

Mechanistic Diversity in Long-Range Regulation of Worker Reproduction in

Polydomous Ant Species

by

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ABSTRACT

Ant colonies provide numerous opportunities to study communication systems that maintain the cohesion of eusocial groups. In many ant species, workers have retained their ovaries and the ability to produce male offspring; however, they generally refrain from producing their own sons when a fertile queen is present in the colony. Although mechanisms that facilitate the communication of the presence of a fertile queen to all members of the colony have been highly studied, those studies have often overlooked the added challenge faced by polydomous species, which divide their nests across as many as one hundred satellite nests resulting in workers potentially having infrequent contact with the queen. In these polydomous contexts, regulatory phenotypes must extend beyond the immediate spatial influence of the queen.

This work investigates mechanisms that can extend the spatial reach of fertility signaling and reproductive regulation in three polydomous ant species. In *Novomessor cockerelli*, the presence of larvae but not eggs is shown to inhibit worker reproduction. Then, in *Camponotus floridanus*, 3-methylheptacosane found on the queen cuticle and queen-laid eggs is verified as a releaser pheromone sufficient to disrupt normally occurring aggressive behavior toward foreign workers. Finally, the volatile and cuticular hydrocarbon pheromones present on the cuticle of *Oecophylla smaragdina* queens are shown to release strong attraction response by workers; when coupled with previous work, this result suggests that these chemicals may underly both the formation of a worker retinue around the queen as well as egg-located mechanisms of reproductive

regulation in distant satellite nests. Whereas most previous studies have focused on the short-range role of hydrocarbons on the cuticle of the queen, these studies demonstrate that eusocial insects may employ longer range regulatory mechanisms. Both queen volatiles and distributed brood can extend the range of queen fertility signaling, and the use of larvae for fertility signaling suggest that feeding itself may be a non-chemical mechanism for reproductive regulation. Although trail laying in mass-recruiting ants is often used as an example of complex communication, reproductive regulation in ants may be a similarly complex example of insect communication, especially in the case of large, polydomous ant colonies.

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CHAPTER 1

INTRODUCTION

Sociality in animal species ranges along a continuum from solitary to eusocial, the most complex form (Wilson, 1975). Within group-living species, a social structure is maintained often resulting in dominance hierarchies and behavior is coordinated for activities such as foraging and reproduction (Clarke and Faulkes 1997; Fournier and Festa-Bianchet 1995; Gese et al. 1996; Pusey 1997; Röseler et al. 1985; Röseler et al. 1984; Rubenstein 1978; Whiteman and Côté 2004; Wittig and Boesch 2003). In advanced social species such as the eusocial Hymenoptera, there is reproductive division of labor where one caste supports colony reproduction and a separate worker caste takes care of all other colony needs (Hölldobler and Wilson 2009). Like other eusocial species, eusocial Hymenoptera maintain division of labor and are known to use aggression and pheromones to maintain the social structure. All of these forms of maintenance are intraspecific communication—the exchange of information between conspecifics that benefits both the signaler and receiver—and is a widely studied topic in species that range from solitary to eusocial (Hölldobler 1999; Ayasse et al. 2001; Uetz and Roberts 2002; Johansson and Jones 2007; Osorio and Vorobyev 2008; Hölldobler and Wilson 2009; Blomquist and Bagnères 2010; Bradbury and Vehrencamp, 2011; Richard and Hunt 2013).

Within solitary insect species, intraspecific communication is used to find a mate, recognize conspecifics, form aggregations, for defense, and for territory marking. As

sociality has evolved from solitary to eusocial, the communication systems used in the context of a solitary insect's natural history have been co-opted for uses beyond what is needed in solitary insects and evolved into the complex communication systems found in eusocial species. Nevertheless, many overlapping characteristics are conserved across species and levels of sociality (Blomquist and Bagnères 2010; Hölldobler 1984). Lipids coating the cuticles of all insects that principally serve to prevent desiccation have been co-opted over evolutionary time for communication (Blomquist and Bagnères 2010; Gibbs 1998). These cuticular hydrocarbons are well studied in their role as pheromones involved in nestmate recognition and fertility signaling in eusocial insects, but they are also present on the cuticles of solitary insects. For example, *Drosophila* use cuticular hydrocarbons to identify conspecifics and for mate selection (Everaerts et al. 2010; Ferveur 2005). Within the order Hymenoptera, the abundance and types of hydrocarbons present on the insects' cuticles are not correlated with the level of sociality exhibited by the species (Kather and Martin 2015).

In the evolution from solitary to social to eusocial insects, aspects of insect physiology were co-opted for communication, and those new communication channels became mechanisms for regulation of social conflict. Thus, the study of large, cohesive, superorganismic insect societies is implicitly a study of the limits and potential of these communication modalities to achieve the regulatory functions necessary for group living. The goal of this research is to understand the maintenance of reproductive division of labor over long-ranges in three polydomous ant species, *Novomessor cockerelli*,

Camponotus floridanus, and *Oecophylla smaragdina*. This section reviews some of the communication modalities available to eusocial insects and the reproductive conflict that they must resolve. Also reviewed are life-history details of polydomous ants, which provide a peculiar challenge to regulatory communication that makes them an ideal system for understanding how long-range reproductive regulation is achieved. Finally, methodological challenges to understanding the role of chemical communication are reviewed.

Background information

Eusociality and reproductive conflict over male production in Hymenoptera

Eusociality has evolved in a diverse group of taxa including naked mole-rats, shrimp, termites, thrips, beetles, honeybees, wasps, and ants (Jarvis and Bennett 1993; Hölldobler and Wilson 1990; Crespi 1992; Kent and Simpson 1992; Duffy 1996; Hölldobler and Wilson 2009). Common among all eusocial species is division of labor into reproductive and non-reproductive castes. In most ant species, members of the non-reproductive caste cannot mate but have retained the ability to activate their ovaries and produce viable haploid offspring (Itow et al. 1984; Lenoir et al. 1988; Bourke 1988; Hölldobler and Wilson 1990; Schilder et al. 1999a; Schilder et al. 1999b; Grasso et al. 2000; Pearcy et al. 2004; Pearcy et al. 2006; Hölldobler and Wilson 2009; Wenseleers and Van Oystaeyen 2011; Kronauer et al. 2012). Because workers have the ability to produce their own sons, there is potential for conflict over male production between the reproductive and non-reproductive castes within a colony (Ratnieks 1988; Ratnieks and

Reeve 1992; Bourke 1999; Beekman et al. 2003; Hammond and Keller 2004; Wenseleers and Ratnieks 2006). The potential for conflict over the production of males stems from the unequal relatedness of workers to their sons, nephews, and brothers. In a monogynous colony where the queen is monandrous (i.e., singly mated), all workers are produced by the queen and have a high relatedness ($r = 0.75$) to each other, but males can potentially be produced by either workers or the queen. Furthermore, each worker has a high relatedness to her sons ($r = 0.5$) and nephews ($r = 0.375$) relative to the relatedness to her brothers ($r = 0.25$) creating the potential for conflict between the workers and queen over the production of males (Hamilton 1964b, 1964a; Ratnieks and Reeve 1992; Bourke and Franks 1995; Ratnieks et al. 2006). However, the potential for conflict decreases when colonies are polygynous (i.e., colonies with multiple reproductive individuals) or polyandrous (i.e., a multiply mated queen) due to the reduced average relatedness of workers to their nephews relative to their brothers making it more beneficial for the an individual worker to help rear her brothers than it would be for her to rear her nephews (Ratnieks 1988; Bourke and Franks 1995). Relatedness of an individual worker to her sons would not change in this scenario, but if many workers in the colony were reproducing, the number of sons she produced would be heavily outnumbered by the number of nephews. Because a worker would be unlikely to differentiate between her sons and her nephews, her effort to rear her own sons would instead be helping to rear the far greater number of nephews, which would re-enforce the benefit of rearing brothers over attempting to rear sons in a polyandrous or polygynous colony. Nevertheless, in

observed monogynous, monandrous eusocial-insect colonies where there is a theorized conflict over male production, workers predominately rear their siblings over their own sons, which suggests that the presence of the potential for conflict over male production does not necessarily lead to actual conflict (Bourke and Franks 1995; Ratnieks and Reeve 1992).

A potential explanation for why workers refrain from producing males in monogynous, monandrous colonies is that they receive greater inclusive fitness gains by helping the queen rear their siblings than they would by rearing their own male offspring. This might be the case if producing their own sons caused a decrease in the number of females produced by the queen (Ratnieks and Reeve 1992; Bourke and Franks 1995). Because of the high relatedness between sisters, the decrease in indirect fitness from lower female production in the colony that may occur if a larger portion of workers began laying eggs might outweigh any direct fitness gains workers would receive from producing their own sons. It may also be difficult or impossible for workers to distinguish between queen-laid male eggs and queen-laid female eggs (Bourke and Franks 1995), and so it would be costly to workers if they destroyed female eggs by mistake when trying to replace queen-laid male eggs with their own male eggs (Nonacs and Carlin 1990). When *C. floridanus* were presented with larvae of a range of instars, they were unable to distinguish between the male and female brood (Nonacs and Carlin 1990). When provided with both male and female larvae positioned outside of the nest, workers retrieved both sexes equally. However, when they were provided with pupae, they

retrieved female pupae significantly faster than male pupae. This result suggests that queens may disguise their brood so that workers are unable to cull queen-produced males and replace them with their own.

Although relatedness predicts that monogynous, monandrous species should experience worker reproduction while polygynous and/or polyandrous species do not, this is not supported by the literature. Worker reproduction appears to be low in general, but its occurrence has been documented in both monogynous and polygynous species (Choe 1988; Bourke and Franks 1995). Because conflict does occur, albeit on a low scale, mechanisms exist for maintaining worker sterility in the colony.

Regulation of worker reproduction by policing

The maintenance of reproductive division of labor requires colony members of a eusocial species to be constrained physiologically or behaviorally in order to prevent cheating. The mechanisms that regulate worker reproduction within colonies vary across species ranging from social to chemical regulation (Hölldobler and Wilson 1990; Monnin 2006; Ratnieks et al. 2006; Kocher and Grozinger 2011). Arguably the most well-documented form of social regulation of reproduction in eusocial species is policing, which occurs in four forms: self-policing, worker policing, egg policing, and queen policing (Ratnieks 1988; Ratnieks and Visscher 1989; Wenseleers et al. 2004). Policing is thought to have evolved in response to conflict over male production in eusocial insects and results in worker acquiescence.

Worker policing—the process of displaying physical aggression toward workers with active ovaries or worker-laid eggs—is a form of physical regulation of worker reproduction seen among Hymenoptera species (Ratnieks 1988; Ratnieks and Visscher 1989; Liebig et al. 1999; Oldroyd 2013). Some Ponerinae and Myrmicinae ant species have social systems in which the reproductive female is succeeded after death by a mated worker that becomes reproductive (a so-called gamergate) (Peeters and Crozier 1988). Gamergates in species of the ant genus *Diacamma* use physical mutilation to prevent workers from mating and reproducing prior to the demise of the current gamergate (Peeters and Higashi 1989; Sommer et al. 1993). As a callow ecloses, special thoracic appendages (so-called gemmae) that are critical for mating are removed by the gamergate or workers preventing her from mating. Workers in the Ponerine ant *Harpegnathos saltator* also demonstrate policing behavior directed toward sisters that have active ovaries; the aggressive behavior results in the deactivation of the newly reproductive worker's ovaries and thereby prevents too many gamergates in a single colony at a given time (Liebig et al. 1999). Policing of workers also occurs in the Myrmicine ant species *Novomessor cockerelli*. Cheating workers of *N. cockerelli* are identified by the development of a fertility signal composed of hydrocarbons present on their cuticle that resemble the fertility signal of the queen, which results in the identified reproductive worker receiving physical aggression from her non-reproductive sisters (Smith et al. 2008, 2009). In addition to workers policing cheating nestmates, queens police reproductive workers as well by behaving aggressively toward them and marking

reproductive workers with a pheromone from their Dufour's gland to stimulate further aggressive behavior toward the marked worker from her nestmates (Smith et al. 2011; Smith et al. 2012). Similar policing mechanisms have been described in numerous other ants and wasps as well (Gobin et al. 1999; Liebig et al. 1999; Monnin and Ratnieks 2001; Iwanishi et al. 2003; Hartmann et al. 2003; Hartmann et al. 2005; Dietemann et al. 2005; Wenseleers et al. 2005a; Wenseleers et al. 2005b; Wenseleers and Ratnieks 2006).

An alternative policing strategy is oophagy—the culling of worker-laid eggs—first documented in the honeybee, *Apis mellifera* by Ratnieks and Visscher (1989). *Apis mellifera* workers are able to distinguish between worker-laid and queen-laid eggs in the comb and cull the worker-laid eggs while leaving the queen-laid eggs unharmed (Ratnieks 1993; Ratnieks and Visscher, 1989). Oophagy in honeybees is a successful response to worker cheating resulting in only 0.12% of males in a queenright i.e., a colony containing a queen) colony being produced by workers. The ability to of workers to accurately identify worker-laid eggs has also been demonstrated in *Apis florea* and *Apis cerana* (Halling et al. 2001; Oldroyd et al. 2001). The cue enabling workers to identify worker-laid eggs is thought to be chemical as there are no morphological differences between queen-laid and worker-laid eggs, and the presence of a queen-laid egg in the same cell with a worker-laid egg reduces the likelihood of culling (Katzav-Gozansky et al. 2001, 2003; Martin et al. 2005). Similar to honeybee workers, workers of some ant species can discriminate between worker-laid and queen-laid eggs and regulate reproduction by oophagy rather than policing of workers. A fertility signal present on a

Camponotus floridanus queen's cuticle is also present on queen-laid eggs. Worker-laid eggs, which lack the fertility signal, are culled by their sisters (Endler, Hölldobler, & Liebig 2007; Endler et al. 2004). The fertility signal present on both the queen and her eggs indicate her level of fertility—stronger, more abundant signals indicating a more fertile queen thereby serving as an honest signal for the workers and regulating worker reproduction (Endler et al. 2006). Like policing of reproductive workers, oophagy occurs in many species of bees, wasps, and ants (Monnin and Peeters 1997; Foster and Ratnieks 2000; Halling et al. 2001; Oldroyd et al. 2001; Foster et al. 2002; D’Ettorre et al. 2004; Saigo and Tsuchida 2004; Wenseleers et al. 2005a; Wenseleers et al. 2005b; Helanterä and Sundström 2007; Bonckaert et al. 2007; Zanette et al. 2012).

Suppression of worker reproduction by queen compounds

Compounds produced by the queen in eusocial-insect colonies can regulate worker reproductive behavior directly by serving as an honest signal to the workers of her presence and fertility resulting in workers refraining from producing their own sons (Seeley 1979; Keller and Nonacs 1993; Villalta et al. 2018; Smith and Liebig 2017). For example, queen honeybees, *Apis mellifera*, produce a queen substance or queen mandibular pheromone that has an suppresses the development of worker ovaries and is spread throughout the colony by workers, termed messengers, in order to ensure it reaches all of the colony members signaling to them that there is a fertile queen present (Butler 1957; de Groot and Voogd 1954; Hoover et al. 2003; Naumann et al. 1991; Seeley 1979). Because honeybee colonies are large, having messenger bees to transmit

the signal of the queen's presence throughout the hive is an integral part of the communication system within the colony (Seeley 1979). Similarly, in the termite *Reticulitermes speratus*, reproductives regulate worker reproduction through the release of a volatile pheromone that inhibits female workers from developing into neotenic (secondary reproductives) (Matsuura et al. 2010).

