in the pygidial gland, often in large amounts in the Dolichoderinae, and are

believed to be used in defense and alarm behavior (Morgan 2008). Iridomyrmecin in particular functions primarily as a deterrent to other insects (Pavan 1952). Recently, dolichodial and iridomyrmecin have been shown to affect two other aspects of colony behavior: necrophoresis and trail-following. Argentine ants, like many other social insect species, engage in necrophoresis, the removal of dead colony members from the nest. Live workers constantly produce and release dolichodial and iridomyrmecin, and extracts of freshly killed workers contain the two iridoids (Choe et al. 2009). The iridoids disappear from the cuticle within ~ 1 h following death, triggering the removal of the dead worker. Although the iridoids gradually disappear from the cuticle within the first hour, there are still detectable levels stored internally in the pydial gland after one hour. It is possible that workers spread the compounds over the cuticle through allogrooming behaviors; if an ant is dead, it can no longer actively excrete its glandular contents or groom itself, and hence the signal dissipates. Dolichodial and iridomyrmecin were also shown to induce trail-following in workers. The iridoids were the most abundant compounds found in the chemical trails of *L. humile* workers, and ants preferentially chose branches marked with iridoids rather than solvent controls (Choe et al. 2012). The finding that queens and workers differed significantly in the relative abundance of dolichodial and iridomyrmecin suggested that these compounds might have additional functions.

Thus, a second major objective of this dissertation was to determine the roles of the iridoids in trail-following by workers. To address this objective, natural dolichodial and iridomyrmecin fractions were isolated by preparative gas chromatography (prep-GC).

A previous study examined trail-following using synthetic iridomyrmecin and *trans, trans*-dolichodial derived from the stick insect *Anisomorpha buprestoides* (Choe et al. 2012). In Chapter 3, I described the separation of milligram quantities of natural dolichodial and iridomyrmecin. This allowed me to compare trail-following responses of workers to each compound individually, over a range of concentrations. It also allowed me to further analyze the chemical properties of iridomyrmecin. Unlike dolichodial, in which the absolute configuration of the molecule has been worked out (Cavill et al. 1976), the naturally produced stereoisomer of iridomyrmecin has not been determined in *L. humile*. Because *L. humile* workers appeared to follow trails marked with iridomyrmecin only, I designed an experiment to test the efficacy of individual stereoisomer of iridomyrmecin compared to the natural product. I was able to identify identified (+)-iridomyrmecin as the major iridoid present in Argentine ant workers, along with minor amounts of (-)-iridomyrmecin and (+)-isoiridomyrmecin. While the natural iridomyrmecin fraction elicited trail-following by worker ants, a similar synthetic blend did not. Closer examination of the natural iridomyrmecin fraction suggested the extract may contain trace amounts of Z9-16:Ald, which would explain the observed activity of the natural fraction but not the synthetic blend.

A third major objective of this dissertation was to determine the active components of the Argentine ant queen pheromone. Queen pheromones are essential for the cohesive structure of social insect colonies. Queen pheromones in ants are generally comprised of CHCs, although some experimental evidence suggests a role for queen-specific venom gland components in queen recognition in the red imported fire ant *S.*

invicta (Vargo 1998; Vargo and Hulsey 2000). Insect cuticular lipids usually occur as components of a complex blend of saturated hydrocarbons (SHCs), unsaturated hydrocarbons, or alkenes (UHCs), and polar constituents (Bello 2014). Components of these complex blends cannot be separated with normal phase liquid chromatography, and are insoluble in aqueous solvent systems typically used with reverse phase chromatography. Because of the difficulties associated with separating the components of these complex CHC mixtures, most of the studies on queen pheromones have focused on identifying individual compounds that are overrepresented in queens, and ascribing functional roles to them based on this overrepresentation. There are two problems with this approach. First, there are often multiple differences in the CHC profiles of queens and workers, and these compounds very likely have additive or synergistic effects that would potentially be overlooked by just looking at chromatographic profiles. Many pheromones, and likely most, consist of two or more compounds that alone are inactive or moderately active (Byers 1992). Second, most studies that have investigated the role of queen pheromones in ants have failed to determine the activity of crude natural extracts (Holman et al. 2010; Van Oystaeyen et al. 2014; but see Vargo 1998). Whereas it is certainly possible that a single compound could function as a chemical signal of queen presence and/or dominance, this could be unequivocally proven by comparing the activity of a crude extract with the putative synthetic pheromone.