The insect cuticle itself can also carry honest signals of queen fertility that can be used to suppress reproduction in workers in close proximity to the queen (Keller and Nonacs 1993; Endler et al. 2004; Liebig 2010; Smith et al. 2012; Van Oystaeyen et al. 2014; Smith and Liebig 2017). For example, reproductives in the termites *Zootermopsis nevada* and *Cryptotermes secundus* exhibit hydrocarbons on their cuticle that are absent on workers' cuticles (Weil et al. 2009; Liebig et al. 2009). It is possible that these reproductive specific hydrocarbons play a role in regulating workers from developing into reproductive neotenic similar to the volatile pheromone released by *R. speratus* reproductives. Potential fertility signals identified as hydrocarbons on the cuticle of reproductives that are absent from the cuticle of workers are found in ant species including *C. floridanus*, *N. cockerelli*, *Pachycondyla inversa*, *Odontomachus brunneus*, *Neoponera apicalis*, *Lasius niger*, and *Harpegnathos saltator* (Heinze et al. 2002; Endler et al. 2004; Endler et al. 2006; Smith et al. 2012a; Smith et al. 2012b; Yagound et al. 2014, 2015; Holman et al. 2013; Holman et al. 2016; Holman et al. 2010). Holman et al. (2013, 2016, 2010) identify the use of 3-methyl-branched alkanes as being necessary and sufficient without the colony cue as background information to regulate reproductive

division of labor within *Lasius niger*, *Lasius flavus*, and *Lasius lasiodes* and suggest that 3-methyl-branched alkanes are a special class of alkane that have evolved within ants specifically to serve as a queen signal. However, work in *Odontomachus brunneus* suggests that this may be a simplification of the mechanism (Smith et al. 2015; Smith and Liebig 2017). Although a single hydrocarbon may be sufficient for some species, it is unlikely to be sufficient for all species of ants.

Queen-laid eggs often have a fertility signal present on the cuticle that mimics the signal present on the cuticle of the queen (Endler et al. 2004; Endler et al. 2006; Smith et al. 2008; Liebig 2010). The fertility signal present on the eggs can serve a dual purpose by allowing workers to distinguish between worker-laid and queen-laid eggs to cull worker-laid eggs as discussed above and to serve as an honest signal of the queen's presence within the colony and her fertility status. When *C. floridanus* workers are provided with queen-laid eggs that contain the hydrocarbons thought to be the fertility signal, workers refrain laying eggs. However, when they are provided with larvae and pupae or no brood, they begin to lay eggs (Endler et al. 2004).

The role of larvae in reproductive regulation

In addition to fertility signals present on the queen and eggs, larvae have been identified in honeybees, bumble bees, and five ant species, including results from work presented in chapter 1 of this thesis, as a mechanism for maintaining reproductive division of labor in eusocial colonies (Jay 1968; Mamsch 1967; Heinze et al. 1996; Villalta et al. 2015; Warner et al. 2016; Ebie et al. 2015; Starkey et al. 2019). Larvae of

the honeybee, *A. mellifera*, regulate worker reproduction within the colony through the use of both volatile and non-volatile pheromones (Jay 1968; Le Conte et al. 1990; Traynor 2014; Traynor et al. 2014). Queenless *A. mellifera* worker groups provided with queen-laid worker larvae are inhibited from developing their ovaries (Jay 1968; Traynor 2014; Traynor et al. 2014). Honeybee larvae contain a group of ten non-volatile esters on their cuticle that inhibit the development of worker ovaries when the workers are isolated from the queen (Le Conte et al. 1990; Mohammedi et al. 1998; Arnold et al. 1993). Methyl linolenate and ethyl palmitate are two of the larval cuticular esters that have been shown to play a critical role in the inhibition of worker ovary development (Mohammedi et al. 1998). In addition to the non-volatile compounds on the cuticle of the larvae, E- β -ocimene is a volatile brood pheromone released by larvae that has been demonstrated to inhibit honeybee worker reproduction (Maisonnasse et al. 2009; Maisonnasse et al. 2010; Traynor 2014; Traynor et al. 2014).

Despite the clear role of larvae in the regulation of honeybee worker reproduction, the role of larvae in other eusocial Hymenoptera is an understudied mechanism. The role of larvae in reproductive regulation of ants has only been documented in five ant species. Larvae inhibit workers in queenless worker groups from laying viable eggs in the ant *Myrmica ruginodis* (Mamsch 1967). Queenless worker groups of *M. ruginodis* produce male offspring, but they do not activate their ovaries when presented with queen-laid larvae in ratios that reflect natural worker-to-brood ratios. In *Aphaenogaster senilis*, larvae possess a contact pheromone on their cuticle that delays workers from laying eggs

when they are separated from the queen but still given queen-produced larvae (Villalta et al. 2015). Larvae also have a negative effect on egg number in queenless groups of the ant *Pachycondyla villosa* (Heinze et al. 1996). When queenless worker groups of *P. villosa* are given queen-laid larvae, there is negative correlation between the number of larvae present in the worker groups and the number of eggs produced by the workers in that group.

The proximate mechanism underlying the larval regulation of worker ovary development in the ant *A. senilis* is a non-volatile contact pheromone on the larvae, but the exact compounds are unknown (Villalta et al. 2015). The mechanisms used by larvae to regulate worker reproduction in *M. ruginodis* and *P. villosa* are still unidentified (Mamsch 1967; Heinze et al. 1996). As in honeybees and *A. senilis*, a non-volatile contact pheromone on the larval cuticle may suppress ovary development (Le Conte et al. 1990; Arnold et al. 1993; Mohammedi et al. 1998; Villalta et al. 2015) or a volatile larval pheromone may be released by the larvae as in honeybees (Maisonasse et al. 2009, 2010). Alternatively, providing nutrients for the larvae may deplete the workers of a pro-fertile substance that would be needed to produce viable eggs (Bier 1954; Pain 1954). The pro-fertile substance described by Pain (1954) and Bier (1954) may be vitellogenin in both honeybees and ants as it is known to play a role in honeybee workers as a proteinaceous pre-cursor to yolk production (Amdam et al. 2003).

Polydomy in ants and novel challenges to reproductive regulation

Some ant species have colonies that are spread among multiple spatially separated nests, which is referred to as polydomy (Hölldobler and Wilson 1977; Hölldobler and Carlin 1989; Snyder and Herbers 2004; Pedersen and Boomsma 1999; Debout et al. 2007). The subnests or satellite nests in polydomous colonies often contain workers, larvae, and pupae (Debout et al. 2007). In polygynous ant species, there can be multiple subnests that contain a queen, and polydomy in these species can serve as a budding process where propagules with daughter queens and workers move to a nearby location instead of a lone foundress forming her own colony in a distant location. In some species, this budding process leads to fissioning into distantly separated colonies over time (Debout et al. 2007). However, monogynous species can also be polydomous with the queen housed in one of the subnests while the other disconnected subnests contain only brood and workers.

In the case of monogynous species, it is thought that polydomy may have evolved as a way to increase foraging success for the colony. Monodomous ant colonies are considered central-place foragers because they forage for food and return it to a consistent central location. Polydomy allows the workers to cover a larger foraging territory and bring food back to the nest quicker without risk of losing it to competitors or being preyed upon. Food can then be redistributed throughout the colony subnests or be utilized within a single subnest, which results in a decentralized foraging strategy that is more successful than monodomous nests in certain environmental conditions (Holway and Case 2000; Pfeiffer and Linsenmair 1998; Schmolke 2009). Alternatively, polydomy

may have evolved to provide insurance to the colony in the form of duplicate nests with brood and workers (Debout et al. 2007; Van Wilgenburg and Elgar 2007). If one subnest is attacked or damaged, surviving workers and brood can relocate along with the queen to an undisturbed subnest. This provides a way to ensure that the colony will not be stranded without a nest and left vulnerable after a disturbance (Debout et al. 2007; Van Wilgenburg and Elgar 2007).

Having a colony spread among multiple subnests may have implications for the maintenance of reproductive regulation in the colony, especially in the case of monogynous species where the workers in the subnests may not come into direct contact with the queen for extended periods of time. There is evidence that workers do sometimes overcome the forces of reproductive regulation and produce males in the subnests separated from the queen, as in the polydomous slave-making ant *Temnothorax americanus* with approximately 70% of males being worker produced (Foitzik and Herbers 2001). Additionally, workers in colonies of the monogynous, polydomous species *Pachycondyla goeldii* have higher ovary activation in the subnests without the queen than those in the subnest with the queen, which may be explained by the distance of the queenless subnests from the queenright subnest (Denis et al. 2007). However, there are species such as *Cataglyphis iberica* where separated workers continue to maintain the integrity of the colony while being spread among multiple subnests (Dahbi et al. 1996, 1997; Dahbi and Lenoir 1998; Dahbi et al. 1999). *Novomessor cockerelli* is another example of a monogynous species that is polydomous, but the queen remains the only

reproductive in the colony in this species as well (Smith et al. 2012). Monogynous polydomous species like these lead to puzzling questions about how the queen's signal is transported among multiple subnests maintaining reproductive division of labor when there is physical separation between the subnests leading to some workers being separated from the queen for extended periods of time. The natural separation between workers and the queen in polydomous, monogynous colonies that nevertheless maintain reproductive regulation makes these species ideal for studying the specific role of communication in the regulatory process.

Practical challenges to understanding chemical communication in eusocial insects

As discussed above, signals in the form of pheromones as well as substances found on the insect cuticle may play a significant role in the regulation of reproductive conflict in a eusocial-insect colony. However, new evidence from chemical ecology suggests that methodological choices in past studies may have obscured the true role of some of these chemicals. Moving forward, experiments must be more carefully constructed so that the ecologically relevant forms of hypothesized chemical signals are used within the ecologically relevant chemical context.

Insect cuticles have a waxy covering formed by long-chain hydrocarbons that prevent desiccation (Lockey 1980, 1985; Gibbs and Pomonis 1995; Gibbs 1998; Martin and Drijfhout 2009; Blomquist and Bagnères 2010). In ants, the hydrocarbons present on the cuticle include alkanes, alkenes, methyl-branched alkenes and alkanes, and dienes with alkanes and monomethylalkanes being the most abundant (Lockey 1980; Martin and

Drijfhout 2009; Blomquist and Bagnères 2010). Along with playing a major role in preventing desiccation, these hydrocarbons have also been co-opted for communication in many insect species. In particular, they play an important role the detection of mates, recognition of conspecifics, recognition of nestmates, foraging and recruitment, group aggregations, and fertility signaling (Blomquist and Bagnères 2010; Howard and Blomquist 2005). In ant species, the cuticular hydrocarbons indicate to colony members who is a nestmate and who is not by the formation of a uniform colony odor across the colony, which is thought to be produced or mixed within the postpharyngeal gland of the ant (Bagnères and Morgan 1991; Soroker et al. 1994; Soroker et al. 1995; Vander Meer et al. 1998; Akino et al. 2004). Workers are thought to use a neural template to distinguish between the colony odor and others by recognizing differences in the composition of hydrocarbons present on the cuticle (Leonhardt et al. 2007).

Cuticular hydrocarbons are also integral in the maintenance of reproductive division of labor by indicating the presence of a queen or queens via a fertility signal unique to their cuticular profile that indicates to workers that they are reproductively active (Peeters et al. 2000; Dietemann et al. 2003; Endler et al. 2004; Endler et al. 2006; Endler et al. 2007; Smith et al. 2008, 2009; Liebig et al. 2009; Lommelen et al. 2010; Smith et al. 2012, 2013; Van Oystaeyen et al. 2014; Smith and Liebig 2017).

Nevertheless, laboratory studies with cuticular hydrocarbons fail to account for the recent evidence of isomer sensitivity. Methyl-branched hydrocarbons produced by insects are most commonly synthesized as the R-isomer form, and this pattern of synthesis is

conserved across insects (Bello et al. 2015). However, most research on the detection of these stereoisomeric hydrocarbons uses racemic mixtures rather than the isomer synthesized by the insect even though there is some evidence suggesting that the insects can detect the differences between the isomers (Leal 1996; Mori 2007; Svensson and Larsson 2008; Millar 2010; Yu et al. 2014). In both scarab beetles and saproxylic beetles, a differential response to enantiomers of pheromones used in their communication systems has been observed (Leal 1996; Svensson and Larsson 2008). Initial evidence of discrimination between pheromone isomers has also been demonstrated in the little fire ant, *Wasmannia auropunctata* (Yu et al. 2014). Yu et al. (2014) tested the response of *W. auropunctata* to the (*S*)-enantiomer and a racemic mixture of the alarm pheromone used in the species. The ants responded to the (*S*)-enantiomer of the pheromone but not the racemic mixture, and it was determined that they only produce the (*S*)-enantiomer of the alarm pheromone. However, their response to the (*R*)-enantiomer alone was not tested.

Additionally, identification and evidence of cuticular hydrocarbons serving as a queen fertility signal resulting in complete inhibition of worker reproduction is still needed. Van Oystaeyen et al. (2014) suggest that a conserved class of hydrocarbons, alkanes and methylalkanes, serve as queen pheromones across multiple evolutionary lineages of eusocial species. However, this result has been disputed by Amsalem et al. (2015) after testing the alkanes proposed by Van Oystaeyen et al. (2014) to be the queen pheromone in *Bombus terrestris* with a related bumble bee, *Bombus impatiens*. *Bombus impatiens* treated with the proposed queen pheromone did not differ in latency to lay eggs

of ovary development from the workers treated with hexane alone. Additionally, the queen pheromones proposed by Van Oystaeyen et al. (2014) were tested alone rather than in the context of the other hydrocarbons that are found on the cuticle of workers and reproductives serving as a colony recognition signal. Smith et al. (2015) have shown that *Odontomachus brunneus* only respond to the queen fertility signal when it is combined with the colony hydrocarbon profile as it would be when occurring naturally on the queen. This discrepancy in the literature leaves many questions unanswered about fertility signals in eusocial Hymenoptera.

Research Rationale

To investigate mechanisms used to maintain reproductive division of labor over long-ranges in polydomous ant species, we studied three species across two ant subfamilies that are all monogynous and polydomous and do not have overlapping ranges. The myrmicine ant *Novomessor cockerelli* is a desert species native to the southwestern United States and northern Mexico (Demarco and Cognato 2015). Colonies are monogynous, monandrous, and can have 1–5 unconnected subnests making them a polydomous species (Hölldobler and Carlin 1989; Smith et al. 2012). The formicine carpenter ant *Camponotus floridanus* is native to Florida, USA, and has colonies that are also monogynous, monandrous, and polydomous (Gadau et al. 1996; King et al. 2013). Finally, the formicine weaver ant *Oecophylla smaragdina* is an arboreal species native to Australasia that is monogynous in most populations and is often polyandrous (Peng et al. 1998; Schlüns et al. 2009). Colonies of this species can each span across many trees and

inhabit upwards of 100 subnests made from weaving leaves together with the silk produced from their larvae (Andersen 1992; Cole Jr. and Jones Jr. 1948; Hölldobler 1983; Hölldobler and Wilson 1977; Lokkers 1990; Marcela et al. 2012; Peng et al. 1998).

Although all three species achieve reproductive regulation across similar polydomous challenges, there are significant differences in their physiology and lifestyle that may affect the mechanisms underlying the regulatory function. Workers of all three species have retained their ovaries and will produce viable male-destined eggs when the queen dies or is artificially removed from the colony (Hölldobler and Wilson 1983; Hölldobler and Carlin 1989; Endler et al. 2004). Unlike *C. floridanus*, *N. cockerelli* and *O. smaragdina* workers maintain active ovaries and produce trophic eggs when the queen is present in the nest (Hölldobler and Wilson 1983; Hölldobler and Carlin 1989). These eggs are used as a food source for the queen and larvae within the colony. Queens of both *C. floridanus* and *N. cockerelli* have queen-specific hydrocarbons present on their cuticle that are also present on the eggs they produce (Endler et al. 2004; Endler et al. 2006; Smith et al. 2008). *Camponotus floridanus* workers cull worker-laid eggs, which are identified by the lack of queen-specific hydrocarbons but are unable to identify egg-laying workers (Endler et al. 2007, 2006). However, *Novomessor cockerelli* worker-laid and queen-laid eggs are chemically indistinguishable, and so worker-laid eggs are not culled (Smith et al. 2008). Instead, workers laying viable eggs are identified by the development a hydrocarbon profile resembling that of the queen and policed by their sisters (Smith et al. 2009). With the exception of work done by Hölldobler and Wilson

(1983) indicating that there is likely a queen-specific chemical profile on the cuticle of workers that inhibits worker production of sons, little is known about the regulation of worker reproduction in *Oecophylla smaragdina*. The similarities and differences of these three ant species make them ideal for the investigation of mechanisms used by eusocial insects to maintain reproductive division of labor over long ranges where workers may not regularly interact directly with the queen.

CHAPTER 2

LARVAL REGULATION OF WORKER REPRODUCTION IN THE POLYDOMOUS ANT *NOVOMESSOR COCKERELLI*¹

Abstract

Although workers in many ant species are capable of producing their own offspring, they generally rear the queen's offspring instead. There are various mechanisms that regulate worker reproduction including inhibitory effects of ant brood. Colonies of the ant *Novomessor cockerelli* are monogynous and polydomous resulting in a large portion of nest workers being physically isolated from the queen for extended periods of time. Some workers experimentally isolated from the queen in laboratory nests lay viable eggs, which develop into males. We investigate the mechanism that regulates worker fertility in subnests separated from the queen by giving queenless worker groups queen-produced larvae, queen-produced eggs, or no brood. Our findings show that larvae significantly delay the time to worker egg-laying, but eggs have no effect. Larval inhibition is a likely mechanism that contributes to the regulation of worker reproduction in *N. cockerelli* because larvae are easily transported to subnests that do not contain a queen.

Introduction

A defining characteristic of eusociality is division of labor into reproductive and non-reproductive castes (Hölldobler and Wilson 2009; Hölldobler and Wilson 1990). In

¹ Ebie, Jessica D., Bert Hölldobler, and Jürgen Liebig. 2015. "Larval Regulation of Worker Reproduction in the Polydomous Ant *Novomessor Cockerelli*." *The Science of Nature* 102 (11–12): 72. <https://doi.org/10.1007/s00114-015-1323-2>.

the majority of ant species, members of the non-reproductive caste have retained the ability to activate their ovaries and produce viable offspring (Bourke 1988; Hölldobler and Wilson 2009; Hölldobler and Wilson 1990). Consequently, regulatory mechanisms exist that inhibit reproduction by the non-reproductive caste and vary across species ranging from social to chemical regulation (Hölldobler and Wilson 1990; Ratnieks et al. 2006; Kocher and Grozinger 2011; Monnin 2006). In some ant species, the queen plays a role in regulating worker reproduction through the presence of a fertility signal on her cuticle or eggs, whereas, others behaviorally regulate worker reproduction through acts such as marking reproductive workers with a chemical initiating policing (Endler et al. 2004; Smith et al. 2012; Monnin 2006).

Novomessor cockerelli is particularly interesting because each monogynous colony is spread among 1—5 unconnected subnests, and a large portion of nest workers may be physically isolated from the queen for extended periods of time (Hölldobler and Carlin 1989; Demarco and Cognato 2015). Additionally, nest workers have active ovaries to produce trophic eggs as a nutrient source for the queen and larvae. When experimentally isolated from the queen in laboratory nests, some workers lay viable eggs that develop into males. Such male production by workers does not occur in natural subnests separated from the queen (Smith et al. 2012). Although *N. cockerelli* workers police reproductive nestmates, they are unable to cull worker-laid eggs, which are chemically indistinguishable from queen-laid eggs (Hölldobler and Carlin 1989; Smith et

al. 2008, 2009). In this study, we investigated the mechanism used to regulate worker fertility in subnests separated from the queen.

Materials and Methods

Study Species. *Novomessor cockerelli* colonies were collected in Portal, Arizona, USA using the method Smith and Haight (2008) describe. Colonies were housed at Arizona State University in plaster nests with a red acetate and glass-covered cavity simulating darkness for the ants. An attached foraging area was provided for food retrieval. Colonies were maintained in an environmental growth chamber at 27°C and a 12:12 h light-dark cycle. They were fed a diet of water, sugar water, Nature's Song sunflower hearts and wild finch seed, and Bhatkar twice per week (Bhatkar and Whitcomb 1970). They also received an alternating diet of pre-killed beetle larvae, *Zophobas morio*, and crickets, *Grylloides sigillatus*, twice per week. Experiments were carried out from April—August 2015 with preliminary experiments in 2013.

Experimental Procedures. Queenless worker groups were created by randomly selecting 60 foraging workers from nest foraging areas and 240 nest workers from nest cavities. These workers were divided into three worker groups of 100 ants each using stratified randomization. Nest workers were randomized independently of foraging workers. This was done for 11 colonies creating 11 groups of 3 queenless worker groups. Experimental nests mimicked those of the natal colony but were smaller in size. Worker groups randomly received one of three treatments: 60 queen-laid eggs, 60 queen-produced larvae, or no brood. The developmental stage and number of brood in the egg

and larvae treatments were checked weekly. Eggs that had developed into larvae were replaced with queen-laid eggs bringing the total egg count to 60. Larvae that had pupated or reached the pharate pupa stage were replaced with queen-produced larvae ranging from first instar to the final instar before transition to pharate pupae returning the larvae count to 60 while the range of larval instars was maintained.

Worker groups were checked weekly for the presence of worker-laid eggs by counting total brood present, and a weekly worker census was taken. Trophic eggs were unlikely to be counted because they are consumed shortly after being produced (B Hölldobler and Wilson 1990). Additionally, *N. cockerelli* trophic eggs are softer and shaped differently than viable eggs. Workers were determined to have laid eggs if total brood number exceeded 60 in the egg treatment or eggs were present in the larvae or no brood treatments. Latency data for groups that had not produced viable eggs at the completion of the 11-week observation period was recorded as 11 weeks.

Data Analysis. Worker mortality data were analyzed with a Cox regression mixed-effects model with treatment as a fixed effect and colony as a random effect (Therneau 2015). Latency data were log transformed to correct for a deviation from normality and were analyzed using a Repeated Measures Analysis of Variance (Fox and Weisberg 2011; R. C. D. Team 2014). Post-hoc analysis was done using a Tukey test corrected for repeated measures (Kutner et al. 2005).

Results

Worker mortality did not differ between treatment groups ($\chi^2 = 2.37$, $df = 2$, $P = 0.31$; Cox mixed-effects model, Egg vs Larvae: $z = 1.50$, $P = 0.13$, Egg vs No Brood: $z = 1.03$, $P = 0.30$; Figure 1). The latency to viable egg production by queenless workers was significantly different between treatment groups (Repeated Measures ANOVA: $F_{2, 20} = 13.39$, $P = 0.0002$; Figure 2). Workers in the larvae treatment group ($\bar{x} = 7.09 \pm 2.80$ weeks) took significantly longer to produce viable eggs than those in both the egg treatment ($\bar{x} = 4.64 \pm 1.12$ weeks) and the no brood treatment ($\bar{x} = 4.09 \pm 0.83$ weeks; Tukey Test: Egg treatment $q = 5.19$, $P = 0.0042$; No brood Treatment $q = 6.84$, $P = 0.00028$). There was no difference in latency to produce viable eggs between the no brood and the egg treatments (Tukey Test: $q = 1.65$, $P = 0.48$).

Discussion

Our results highlight the role of larvae in regulating worker reproduction in a polydomous ant species and corroborate the potential importance of larvae in inhibiting worker reproduction in eusocial colonies. Inhibition of worker reproduction in the queen's presence enhances colony level reproduction and is ultimately the result of indirect fitness gains outweighing direct fitness gains to workers from producing their own male offspring. In the case of the polydomous species *N. cockerelli*, workers are unlikely to stay permanently in the subnests, and so larval delay in addition to worker policing and queen marking likely explain the regulation of worker reproduction. We have observed frequent movement between subnests in the field with nestmates often

being carried from one nest to the other (personal observation). The role of larvae in *N. cockerelli*, is analogous to the transport of the queen substance by *A. mellifera* messenger bees that spread the queen's fertility signal to hive nestmates (Seeley 1979). The fertility signal present on queen-laid eggs in the ant species *C. floridanus* also serves to indicate the presence of a reproductive individual throughout the colony (Endler et al. 2006, 2004). By transporting queen-produced larvae to subnests of the polydomous colony (personal observation), *N. cockerelli* workers similarly spread information about the presence of a fertile queen. In a prior study, Hölldobler and Carlin (1989) show that queenless *N. cockerelli* worker groups do produce viable eggs in the presence of queen-laid brood; however, the exact incubation time until production of viable eggs in queenless groups began was not recorded. The current study extends that result by controlling for the stage of brood and incubation time.

The larval regulatory effect shown in this study has only been observed in three ant species and one bee species, and this is the first example of larval regulation in a polydomous species. In the absence of a queen, larvae have been shown to suppress worker reproduction in *Myrmica ruginodis*, delay worker reproduction in *Aphaenogaster senilis*, and decrease egg production in *Pachycondyla villosa* (Heinze et al. 1996; Mamsch 1967; Villalta et al. 2015). Similarly, honeybee, *Apis mellifera*, larvae have a volatile pheromone and esters on their cuticle that have a complete inhibitory effect on worker ovary development (Arnold et al. 1993; Le Conte et al. 1990; Maisonnasse et al. 2010).

The proximate mechanism underlying larval delay in *N. cockerelli* worker ovary development is still unclear. As in honeybees (Arnold et al. 1993; Le Conte et al. 1990) and *A. senilis* (Villalta et al. 2015), a non-volatile contact pheromone on the larval cuticle may suppress ovary development. A volatile larval pheromone released by the larvae is also possible, as this mechanism is present in honeybees (Maisonasse et al. 2010). Alternatively, trophic egg production may deplete workers of protein or other nutrients needed to produce viable eggs (Bier 1954; Pain 1954).

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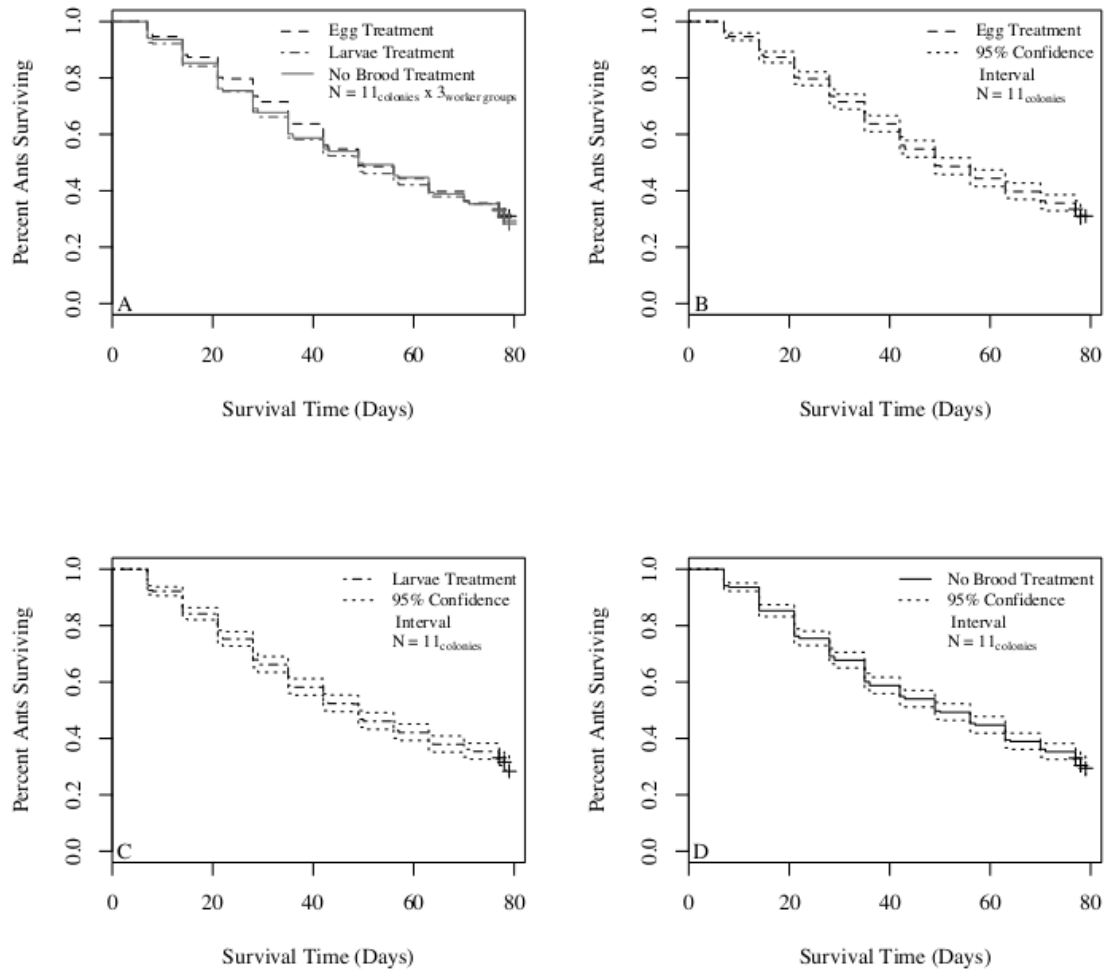


Figure 1. Survival analysis of *Novomessor cockerelli* worker mortality (A) The three queenless worker group treatments (Egg, Larvae, and No Brood) did not differ in worker mortality during the course of the study (Cox mixed-effects model: $\chi^2 = 2.37$, $df = 2$, $P = 0.31$). (B—D) Percent survival of ants in three queenless worker group treatments is shown with 95% confidence intervals.