Furthermore, to accurately identify pheromonal activity, the activity of a crude extract must be confirmed in the absence of other signal modalities, such as visual or tactile cues, when subjected to a bioassay that is robust and reproducible. If the activity of

the crude extract is the same as the activity observed in colonies containing live queens, this provides hard evidence for the role of a pheromone. In Argentine ants, laboratory colonies in which queens have been removed will rear new queens from existing brood. The addition of freshly killed queens to queenless colonies inhibits the production of new queens, but not when freshly killed queens have been extracted with pentane (Vargo and Passera 1991). While this strongly suggests that workers recognize queens by a chemical signal, it was necessary to show that the extract was able to elicit the required behaviors in order to confirm the signal was indeed chemical in nature. I addressed this issue in experiment one of Chapter 4, in which I compared the activity of a crude hexane extract of queens with that of a recombination of fractions containing saturated hydrocarbons (SHCs) and unsaturated hydrocarbons (UHCs), and more polar constituents. The results indicated that a crude hexane extract of queens was identical its ability to inhibit queen production in queenless colony fragments compared to queen-right colonies. Moreover, through recombination of SHCs, UHCs, and polar constituents, I found that SHCs and UHCs were sufficient to replicate the activity of the total recombined extract (SHCs, UHCs, and polar constituents). The next step was to identify which SHCs and UHCs were required to elicit bioactivity. In a second experiment (Chapter 4), I separated SHCs and UHCs into subsets based on carbon chain length, and recombined them in various combinations in order to compare their bioactivities. Unfortunately, the results were inconclusive due to the failure of the control colonies to produce new queens. Although experiment two was not successful in further narrowing down the active queen

pheromone blend, the methodology will be useful in other such systems where there are homologous CHCs that are unique to queens.

The seasonal nature of queen production in Argentine ants in southern California presents a significant challenge to studying the effects of queen pheromones. Studies on the effects of queen pheromones have to be conducted early in the spring to ensure the sensitivity of the bioassay. Currently, queen production in laboratory colonies in which queens have been removed is the only method used to determine whether or not workers recognize the presence of a queen (Vargo and Passera 1991; Chapter 4). This presents three main challenges when investigating queen pheromones. First, the large number of chemical variables makes establishing a sufficient number of experimental colonies logistically difficult. Queens are qualitatively different from workers in the presence of at least 14 compounds (Chapter 2). Even when these compounds are broadly subdivided by their chain lengths, the large number of combinations is rather unwieldy, especially when trying to use enough replicates per treatment to establish statistical significance ($n \geq 10$). Second, the time required for queenless colonies to rear new queens is between two and four months (Keller et al. 1989; Vargo and Passera 1992; Chapters 4 & 5). Challenges one and two give rise to a third issue: a large number of queens are needed to prepare extracts for so many treatment groups and replicates. Preparing a sufficient quantity of extract represents a significant bottleneck in determining *L. humile* queen pheromone activity. While it is not impossible to collect a sufficient number of queens, it requires a substantial investment of time and resources.