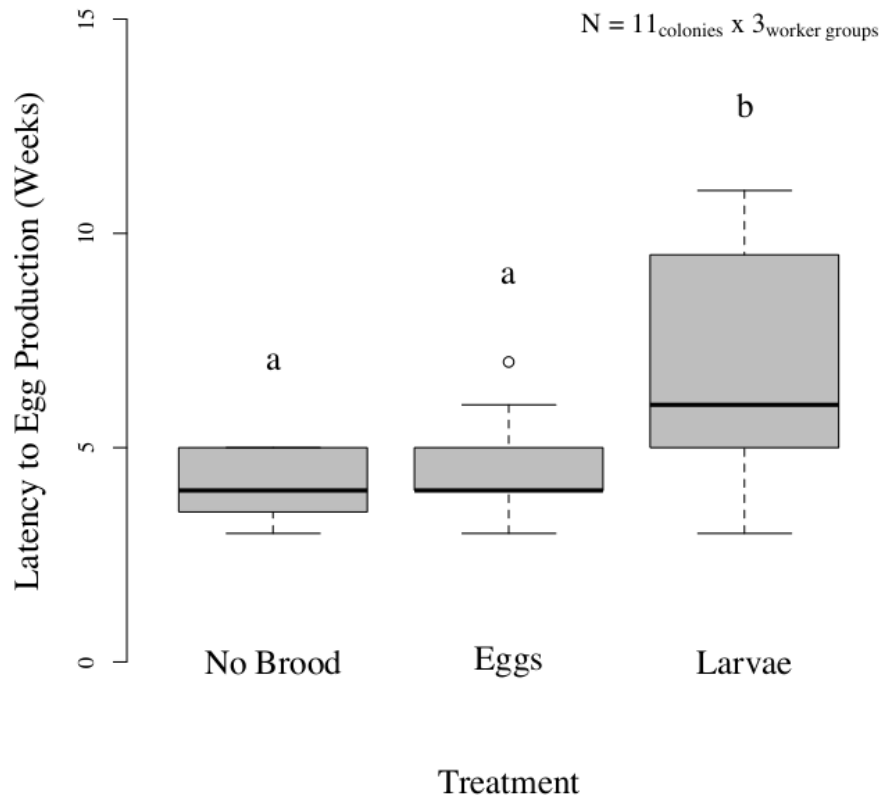


Figure 2. Latency of workers to produce viable eggs. Queenless worker groups given queen-produced larvae had significantly longer latency to produce viable eggs than queenless worker groups provided with queen-laid eggs or no brood (Repeated Measures ANOVA: $F_{2,20} = 13.39$, $P = 0.0002$). The horizontal bar in the box represents the median time (weeks) until production of viable eggs. The lower and upper edges of the box represent the 25th and 75th quartiles, and the whiskers represent the 1.5 interquartile range. Data falling outside of the 1.5 interquartile range are represented by an open circle.

CHAPTER 3

QUEEN-SPECIFIC ENANTIOPURE CUTICULAR HYDROCARBON DISRUPTS NESTMATE RECOGNITION PATTERNS

Abstract

When a highly fertile *Camponotus floridanus* is introduced into a foreign, mature colony, she does not receive aggression from the workers of the colony. However, an incipient queen introduced into a foreign, mature colony will receive direct aggression from the workers (Moore and Liebig 2010). Although cuticular hydrocarbons have been implicated in the reproductive signaling of *Camponotus floridanus* as a releaser pheromone that inhibits worker aggression toward a fertile, foreign, the specific chemicals and the possible role of stereochemistry is still unknown. 3-methylheptacosane (3-MeC27), the most abundant component of *C. floridanus* queen-specific cuticular hydrocarbons, was tested to confirm its role as a releaser pheromone involved in maintaining reproductive division of labor in this eusocial species. Additionally, the effectiveness of the racemic mixture of 3-MeC27 and the two stereoisomers was also tested. Major workers from foreign colonies were coated in the 3-methylheptacosane, either the (*R*)- or (*S*)-enantiomer, *n*-pentane, *n*-nonacosane, or remained unaltered and were placed inside a foreign colony nest within an observation arena with twelve workers from that colony. Duration of aggressive behaviors (any behavior other than antennation including biting, pulling, and grappling) directed toward the treated ant was recorded and analyzed. Duration of aggression toward workers treated with (*R*)-3-MeC27 did not differ

from duration of aggression toward nestmate controls or other treatments. However, the aggression was reduced relative to the foreign control, *n*-pentane, *n*-nonacosane, 3-MeC27, and (S)-3-MeC27, and was significantly less than the pentane control. These results support the role of 3-MeC27 as a releaser pheromone that interferes with nestmate recognition and suggest that there may be differences in response to the stereoisomers.

Introduction

Broadly across social animals, signaling has evolved to indicate information about an animal, disseminate information about a particular situation or context, or organize groups of individuals (Bradbury and Vehrencamp 2011). Signaling modalities can span visual, acoustical, and chemical modalities. To the human observer, some modalities may be more conspicuous than others, but even those that are easiest to observe may have a cryptic function if any at all. This is particularly the case for chemical signaling, where compounds may differ not only in their chemical composition but their conformational configuration as well. The many degrees of freedom on which chemical signaling mechanisms may be built makes them ideal candidates for studying communication in tightly integrated social groups with highly functional olfactory systems.

Advanced social species such as eusocial species are particularly well-suited for studying communication and signal evolution because of the maintenance of reproductive division of labor within their colonies. Eusocial species' colonies are divided into reproductive and non-reproductive castes, which are maintained using varying modes of communication (Hölldobler and Wilson 2009; Hölldobler and Wilson 1990; Bourke and

Franks 1995). In eusocial Hymenoptera species, one or several reproductive queens produce all of the offspring for the colony while the non-reproductive caste of workers performs all other colony tasks (Hölldobler and Wilson 2009). However, workers of many eusocial Hymenoptera species have retained the ability to activate their ovaries and produce viable, typically male-destined eggs. This occurs when a colony's queen dies or is artificially removed from the colony (Bourke 1988; Hölldobler and Wilson 2009; Hölldobler and Wilson 1990). The ability of the workers to produce viable, male-destined eggs, which can lead to decreased colony efficiency (Cole 1986; Hartmann et al. 2003; Hillesheim et al. 1989; Landolt et al. 1977; Pamilo 1991), has resulted in the evolution of both restorative mechanisms for addressing cheating in colonies and preventative mechanisms that allow the queen to communicate her presence and fertility to the rest of the colony (Peeters and Liebig 2009; Hart et al. 2004; Bourke 1988; Wenseleers et al. 2004; Smith and Liebig 2017). The restorative mechanism used to correct for cheating workers varies by species but often includes either direct policing of workers that have activated their ovaries or the culling of worker-laid eggs (Smith et al. 2012; Smith et al. 2009; Endler et al. 2004). In addition to the evolution of mechanisms to address cheating individuals within a colony, some species have evolved maintenance mechanisms, which often include the presence of queen-specific cuticular hydrocarbons that are present on both the queen and her eggs and play a role in the colony as both a releaser and a primer signaling the presence of a fecund queen to workers reducing the likelihood that they will activate their ovaries to produce sons rather than rearing their

siblings (Smith and Liebig 2017; Van Oystaeyen et al. 2014; Peeters and Liebig 2009; Liebig 2010).

In *Camponotus floridanus*, a eusocial, monogynous ant species native to the southeastern United States, the queen-specific hydrocarbons present on the cuticle of the queen and the queen-laid eggs regulate worker reproduction and indicate the presence of the queen to facilitate workers tending to and grooming her (Endler et al. 2007, 2006, 2004; Moore and Liebig 2010, 2013). The queen-specific compounds present on *C. floridanus* queens inhibit worker production of male-destined eggs when presented to queenless worker groups on eggs, and its absence on worker-laid eggs leads to worker culling of the eggs (Endler et al. 2004).

The dominant component of the queen-specific hydrocarbon profile is 3-methylheptacosane (3-MeC27) (Endler et al. 2004). 3-methylheptacosane has yet to be tested independently of the other component parts of the queen-specific hydrocarbon profile to determine if it is sufficient on its own to act as a releaser or primer pheromone within the colony or if other components of the queen-specific profile are necessary. Because 3-MeC27 is a methyl-branched hydrocarbon it exists in two stereoisomeric forms occurring as either the (*R*)- or (*S*)-enantiomer. There is evidence in other insect species that these enantiomers can be detected and even play different roles in the communication system of the organism (De Narbonne et al. 2016; Mori 1998, 2007; Wood et al. 1976; Yu et al. 2014).

Camponotus floridanus workers are able to distinguish between the enantiomers of the 3-MeC27 compound (Sharma et al. 2015). Although the (*R*)-enantiomer is the most commonly occurring stereoisomer of methyl-branched cuticular hydrocarbons in insects (Bello et al. 2015) most studies that examine the role of queen cuticular hydrocarbons in regulating worker reproduction use a racemic mixture of queen-specific compounds rather than the enantiomerically pure compounds (with the exception of work done with *Lasius niger* by De Narbonne et al. (2016)).

Previous work has demonstrated that the queen specific compounds present on the cuticle of *C. floridanus* queens can not only inhibit worker reproduction but also serve as a releaser pheromone thereby initiating or deterring particular behaviors in response to a queen. Moore and Liebig (2010) showed that a queen of an incipient *C. floridanus* colony will receive aggression after being introduced into an established foreign colony. However, workers of mature colonies do not express aggression toward highly fertile foreign queens that express larger amounts of the queen-specific hydrocarbons relative to a queen of an incipient colony (Moore and Liebig 2010). The lack of aggression toward a highly fertile queen is likely due to the potential cost to the colony if workers mistakenly attack their own queen. It is unlikely that a mature colony will encounter a foreign, highly fertile queen in natural situations, and so the risk of attacking their own queen outweighs the potential benefit of behaving aggressively toward a foreign queen. However, an incipient queen is easier to distinguish from a mature colony's own highly fertile queen due to differences in abundance of the queen-specific cuticular hydrocarbons resulting in

a lower risk of mistaking the colony's queen as a foreign queen. Here we investigate the specific role of the queen-specific cuticular hydrocarbons, particularly 3-MeC27, as a releaser pheromone. We tested the hypothesis that 3-MeC27 signals queen presence to *C. floridanus* workers as a releaser compound by reducing aggression toward a foreign individual while also testing the effectiveness of the enantiomerically pure forms of 3-MeC27 and the racemic mixture as a releaser pheromone. Because it has been demonstrated that context is important for the signal to be interpreted correctly by the workers, we chose to present 3-MeC27 to workers in the context of a worker-profile background albeit a foreign worker in order to replicate the study methods used by Moore and Liebig (2010).

Materials and Methods

Animals: Mature colonies of *Camponotus floridanus* collected as incipient colonies from the Florida Keys were used for this study. *Camponotus floridanus* are composed of one queen and major and minor worker castes. Major and minor workers are easily distinguishable from each other based on size and morphology. Majors are larger with a squarer-shaped head than minors. All colonies were paired within the population they were collected from. Population 1 occurs north from Duck Key, and population 2 occurs south from Grassy Key. Colonies were housed in a USDA regulated facility at Arizona State University, Tempe, AZ inside plastic containers (38.1 cm long x 25.4 cm wide x 8.26 cm deep) with dental-plaster floors and molded cavities covered by glass. Sides of the plastic containers were coated with Fluon, an industrial lubricant, to

prevent ants from escaping. A gasket sealed lid with a mesh-covered opening was placed on top of the plastic containers to ensure ants did not escape. Colonies were fed pre-killed beetle larvae, *Zophobas morio*, and agar-based protein and carbohydrate cakes (Bhatkar & Whitcomb 1970) twice weekly in addition to receiving water and 20% sugar water.

Behavior Assays: Colonies were randomly paired within their respective population (population 1 occurring north of Duck Key and population 2 occurring south from Grassy Key). Colonies were paired within in their populations to control for any hydrocarbon differences between populations. All assays were two-directional for each pairing (i.e., each colony had majors treated with all hydrocarbon treatments and introduced to the other colony). Behavior assays were conducted inside the colony's nest where a circular observation ring (8.4 cm diameter x 3.5 cm high) coated on the inner and outer surfaces with Fluon, a liquid lubricant that prevents climbing, was placed on top of the red acetate and glass covering the nest cavity. For each trial a new set of 4 majors and 8 minors was isolated in the observation ring within their colony, where they were allowed to acclimate for 3 minutes prior to the introduction of the hydrocarbon treated ant. The treated major ant received one of six treatments: (1) nestmate control (negative control), (2) foreign control (positive control), (3) *n*-pentane treated foreign major (controls for the use of pentane to dissolve the other hydrocarbon compounds), (4) *n*-nonacosane (c29) treated foreign major (controls for the alteration of the worker cuticular hydrocarbon profile), (5) 3-methylheptacosane (3-MeC27) treated foreign major (tests the effect of the racemic mixture of 3-MeC27), (6) (*R*)-3-methylheptacosane (*R*)-3-MeC27)

treated foreign major (tests the effect of the (*R*)-3-MeC27 enantiomer), (7) (*S*)-3-methylheptacosane (*S*-3-MeC27) treated foreign major (tests the effect of the (*S*)-3-MeC27 enantiomer). Following chemical application, majors were placed in a 10 mL beaker and placed on ice for one minute to chill but not anesthetize them to reduce the likelihood that the treated ant would initiate aggression when introduced into the observation ring. Assays were recorded using a Samsung Galaxy smartphone or an Apple iPhone. Recording of the assay began with the recording of an index card with a random alphanumeric id number used later to blind the researcher to the treatment during analysis followed by the introduction of the treated major into the observation ring. The treated major was introduced into the observation arena in an area less populated with ants to avoid touching subject ants during the introduction of a new individual. Assays were recorded for 2 min and 30 s while ensuring no vibrations occurred to the table or nest that may have influence the behavior. The first 30 s were not analyzed to allow for a short acclimation time to any disturbance from the introduction of a new individual into the observation arena. During the behavior assay, the experimenter wore a face mask to avoid breathing on the workers, which can cause agitation in ant colonies due to the introduction of CO₂ (personal observation). At the conclusion of the trial, the treated ant was removed and euthanized by being placed in a -30°C freezer. The 12 ants in the arena were removed and kept isolated from the colony until all trials with that colony were finished to prevent use of individual ants in multiple trials. Any assays where aggression was initiated by the focal were excluded from analysis.

Chemical Application: The (*R*)-3-MeC27 and (*S*)-3-MeC27 were synthesized by Dr. Jocelyn Millar at the University of California Riverside, Riverside, California. The racemic mixture of 3-MeC27 was created by mixing equal parts (*R*)-3-MeC27 to (*S*)-3-MeC27. For experiments, the amount of chemical added to the cuticle of the focal ants was determined by use of gas chromatography to calculate the ratio of 3-MeC27 relative to the colony profile compounds found on queen-laid eggs sampled from 6 queenright *C. floridanus* colonies. The total area under the major hydrocarbon peaks (*n*-nonacosane, *n*-triacontane, *n*-hentriacontane, *n*-dotriacontane, *n*-tritriacontane) present on the sampled queen-laid eggs was calculated from the gas chromatography results. Then, the average ratio of (*R*)-3-MeC27 to the summed area was calculated for the six queen-laid egg samples and divided by 1.5 to amplify the fertility signal. The signal was amplified to more accurately estimate the amount of (*R*)-3-Me-C27 found on the cuticle of mature queen without sacrificing queens to calculate the actual amount. The calculated amount of 3-MeC27 on single *C. floridanus* egg after amplification is 0.026 µg. For this study, we used 10.4µg of all hydrocarbons, which is equivalent to the amount present on 400 eggs. We do not know the amount of 3-MeC27 present on a highly fertile queen and did not have queens available to sacrifice to determine this, and so this amount was chosen based on positive behavior responses observed during preliminary trials.

All solutions were mixed in a concentration of 2 µg hydrocarbon: 1µl *n*-pentane to reduce the amount of *n*-pentane to which ants were directly exposed. Six majors were randomly selected from the foreign queenright colony paired with the host colony. One

additional major was selected from the colony that would host the behavior assays. Five of the foreign majors had 5.2 μ l (10.4 μ g of hydrocarbon) of the respective chemical treatment applied directly the dorsal side of their heads, thoraxes, and gasters using a 10 μ L microsyringe. Neither nestmate control nor foreign control majors had chemical compounds applied to their cuticle. Ants were then placed in individual, clean 10 mL beakers with labeled aluminum foil covering the opening. The unmanipulated control ants were also placed in a beaker to control for time spent in a beaker and outside of the colony. Ants remained in the beakers to recover and groom themselves for 45 min prior to beginning the behavioral assays.

Video Analysis: Videos were randomized by changing the file name to the trial code. This ensured JE was blind to the treatments and prevented her from detecting any patterns as she scored the videos. Behavior assays were scored for duration of trial where the focal ant was aggressed in one of the following ways. Aggression was considered any behavior other than antennating and included pulling, biting, mandibular holding, and grappling. Antennating was defined as the tapping of an individual's antennae against the focal ant. Pulling was scored when a worker moved the focal ant by closing her mandibles around one of the focal ant's body parts then dragging the focal ant while walking. A worker was scored as biting if she repeatedly closed her mandibles around a body part of the focal ant then opened her mandibles again in succession without other behaviors occurring between incidences. Mandibular holding occurred when a worker closed her mandibles around a body part of the focal ant and remained in that position for

more than one second. Grappling was scored if a worker closed her mandibles around the petiole of the focal ant and the focal ant reciprocated resulting in a wrestling-style movement. These data were recorded using the open source software BORIS (Friard and Gamba 2016). Although duration of each behavioral event was recorded, the duration of the behavior assay was converted into the proportion of time when any aggressive behavior occurred for analysis.

Data Analysis: All data were analyzed in RStudio (Rstudio Team 2018) using a linear mixed-effects model from the lme4 (Bates et al. 2015) and lmerTest (Kuznetsova et al. 2017) packages. The data, proportion of time during which focal ant received aggression, were logit transformed to achieve normality and analyzed using a linear mixed effects model with treatment as a fixed effect and colony id as a random effect. A general linear hypotheses post hoc test for linear mixed-effect models from the multcomp package (Hothorn et al. 2008) was used following the initial analysis to explore pairwise relationships between treatments.

Results

Of the 140 trials run, five trials were excluded from the analysis due to aggression initiated by the focal ant. The main effect of treatment had a significant effect on the proportion of the behavior assay during which the subject ants directed acts of aggression at the focal ant (linear mixed-effects model: $n = 160$, $F = 6.76$, $df = 6$, $p < 0.001$; Figure 3). A general linear hypotheses post hoc test (glht) for linear mixed-effects models (Hothorn et al. 2008) was used to investigate pairwise treatment differences. The

nestmate control treatment ($n = 22$, $\bar{x} = 0.31$) had a significantly smaller proportion of the behavior assays during which aggression occurred than the (*S*)-3-MeC27 treated foreign major treatment ($n = 22$, $\bar{x} = 0.69$, glht: $z = 4.19$, $p < 0.001$), 3-MeC27 treated foreign major treatment ($n = 22$, $\bar{x} = 0.76$, glht: $z = 4.44$, $p < 0.001$), nonacosane treated foreign major treatment ($n = 22$, $\bar{x} = 0.72$, glht: $z = 4.53$, $p < 0.001$), *n*-pentane treated foreign major treatment ($n = 22$, $\bar{x} = 0.80$, glht: $z = 5.30$, $p < 0.001$), and foreign control treatment ($n = 27$, $\bar{x} = 0.73$, glht: $z = 4.66$, $p < 0.001$). However, the proportion of the behavior assay when aggressive acts occurred toward the focal ant was not significantly different between the nestmate control treatment ($n = 22$, $\bar{x} = 0.31$) and the (*R*)-3-MeC27 treatment ($n = 23$, $\bar{x} = 0.52$, glht: $z = 2.36$, $p = 0.21$).

Although there was no difference in aggression directed toward the focal ant between the nestmate control and the (*R*)-3-MeC27 treated foreign major treatments, the (*R*)-3-MeC27 treated foreign major treatment ($n = 23$, $\bar{x} = 0.52$) also did not differ significantly in the amount of aggression received by the focal ant from the foreign control ($n = 27$, $\bar{x} = 0.73$, glht: $z = 2.24$, $p = 0.27$), (*S*)-3-MeC27 treated foreign major ($n = 22$, $\bar{x} = 0.69$, $z = 1.87$, $p = 0.27$), 3-MeC27 treated foreign major ($n = 22$, $\bar{x} = 0.76$, $z = 2.12$, $p = 0.34$), or nonacosane treated foreign major ($n = 22$, $\bar{x} = 0.72$, $z = 2.21$, $p = 0.29$) treatments. However, the proportion of the behavior assay during which aggression occurred was significantly lower in the (*R*)-3-MeC27 treated foreign major ($n = 23$, $\bar{x} = 0.52$) than the *n*-pentane treated foreign major treatment ($n = 22$, $\bar{x} = 0.80$, $z = 2.99$, $p = 0.045$).

Discussion

The addition of (*R*)-3-MeC27 to *C. floridanus* majors reduces the level of aggression elicited by non-nestmates; when the treated major is introduced into a foreign nest, the level of received aggression is not significantly different to levels experienced by a nestmate that is temporarily removed and then reintroduced into the colony (Figure 3).

The levels of aggression encountered by (*R*)-3-MeC27-treated foreign majors are apparently intermediate – not significantly different than aggression levels toward either a nestmate or foreign control major – whereas the nestmate control did differ significantly in the level of aggression received compared to the foreign control, *n*-nonacosane treated foreign major, *n*-pentane treated foreign major, 3-MeC27 treated foreign major and (*S*)-3-MeC27-treated foreign major. These findings support the role of (*R*)-3-MeC27 as a key component of the queen-specific cuticular hydrocarbons in the ant *Camponotus floridanus*. Further study is needed to clearly determine whether both isomeric forms of the methyl-branched hydrocarbon work equally well as a releaser pheromone or if the (*R*)-enantiomer is the most effective form of the methyl-branched hydrocarbon given the ability of workers to distinguish between the isomers demonstrated by Sharma et al. (2015).

Previous work has shown that *Camponotus floridanus* queens possess a queen-specific cuticular hydrocarbon profile that serves as both releaser and primer pheromones within the colony and help to maintain reproductive division of labor within the colony.

These results corroborate the findings by Moore and Liebig (2010) demonstrating that colonies accept foreign, mature queens, which have larger amounts of 3-MeC27 on the cuticle than incipient queens that received aggression when placed in foreign colonies. The isolation of 3-MeC27 in this study suggests that this compound, when coupled with the context of a *C. floridanus* colony profile, elicits similar results as those found by Moore and Liebig (2010). These findings support previous work showing that a single, methyl-branched alkane is sufficient (Van Oystaeyen et al. 2014) to act as a releaser pheromone when in the context of the colony profile (Smith et al. 2015).

The effectiveness of 3-MeC27 as a primer pheromone in *Camponotus floridanus* still needs to be confirmed with future work. However, this work validates previous work showing that the queen-specific hydrocarbons present on the cuticle of *C. floridanus* queens serve as a fertility signal in this eusocial species. Furthermore, it provides evidence that reproductive signaling may have evolved to be sensitive to stereochemistry, providing yet another degree of freedom on which communication can diversify in social insects.

Acknowledgments

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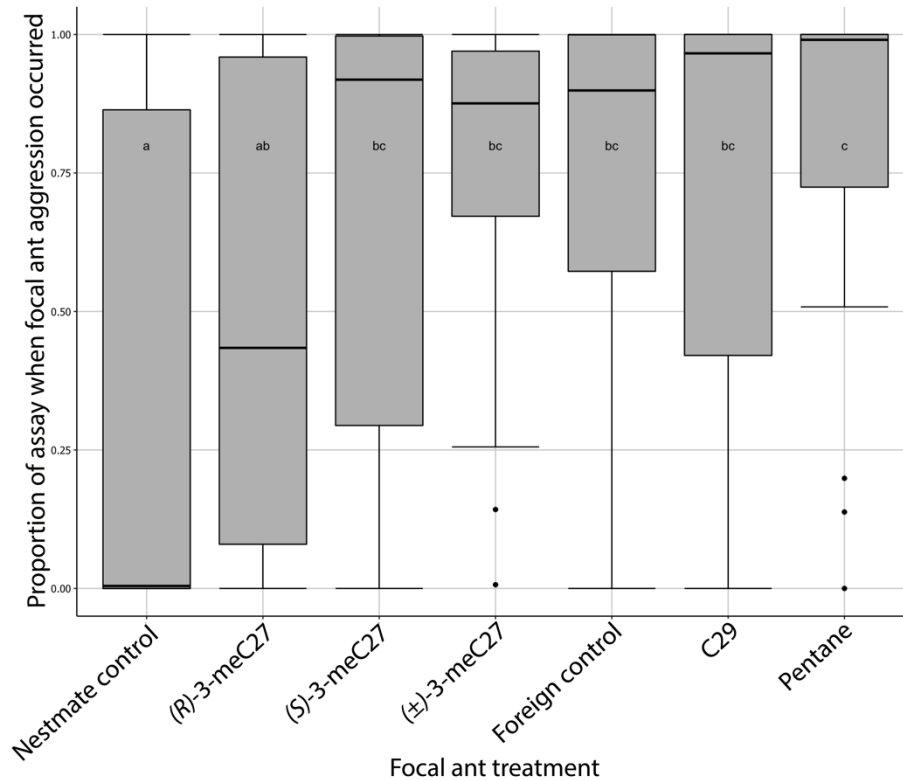


Figure 3. Comparison of aggression directed at foreign workers with manipulated cuticular hydrocarbon profile. The proportion of the time aggression was directed at a foreign major treated with (*R*)-3-MeC27, (*S*)-3-MeC27, 3-MeC27 (racemic mixture), C29, pentane, or remained untreated receive directed aggression compared to a nestmate control (linear mixed-effects model: $n = 160$, $F = 6.76$, $df = 6$, $p < 0.001$). The horizontal bar in the box represents the median proportion of time aggression directed at a foreign major occurred. The lower and upper edges of the box represent the 25th and 75th quartiles, and the whiskers represent the 1.5 interquartile range. Data falling outside of the 1.5 interquartile range are represented by solid dots. Letters indicate results of a linear mixed-effects model pairwise comparison post hoc test.

CHAPTER 4
REGULATION OF WORKER REPRODUCTION IN THE POLYDOMOUS ANT

OECOPHYLLA SMARAGDINA

Abstract

The signaling of the presence of a queen in eusocial species is important in maintaining reproductive division of labor, a key characteristic of eusocial species. In polydomous species, such as *Oecophylla smaragdina*, where colonies are spread between multiple satellite nests the queen's presence must be communicated over long distances. We studied the possible mechanisms used by *O. smaragdina* queens as releaser pheromones within the colony, which can provide insight into potential primer pheromones as well. We found that queen cuticular hydrocarbons and queen volatile hydrocarbons released an attractant response from workers. This response was also observed when the cuticular hydrocarbons presented to the workers were from a foreign queen. Analysis of the hydrocarbons has shown that the cuticular hydrocarbon profile of the queen and workers are distinct although the volatile pheromones released by the queen and workers are indistinguishable from each other. These results provide insights into long-range communication by indicating potential mechanisms, queen-specific cuticular hydrocarbons, that may also be present on *O. smaragdina* queen-laid eggs and may be used to distribute the queen signal throughout a polydomous colony while also adding to the growing body of literature examining how the reproductive division of labor is maintained in eusocial insects.

Introduction

Long-range communication in animals requires adaptations in communication modalities, such as acoustic, visual, and chemical, that allow for a sustained signal that can be reliably perceived by the receiver from large distances. Challenges with long-range communication include preventing signal decay at an insufficient distance and allowing for signal detection in a noisy environment (Brumm and Slater 2006; Fleishman 1988; Naguib and Wiley, 2001; Natu et al. 1983; Ord 2012; Ord and Stamps 2008; Symonds et al. 2011). Animals that communicate across long ranges have evolved adaptations such as the use and detection of low-frequency waves in elephants (O'Connell-Rodwell 2007; Garstang 2004), real-time adjustments to the signal based on the distance to the receiver in zebra finches (Brumm and Slater 2006) and *Anolis* lizards (Fleishman 1988), and antennae adapted for detecting sex pheromones over long distances in moths (Symonds et al. 2011).

The mechanisms used for long-range communication are less well studied in eusocial insects such as polydomous ant species where a single colony may be distributed between several to over one hundred spatially separate nests that each include workers and brood. In some of these polydomous ant species, there is only a single queen residing in the colony resulting in potentially long periods of time when workers will not interact with the queen (Debout et al. 2007). Because in many species of ants workers retain their ovaries and may begin producing male-destined eggs when the queen dies, mechanisms to signal the presence of a fertile queen to the workers and regulate reproductive division

of labor have evolved (Smith and Liebig 2017; Ebie et al. 2015; Endler et al. 2004; Bourke 1988; Bourke 1999; Heinze 2004; Beekman and Ratnieks 2003). These mechanisms maintain reproductive division of labor within the colony, a key characteristic of eusocial species, into reproductive and non-reproductive castes (Hölldobler and Wilson 2009).