An alternative to queen development in queenless laboratory colonies might be to use individual queen fecundity in colonies exposed to various concentrations of queen extract. In highly polygyne ant societies, there is an inverse relationship between queen number and individual fecundity (Vargo and Fletcher 1989; Vander Meer et al. 1992). If monogyne experimental colonies were established, one could establish treatment groups of increasing queen extract concentrations (ex. 1 qe, 3 qe, 5 qe, 10 qe) and measure the egg-laying rates of these queens. Queens in colonies with high concentrations of queen extract would be predicted to display reduced oviposition rates. Such a bioassay would address two of the three issues identified for queen production in queenless colonies. The time required to conduct these experiments would be only 5-10 days, a substantial decrease from the 60-120 days required for queen production. A reduction in bioassay length would also reduce the amount of extract needed to conduct the experiments. Additionally, this bioassay could potentially be conducted year-round. If viable, this bioassay would allow potential queen pheromones to be screened much more quickly and accurately, while also using less extract in the process.

The failure of experiment two in Chapter 4 prompted further development of our bioassay. Although queenless laboratory colonies of Argentine ants will produce queens at any time of the year, they do so much more frequently if the brood and workers have overwintered (Vargo and Passera 1992). Because Argentine ant workers are completely sterile and incapable of producing eggs, it is not possible to use worker ovarian activity or reproduction as a proxy for recognition of a queen; the production of queens in queenless

colony fragments is currently the most robust assay for establishing queen pheromone activity.

The goal of Chapter 5 was to determine if dietary supplementation in laboratory colonies established from non-overwintered ants would increase the production of queens. Dietary supplementation has been found to increase queen production in several species of ants, including *L. humile*, which triggers a developmental switch from worker to queen phenotype (Wheeler 1986; Aron et al. 2001; Smith et al. 2008; Cridge et al. 2015). Larvae cannot feed themselves; it is up to workers to provide selected offspring with the nutritional requirements to develop as queens, if indeed the developmental switch is governed solely by worker provisioning of larva. A previous study showed that there was no difference in queen production between colonies fed intermediate and high levels of insect protein (1 maggot larva vs. 5 maggot larvae per week). However, Argentine ants only feed on insect hemolymph within the first 1-2 h while it is still liquid. Using an enriched liquid diet, I attempted to induce workers to provision larvae with an abundance of protein in a form that is more readily consumed, and thus circumvent the seasonal effect of overwintering by coercing workers into feeding larvae an enriched diet.

Alternatively, diet could affect the maternal state of the queen, which in turn affects the nutrients packaged into her eggs (Wheeler 1986; Libbrecht et al. 2013). In the harvester ant *Pogonomyrmex rugosus*, overwintering affects maternal JH levels, which affect the developmental fate of offspring produced after the overwintering period. If *L. humile* queens receive an enriched diet that affects JH levels, the subsequent offspring may be more likely to develop into queens. In Chapter 5, I showed that enriched diets

have a significant effect on maternal JH levels, and that elevated JH levels showed a negative relationship with queen production. Only colonies with queens fed a standard diet produced new queens. This suggests that the relationship between JH titer and queen production is complex, and may involve ecdysone as well. Future studies that incorporate artificial manipulation of hormone levels will be necessary to determine if the observed relationship is due to JH levels alone, or whether dietary supplementation affected other pathways responsible for caste determination that were not detected in my study. Insulin/insulin-like signaling (IIS) and vitellogenin (Vg) have also been linked with caste differentiation in *P. rugosus* (Libbrecht et al. 2013). Additional studies will need to be conducted on *L. humile* queens to determine what correlation, if any, there is between these pathways and the potential of offspring to develop into queens. Hormone titers of individual queens are miniscule (~ 2 pg/queen, Chapter 5) due to their small size, making them difficult to study. Future research on the effects of environment (diet, temperature, season, etc.) on queen/larval physiology might consider using genes correlated with hormone production, or other physiological correlates, in order to circumvent this issue. For example, in *P. rugosus*, queens that are overwintered have elevated JH levels compared to non-overwintered queens, and are more likely to produce queens (Libbrecht et al. 2013). Overwintering is also correlated with increased expression of genes associated with insulin signaling, JH biosynthesis, and vitellogenesis. If the genes could be used as a proxy for environmental effects instead of circulating JH titer, it may be possible to conduct assays using colonies with one or a few queens.

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