Queen presence is signaled to workers in many species through the production of queen specific pheromones, which are often cuticular hydrocarbons that when present inhibit workers from producing their own viable, male-destined eggs (Oi et al. 2015; Smith and Liebig 2017; Van Oystaeyen et al. 2014). Often, the queen-specific hydrocarbons are also found on the queen-laid eggs and have been shown to inhibit worker egg-laying (Endler et al. 2004). However, little is known about the mechanisms used to extend the signals that maintain reproductive division of labor over the distances occupied by colonies of polydomous species. Although the queen may possess a suite of cuticular hydrocarbons on her cuticle that signal her fertility, a mechanism that extends the range of the signal is necessary so that her presence in the colony is signaled to workers that may not encounter her for extended periods of time while in satellite nests. To date, the only work studying long-range regulation of worker reproduction was done in *Novomessor cockerelli*, known for inhabiting 1–5 nests per colony, and found that the presence of larvae, which are transported between satellite nests, delays worker egg laying (Ebie et al. 2015).

Although queen-specific hydrocarbons have been shown to inhibit worker reproduction in some species, other mechanisms such as worker policing of reproductive or potentially reproductive workers (Liebig et al. 1999; Helanterä and Sundström 2007; Kikuta and Tsuji 1999; Smith et al. 2009), culling of worker-laid eggs (D'Ettorre et al. 2004; Endler et al. 2004), and egg and larval inhibition are present in other species (Ebie et al. 2015; Endler et al. 2004; Heinze et al. 1996; Mamsch 1967; Villalta et al. 2015; Warner et al. 2016) and may facilitate long-range communication in polydomous species. The variation in mechanisms regulating worker reproduction in single-nest ant species indicates that the mechanisms for regulating worker reproduction in polydomous species may also be diverse and vary between species. This is especially likely given the large variation in the number of satellite nests inhabited by polydomous colonies across species.

Oecophylla smaragdina is an arboreal, polydomous species of ant native to Australasia known for using silk produced by their larvae to weave together tree leaves forming nests. They can inhabit one to upwards of ten mature trees and have been documented as having 500,000 workers and well over 100 nests per colony with many containing all developmental stages of brood in addition to major and minor workers (Andersen 1992; Cole Jr. and Jones Jr. 1948; Hölldobler 1983; Hölldobler and Wilson 1977; Lokkers 1990; Marcela et al. 2012; Peng et al. 1998). *Oecophylla smaragdina* populations in the Northern Territory, Australia have been documented as being polygynous, but monogyny appears to be the dominant colony structure in other studied

populations of this species (Peng et al. 1998; Schlüns et al. 2009). Workers in this species have retained their ovaries and regularly produce trophic eggs to nourish the larvae and queen (Hölldobler and Wilson 1983). Previous work has shown that artificially removing the queen from the colony results in workers transitioning to the production of viable, male-destined eggs after one to two months (Hölldobler and Wilson 1983). Additionally, we have observed the production of males in most satellite nests of wild colonies in Townsville, Queensland, Australia when revisiting colonies that queens were removed from four months earlier (personal observation). The ability of these workers to produce male offspring when the queen is absent from the colony, suggests that there is a mechanism in place that signals the presence of the fertile queen over the long distances spanned by the colony and its many satellite nests. Results from Hölldobler and Wilson (1983) demonstrated that even the presence of a deceased queen is sufficient to inhibit worker egg laying for at least 3.5 months, which suggests that the mechanism could be a queen specific pheromone present on the queen's cuticle that may also be present on the queen-laid eggs. The distribution of queen-laid eggs throughout the colony's many satellite nests may distribute the signal indicating the queen's presence similarly to the dispersal of the queen mandibular pheromone in honeybee colonies (Seeley 1979) and inhibit worker egg laying as it does in *Camponotus floridanus* (Endler et al. 2004). In addition to serving as a primer within the colony, the queen-specific hydrocarbons may also function as a releaser pheromone causing a clustering of workers to gather around the queen forming a large retinue or cluster around the queen (Hölldobler and Wilson

1983; Van Itterbeeck et al. 2015). Here we test the potential role of queen cuticular and volatile hydrocarbons as a signal of the queen's presence in the colony in the form of a releaser. Specifically, we test whether queen-specific odorants, cuticular hydrocarbons and volatile compounds, are attractive to workers

Materials and Methods

Study animals: Mature, monogynous, queenright *O. smaragdina* colonies were collected from trees in residential areas of Townsville, Queensland, Australia. For the initial set of choice assays, colonies were housed in a lab at James Cook University, Townsville, Queensland, Australia in artificial trees constructed from a vertically oriented wooden dowel rod with small tubes connects to it with electrical tape. The artificial trees were contained in plastic bins coated with vegetable oil and talcum powder, which prevented escape. Colonies were fed pre-killed crickets, *Gryllus sp.*, twice weekly in addition to receiving water and 20% sugar water *ad libitum*.

Colonies used in the fiber choice assays consisted of queenright colonies that were transported to Arizona State University, Tempe, Arizona, USA where they were housed in a USDA regulated facility in artificial fabric trees contained in plastic bins coated with vegetable oil and talcum powder. Colonies were housed at 75% humidity and 25°C, and they were fed pre-killed crickets, *Gryllus sp.*, twice weekly in addition to receiving water and 20% sugar water *ad libitum*.

Odorant Binary Choice Assays:

Treatments: Worker groups were presented with a choice between two odorants. The odorant pairs were kept consistent creating 10 treatments. The negative control for all pairs was either the odorant of 10 nestmate workers or no odorant. The negative controls were paired with the odorant of a foreign queen and 10 of her workers to test for the attractiveness of the smell of a queen, 10 foreign workers to serve as a positive control, or one of the three developmental stages of brood (eggs, larvae, or pupae) to test for an odorant that may be present on the brood and serve to extend the queen signal throughout the colony. The specific binary pairings were (1) the odorant of a foreign queen plus 10 foreign workers versus no odorant, (2) the odorant of a foreign queen plus 10 foreign workers versus the odorant of 10 nestmate majors, (3) the odorant of 100 foreign, queen-laid eggs vs no odorant, (4) the odorant of 100 foreign, queen-laid eggs versus odorant of 10 nestmate majors, (5) the odorant of 100 foreign, queen-produced larvae vs no odorant, (6) the odorant of 100 foreign, queen-produced larvae versus odorant of 10 nestmate majors, (7) the odorant of 10 foreign, queen-produced pupae vs no odorant, (8) the odorant of 10 foreign, queen-produced pupae versus odorant of 10 nestmate majors, (9) the odorant of 10 nestmate majors vs no odorant, (10) the odorant of 10 foreign majors versus odorant of 10 nestmate majors.

Odorant treatments were created by placing the foreign queen and 10 workers, 100 eggs, 100 larvae, 10 pupae, 10 nestmate major workers, 10 foreign major workers, or no odorant inside of the 300 mL glass mason jar (top diameter: 6.5 cm, bottom

diameter: 7.5 cm) on top of a 7 cm plastic disk wrapped in plastic wrap for one hour to allow for the collection of odorants on the plastic wrap. After an hour, ants and brood were removed from the jars. Jars that had a queen in them were checked for eggs that may have been laid during the hour, and any eggs that were found were removed. All jars were sealed with plastic wrap, with the exception of a small 1 cm x 1 cm hole cut into the center of the plastic wrap seal, and immediately presented to workers for the choice assays.

Behavioral choice assays: Workers were collected from a queenright colony nest containing brood and brought into the lab on the day of the behavioral assay, and a new colony was used for each replicate. One thousand workers were randomly selected from the colony and distributed into groups of 100 (25 minors and 75 majors) using stratified randomization. An additional 50 workers were collected from the same nest to create each of the worker-only treatments of 10 workers per treatment.

For each replicate, 100 workers were placed into the center of a 30 L plastic bin (33 cm wide x 44 cm long x 21.5 cm tall) that served as the behavioral choice arena. Inside of the arena were two identical 300 mL glass mason jars each containing one of the 10 treatments. The jars were located equidistant from the center of the arena and covered with plastic wrap containing a small 1 cm x 1 cm hole to enable workers to enter and exit the jars. Once the workers were introduced into the bin, they were given 24 h to make a choice between the two jars. Ants were considered to have chosen a jar if they were located inside of the jar at the end of the 24 h period when the number of ants

located inside each jar and the number of ants that did not select a jar were counted. Each colony received each of the 10 treatments, and the room location of the arena that contained each treatment was randomized for each colony to control for any effects of location inside the room. Additionally, the side of the arena where each treatment was located was alternated to control for preference in arena side. All jars and arenas were cleaned with unscented dish soap, deionized water, and hexane (glass jars only) between trials.

Data analysis: The data were analyzed in RStudio (Rs. Team, 2018) with a mixed effect binomial logistic regression with treatment as a fixed effect and colony, queen colony, and behavior arena as random effects using the glmmTMB package (Brooks et al. 2017).

Cuticular and Volatile Hydrocarbon Binary Choice Assays:

Cuticular hydrocarbon collection: Queen hydrocarbon profiles were collected by stroking the gaster of fertile queens with the fiber of a polydimethylsiloxane (film thickness: 30 μ m, pH: 2–11, maximum temperature: 280°C, Supelco) solid phase microextraction fiber (SPME fiber) for 3 minutes while rotating the fiber to ensure maximum extraction. Worker hydrocarbon profiles were collected using the same process with major workers from the same colony as the sampled queen. One fiber was used to sample five workers for 3 min/worker to ensure abundance was similar between the queen and worker treatments.

Volatile hydrocarbon collection: The volatile hydrocarbons released by a queen and 10 major workers or the volatile hydrocarbons released by 10 major workers were collected using 24 h headspace collection where the ants were placed into a clean 250 mL glass beaker and isolated to the bottom half of the beaker using a wire mesh disk. The beaker was covered with aluminum foil, and two polydimethylsiloxane (film thickness: 30 μ m, pH: 2–11, maximum temperature: 280°C, Supelco) SPME fibers were inserted through the aluminum and extended into the beaker in the space above the wire mesh disk for 24 h. One of the two SPME fibers was used for the behavioral assay, and the other was used for GC-MS compound analysis.

Binary choice assays: Fifty major workers were randomly selected from the same colony as the sampled queen. Workers were placed in a -30°C freezer for one minute to slow them down and make transfer into the petri dish that served as the behavioral assay arena possible. Workers were placed inside of a Pyrex 150 mm x 15 mm glass petri dish with a glass lid. The base of the petri dish was covered with a 140 mm piece of filter paper to reduce transfer of any chemicals from the workers to the petri dish. The petri dish was washed with Alconox soap and deionized water then rinsed with hexane between trials. The filter paper was refreshed for each trial. After being placed in the petri dish, workers were left undisturbed for 10 min to allow for acclimation.

The petri dish had two small holes just larger in diameter than the SPME fibers drilled into the sides of the dish at the point of the largest diameter of the dish. Following the 10 min acclimation period for the workers, the fibers were inserted into the dish and

opened to expose the workers to the collected hydrocarbon profiles. Workers were given the choice between the SPME fiber with the queen cuticular hydrocarbon profile and the SPME fiber with the worker cuticular hydrocarbon profile or the SPME fiber with the worker volatile hydrocarbons and the SPME fiber with the queen and worker volatile hydrocarbons. The location of the two hydrocarbon treatments were alternated between trial to control for any room stimuli that may have caused the workers to explore one side of the arena more than the other. The workers were video recorded from directly above for 3 min and a random alphanumeric code was assigned to enable the videos to be analyzed by a blind observer. Following the behavior assay, SPME fibers were analyzed using an Agilent 6980N series gas chromatograph (GC) containing a DB-1MS non-polar capillary column (30 m \times 0.25 mm \times 0.25 μ m) connected to an Agilent 5975 mass spectrometer. For analysis the injection port was held at 250 $^{\circ}$ C, and the transfer line was held at 300 $^{\circ}$ C. The initial GC column temperature was held at 60 $^{\circ}$ C for 2 minutes and then increased to a maximum of 300 $^{\circ}$ C at a rate of 10 $^{\circ}$ C/min and held at the maximum temperature for 20 minutes. Helium was used as the carrier gas at a rate of 1 mL/min.

Data analysis: Videos were analyzed by converting them into an image stack using Adobe Photoshop software and then analyzed in Fiji (Schindelin et al. 2019). Using Fiji, a 20 mm x 10 mm rectangle centered around each of the fibers was overlaid on the images. ImageJ was then used to measure pixel density inside of the rectangle for each image. Because the filter paper was white, the program was able to easily distinguish between the presence or absence of workers surrounding the fiber using pixel density.

Mean pixel density for the treatments was analyzed in RStudio (Rstudio Team 2018) using a paired t-test for both the volatile and cuticular hydrocarbon behavioral assays. Hydrocarbons collected from the cuticula of ants and from headspace were tentatively identified using mass spectra and retention times. Non-metric multidimensional scaling using Bray-Curtis distances with the package *vegan* (Oksanen et al. 2019) in RStudio (Rstudio Team 2018) was used to analyze these data.

Results

Odorant binary choice assays:

The foreign queen odorant versus no odorant treatment had a significant effect on the jar choice workers made (mixed effect binomial logistic regression: $n = 11$, $z = 2.67$, $p = 0.0077$) where $\bar{x} = 68$ workers chose the foreign queen odorant treatment and $\bar{x} = 1$ worker chose the no odorant treatment. The foreign queen odorant versus nestmate workers treatment also had a significant effect on the jar choice workers made (mixed effect binomial logistic regression: $n = 11$, $z = 2.30$, $p = 0.022$) where $\bar{x} = 69$ workers chose the foreign queen odorant treatment and $\bar{x} = 4$ workers chose the no odorant treatment. Worker choice was not significantly different in any of the remaining comparisons (mixed effect binomial logistic regression; foreign, queen-laid eggs odorant vs no odorant: $n = 11$, $z = 0.82$, $p = 0.41$; foreign, queen-laid eggs odorant vs nestmate odorant: $n = 11$, $z = 1.27$, $p = 0.21$; foreign, queen-produced larvae odorant vs no odorant: $n = 11$, $z = -0.62$, $p = 0.53$; foreign, queen-produced larvae odorant vs nestmate odorant: $n = 11$, $z = -1.27$, $p = 0.20$; foreign, queen-produced pupae odorant vs no: $n = 11$,

$z = -0.71, p = 0.48$; foreign, queen-produced pupae vs nestmate odorant: $n = 11, z = -1.15, p = 0.25$; nestmate odorant vs no odorant: $n = 11, z = 0.936, p = 0.35$; foreign worker odorant vs nestmate odorant: $n = 11, z = -0.85, p = 0.39$; Figure 4).

Cuticular and Volatile Hydrocarbon Binary Choice Assays:

During the cuticular hydrocarbon binary choice assays SPME fibers with queen cuticular hydrocarbon extract had significantly more workers staying within the 10 mm x 20 mm focal area surrounding the fiber for a longer duration based on mean pixel density than the SPME fibers with worker cuticular hydrocarbon extract (paired t -test: $t = -2.36, df = 9, p = 0.043$; Figure 5). Similarly, SPME fibers with queen and worker volatile hydrocarbons extracted using headspace had significantly more workers staying within the 10 mm x 20 mm focal area surrounding the fiber for a longer duration based on mean pixel density than the SPME fibers with only worker volatile hydrocarbons extracted using headspace (paired t -test: $t = -2.52, df = 10, p = 0.030$; Figure 6).

Cuticular hydrocarbons and hydrocarbons from headspace collection that accounted for 1% or greater of the total profile and were present in at least half of the samples of either the queen or worker classes were tentatively identified using mass spectra and retention indices (Figures 7 and 8) and analyzed using non-metric multidimensional scaling analysis. There was a clear separation between the cuticular hydrocarbons found on the queen cuticles and the worker cuticles (non-metric multidimensional scaling, stress = 0.04; Figure 9) with shorter chain hydrocarbons found only in the workers while longer chain alkenes and alkanes were present in both the

queen and worker profiles (Figures 7 and 8). Hexacosane was present in all queen samples but absent from all worker profiles. However, no separation between groups was found between hydrocarbons collected from queen plus worker headspace and worker headspace (non-metric multidimensional scaling, stress = 0.09).

Queen egg structure:

During the collection of eggs for the binary choice assays, it was observed that physogastric queens lay eggs in long chains enclosed by a tissue sheath or attached to an adhesive post (Figure 10). This was observed most frequently when queens were housed with few to no workers. When workers were present in high numbers, the eggs were removed from the sheath and post.

Discussion

The colony structure of *Oecophylla smaragdina* provides an excellent opportunity to study long-range regulation of worker reproduction in a eusocial insect due to the large number of workers in each colony and the number of nests inhabited by a single colony. Previous work has demonstrated that *O. smaragdina* queens are highly attractive to their workers and inhibit worker reproduction when present in the colony. To understand how the signal of the queen's presence is distributed across the colony to workers that may not come into frequent contact with the queen, we first investigated the mechanism that elicits such strong behavioral responses from the workers.

Here we demonstrated that hydrocarbons present on the cuticle of the queen are sufficient to attract workers to the source even when the queen they were collected from

is a foreign queen rather than the workers' own queen, which lends strong support to the presence of queen-specific compounds that signal the queen's presence and fertility to the workers. The attractiveness of the cuticular hydrocarbons may explain the larger retinues that are observed surrounding the queen (Hölldobler and Wilson 1983; Van Itterbeeck et al. 2015) and are likely responsible for the regulation of worker reproduction when the queen is present as observed by Hölldobler and Wilson (1983). Additionally, this finding lends further support to a growing body of literature demonstrating that queen-specific hydrocarbons play a role in the maintenance of reproductive division of labor in eusocial insects (Liebig et al. 2005; Peeters and Liebig 2009; Smith and Liebig 2017; Van Oystaeyen et al. 2014). Through future work we will identify the active compounds that initiate worker attractiveness in addition to determining whether a single compound is sufficient to elicit a response as suggested by some (Holman 2018; Van Oystaeyen et al. 2014) or the active compound is only sufficient in the context of the colony recognition cues as shown in other species (Smith and Suarez 2018; Smith et al. 2015).

Although there was no significant effect of hydrocarbons from the queen-produced eggs on worker attractiveness, workers did show a stronger preference for the hydrocarbons collected from queen-laid eggs than those collected from queen-produced larvae, queen-produced pupae, and nestmate workers. This response suggests that it is still possible that the queen's signal is transmitted throughout the colony via the distribution of queen-laid eggs throughout the many satellite nests present in the colony (Marcela et al. 2012; Schlüns et al. 2009). Ongoing work is examining this potential role

of eggs as a transport mechanism for the queen-specific fertility signal as a primer pheromone.

In addition to the releaser effect of the queen cuticular hydrocarbons, there was a significant effect of the volatile pheromones released by the queen on worker attraction. However, we were unable to identify any differences between the queen-produced and worker-produced volatile compounds, and so this response may instead be due to abundance of the compound produced rather than the compound itself resulting in a threshold response. The volatile collection method did not account for potential quantitative differences rather than qualitative differences between the volatiles released by the queen and workers, and so there was a larger abundance of the compounds in the queen produced headspace samples. It is also possible that our analysis of the volatile compounds released by the queen failed to detect an active component that would explain the observed worker behavior and may be partially responsible for the formation of the worker retinue surrounding the queen while the queen-specific cuticular hydrocarbons maintain the presence of the retinue.

Our work has provided additional insights into the maintenance of reproductive division of labor in eusocial insect species with large colony sizes. Additionally, it begins to answer questions about how long-range communication that regulates worker reproduction in these large, distributed colonies where workers adjust their behavior and physiology based on the presence of one queen among a colony of up to 500,000

individuals when many of those individuals will not directly encounter the queen for extended periods of time.

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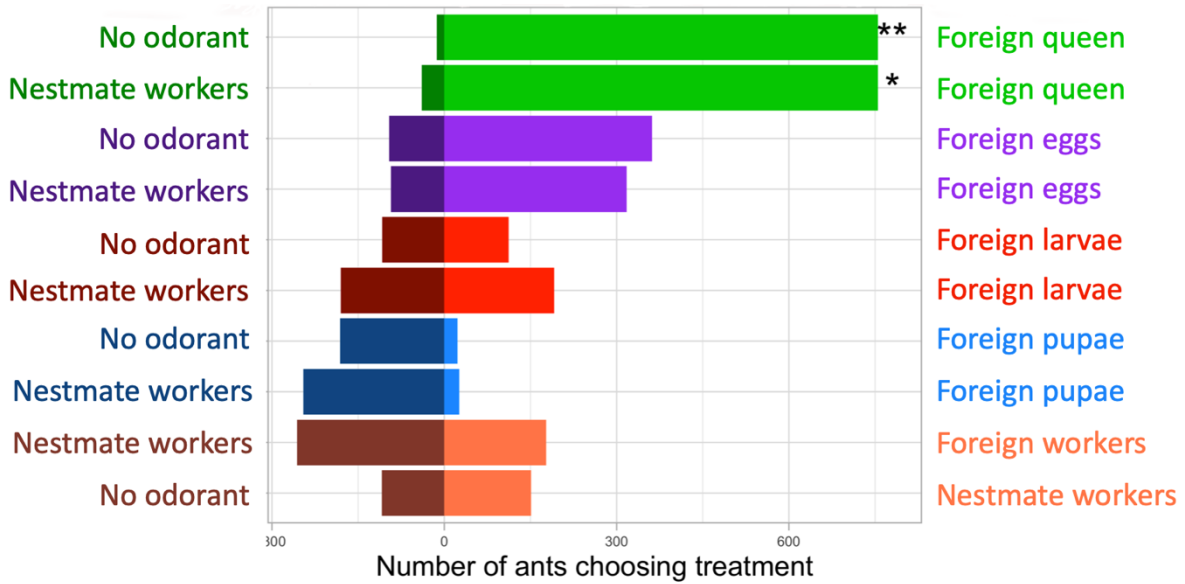


Figure 4. Comparison of worker attraction to queen odorant, worker odorant, brood odorant, and no odorant control. Queenless *O. smaragdina* worker groups given a choice between two jars containing ten different pairs of odorants. After 24 h, the location of the workers was recorded as a choice. Significantly more workers chose the jar containing a foreign queen odorant over a jar containing the odorant of nestmate workers (mixed effect binomial logistic regression: $n = 11$, $z = 2.30$, $*p = 0.022$) and the jar containing the odorant of a foreign queen over a jar with no odorant (mixed effect binomial logistic regression: $n = 11$, $z = 2.67$, $**p = 0.0077$). There was no significant effect of any other treatment on worker choice.

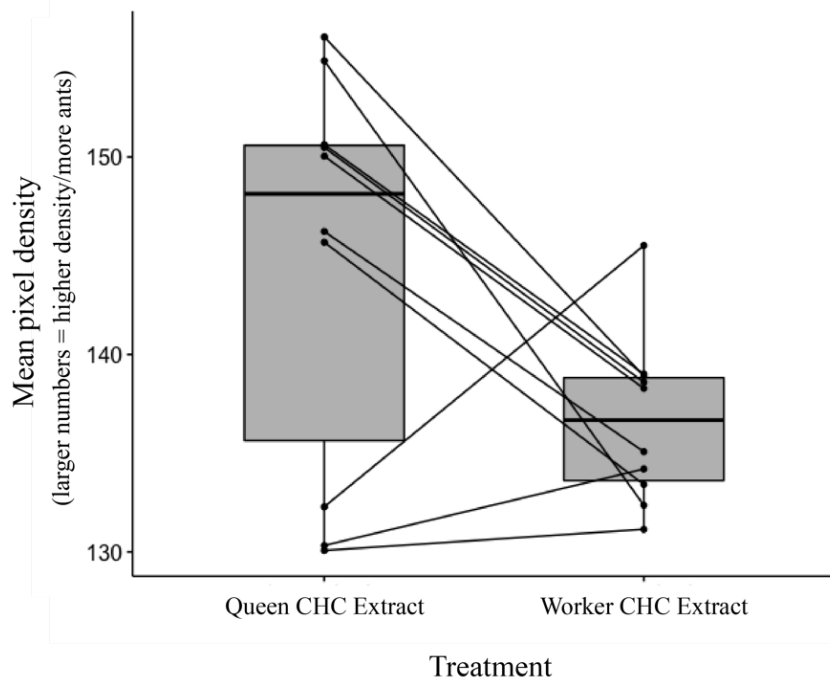


Figure 5. Comparison of worker attraction to queen vs worker cuticular hydrocarbons. *Oecophylla smaragdina* workers were presented with two solid phase microextraction (SPME) fibers. One SPME fiber contained the cuticular hydrocarbon extract of the workers' queen, and the other contained the cuticular hydrocarbon extract of five nestmate workers. During a 3 minute behavioral assay, pixel density within a 10 mm x 20 mm focal area surrounding the fiber was measured. Pixel density was significantly higher near the fiber containing the queen cuticular hydrocarbon extract (paired t -test: $t = -2.36$, $df = 9$, $p = 0.043$) indicating a larger number of workers spent a longer duration of time near that fiber. The horizontal bar in the box represents the median pixel density. The lower and upper edges of the box represent the 25th and 75th quartiles, and the whiskers represent the 1.5 interquartile range. Lines are used to connect paired data points.

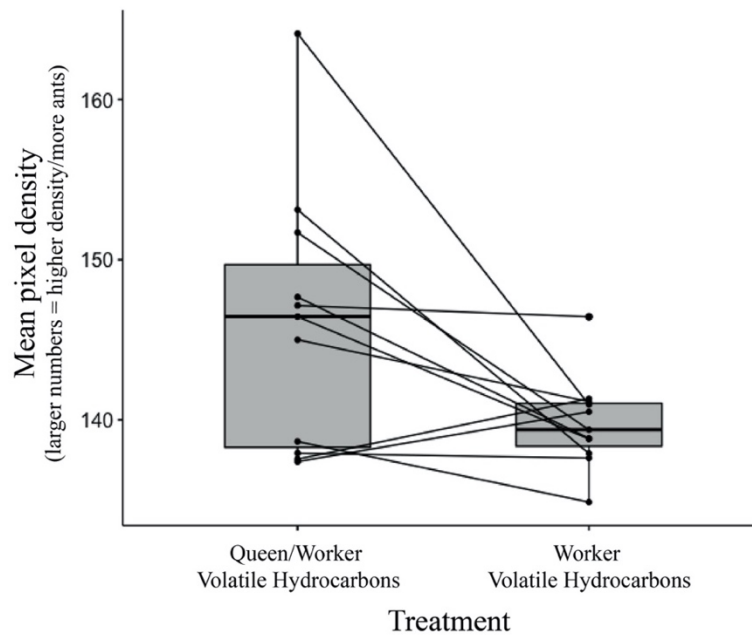


Figure 6. Comparison of worker attraction to queen plus worker volatiles vs worker volatiles. *Oecophylla smaragdina* workers were presented with two solid phase microextraction (SPME) fibers. One SPME fiber contained the volatile hydrocarbons released by the workers' queen and 10 of their nestmates, and the other contained the volatile hydrocarbons released by 10 nestmate workers. During a 3 minute behavioral assay, pixel density within a 10 mm x 20 mm focal area surrounding the fiber was measured. Pixel density was significantly higher near the fiber containing the queen and nestmate volatile hydrocarbons (paired t -test: $t = -2.52$, $df = 10$, $p = 0.030$) indicating a larger number of workers spent a longer duration of time near that fiber. The horizontal bar in the box represents the median pixel density. The lower and upper edges of the box represent the 25th and 75th quartiles, and the whiskers represent the 1.5 interquartile range. Lines are used to connect paired data points.

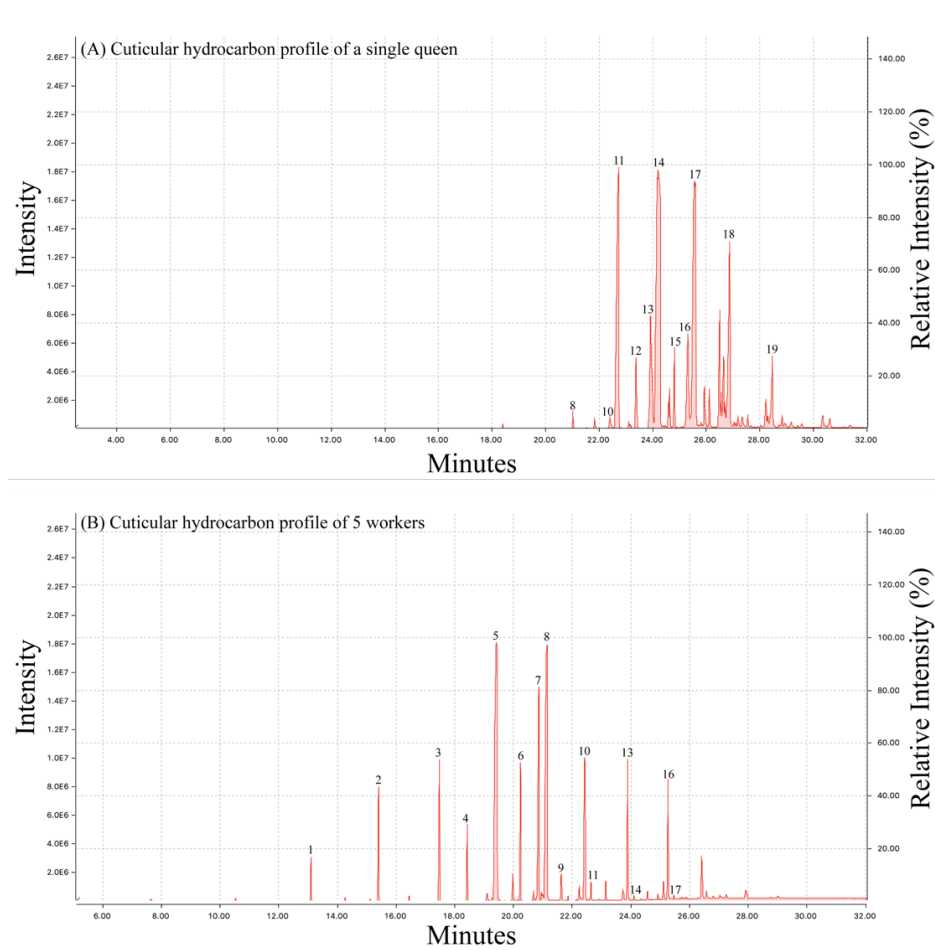


Figure 7. Comparison of cuticular hydrocarbon profile of queen vs worker *Oecophylla smaragdina*. Cuticular hydrocarbons that occurred in at least 1% concentration on half of either the workers or queens sampled were tentatively identified using mass spectra and retention indices. A representative queen profile (A) and worker profile (B) are shown here with peaks labeled. Peaks are identified as follows: 1, Pentadecane; 2, Heptadecane; 3, Nonadecane; 4, Eicosane; 5, Heneicosane; 6, Docosane; 7, x-Tricosene; 8, Tricosane; 9, x-Tetracosene; 10, x-Pentacosene; 11, Pentacosane; 12, Hexacosane; 13, x-Heptacosene; 14, Heptacosane; 15, Octacosane; 16, Nonacosene; 17, Nonacosane; 18, Hentriacontane; and 19, Tritriacontane.

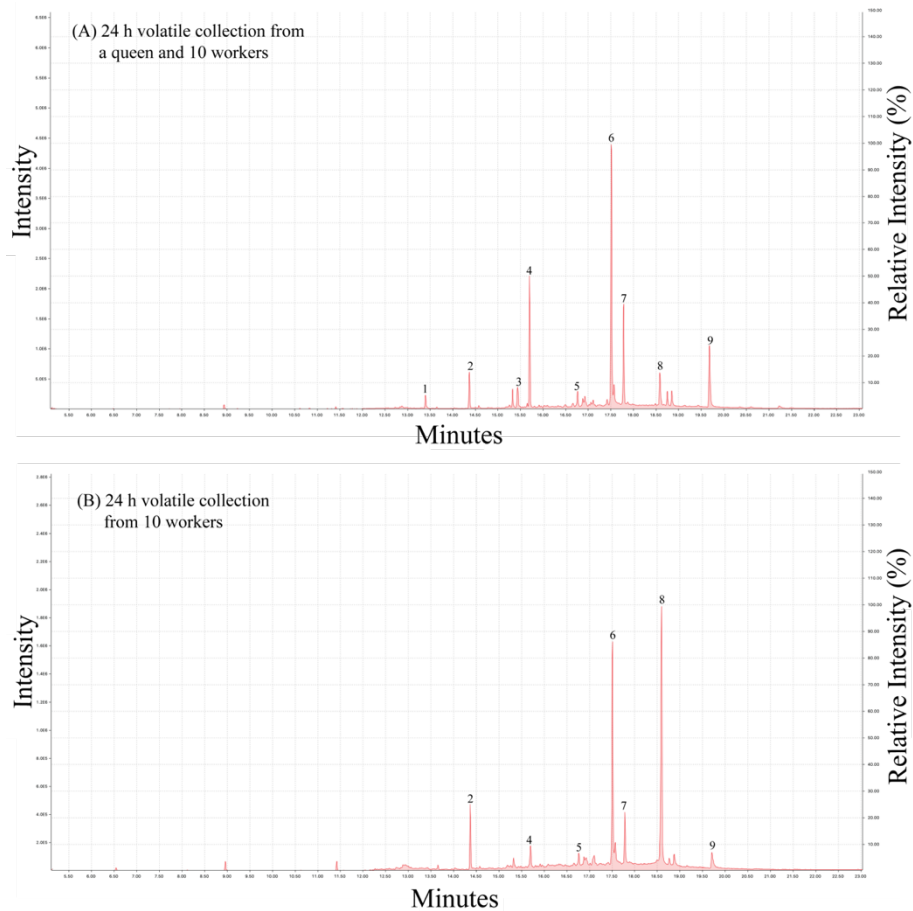


Figure 8. Comparison of queen plus worker volatiles vs worker volatiles for *Oecophylla smaragdina*. Volatile hydrocarbons that occurred in at least 1% concentration in half of either the workers or queens plus workers sampled using 24 h volatile collection were tentatively identified using mass spectra and retention indices. A representative queen and 10 workers profile (A) and 10 workers profile (B) are shown here with peaks labeled. Peaks are identified as follows: 1, Pentadecane; 2, Unknown; 3, x-Heptadecene; 4, Heptadecane; 5, Octadecane; 6, x-Nonadecene; 7, Nonadecane; 8, Unknown; 9, Heneicosane.

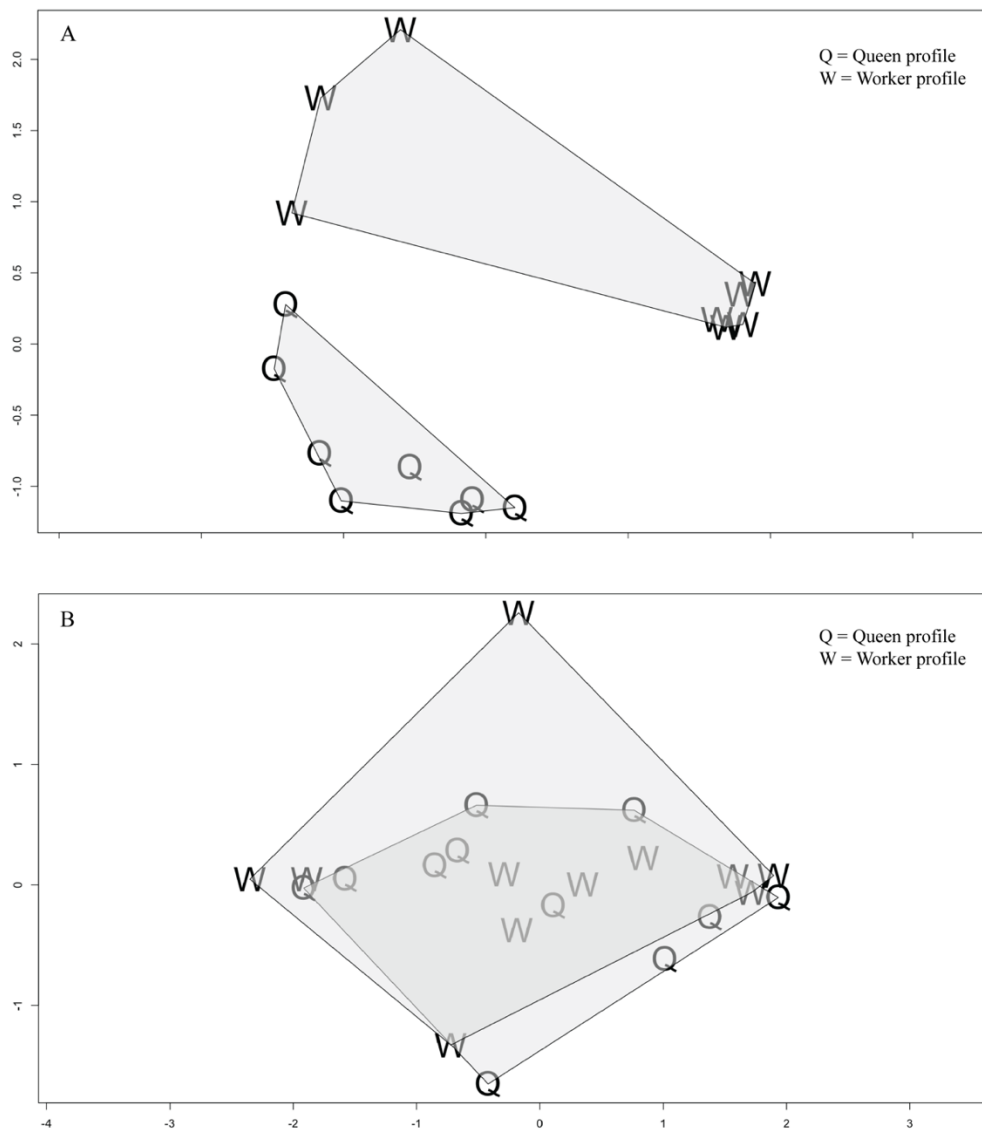


Figure 9. Clustering of worker vs queen cuticular hydrocarbons and volatiles. Non-metric multidimensional scaling analysis was used to analyze similarities between *O. smaragdina* queen and worker cuticular hydrocarbons (A) and volatile hydrocarbons (B). There is a clear separation of cuticular hydrocarbons by group (queen or worker). However, there is no separation between queens and workers for volatile hydrocarbons.

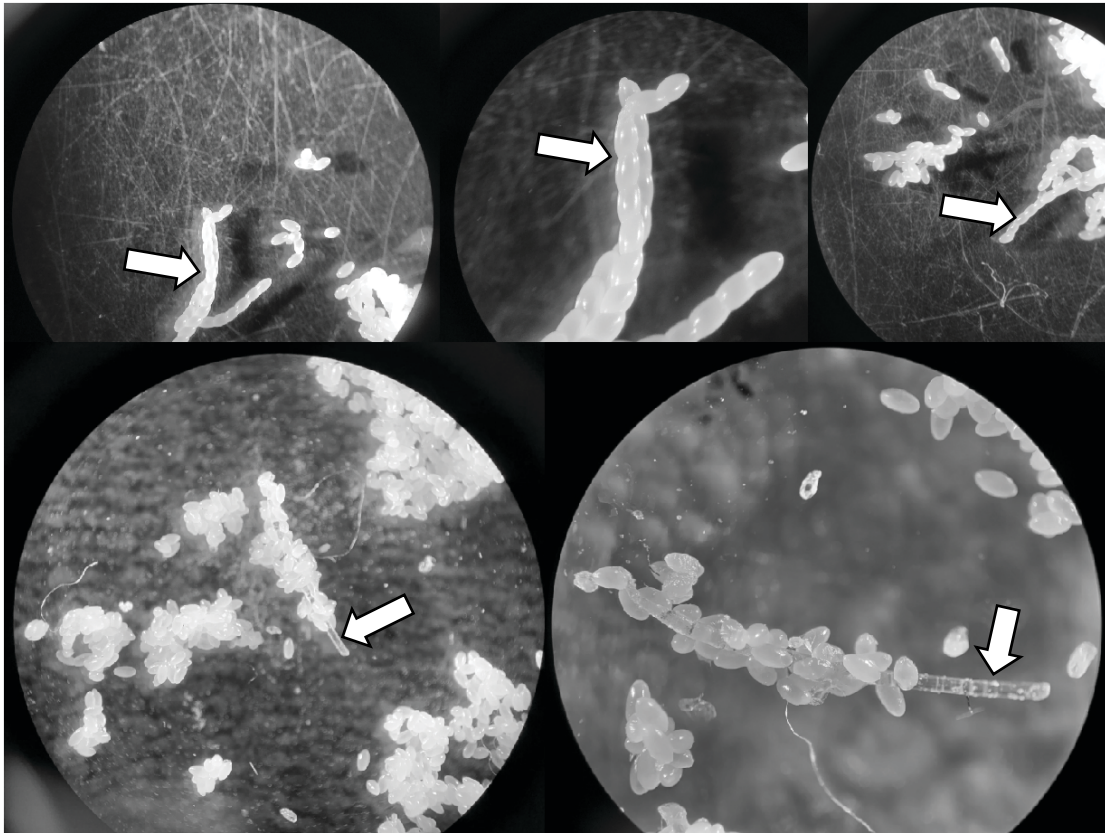


Figure 10. Queen-produced egg clustering structures. Physogastric *O. smaragdina* queens were observed laying eggs in long chains contained in a clear, thin sheath (indicated by white arrows in top three images) long clusters attached to a sticky post present in the center of the cluster (indicated by white arrows in the bottom two images: when housed with few workers. Images were taken with an Apple iPhone through a stereo microscope.

CHAPTER 5

CONCLUSIONS

Whereas most research studying mechanisms used to regulate reproductive division of labor in eusocial species has focused on colonies that inhabit a single nest with connected chambers where workers may encounter the reproductive individual regularly, other cases exist where the presence of a queen is not sufficient to maintain the colony structure (Heinze et al. 1996; Mamsch 1967; Seeley 1979; Starkey et al. 2019; Villalta et al. 2015; Warner et al. 2016). Here, we have established for the first time that polydomy creates pressure for the evolution of an extended regulatory phenotype expressed beyond the queen's immediate presence. Just as the spatial challenges of recruiting large numbers of foraging ants to food caches have led to the use of pheromone deposition for long-range coordination among ants, the long-range challenge of coordinating reproduction in polydomous ants has led to the use of communication mechanisms spatially separated from the queen.

We see cases other than polydomy where similar extended phenotypes for reproductive regulation exist. One such example has been shown in the bumble bee, *Bombus impatiens*, where colonies exist for a single season. While the queen is alive in the colony, workers forgo egg-laying to rear their siblings. However, after the queen dies, workers activate their ovaries and produce male offspring (Cnaani et al. 2002). The presence of the *B. impatiens* queen in the colony and the presence of larvae in the colony have both been shown to inhibit worker egg-laying. However, there is no evidence to

support a chemical signal present on the queen (Amsalem et al. 2017). Instead, the signal of the queen's presence in the colony is her larvae, which inhibit workers from laying their own male-destined eggs (Starkey et al. 2019). The inhibition of worker reproduction by larvae may delay worker reproduction after the queen's death ensuring that her offspring receive provisioning and care before care is switched to worker-produced brood. The annual colony cycle of *B. impatiens* appears to have created the space for additional regulatory mechanisms beyond the queen (i.e., her brood are a stand-in for her presence even after her death).

In polydomous species, reproductive regulation is also necessary without the queen's direct presence; signals of queen's presence must extend over long distances to workers that may not encounter the queen for extended periods of time (Debout et al. 2007). In chapter 2, we show the importance of larvae, which are often overlooked within the Hymenoptera colony structure (Schultner et al. 2017), in serving as such a distributed signal of the queen's presence throughout the colony in the polydomous ant *Novomessor cockerelli*. The use of larvae and not eggs for reproductive regulation in *N. cockerelli* is consistent with prior observations that queen-specific hydrocarbons present on queen-laid eggs are an unreliable signal because worker-laid eggs also contain the same signal (Smith et al. 2008). In chapter 3, we extended existing work demonstrating the importance of queen-specific cuticular hydrocarbons in regulating colony reproductive division of labor in the polydomous ant *Camponotus floridanus* (Endler et al. 2006, 2004) by testing the dominant component of the queen-specific

cuticular hydrocarbon profile, 3-MeC27, and the stereoisomers of that compound. 3-MeC27 is the dominant component of the queen-specific cuticular hydrocarbons present on the queen and her eggs, which inhibit worker egg-laying and allows for acceptance of a foreign queen by workers (Endler et al. 2006, 2004; Moore and Liebig 2010). We showed that 3-MeC27 disrupts normal aggressive behavior toward foreign workers, which suggests that this compound when coupled with a colony background is sufficient to reduce aggression toward a potential queen to avoid the high cost of mistakenly harming the colony's own queen. We also find some evidence supporting Sharma et al.'s (2015) finding that *C. floridanus* workers can distinguish between the two stereoisomers suggesting that chemical structure should be considered when testing chemical communication in animals. In chapter 4, we demonstrate that the cuticular hydrocarbons and volatile pheromones produced by an *Oecophylla smaragdina* queen serve as attractant pheromones for the workers. This work coupled with the findings of Hölldobler and Wilson (1983) provide strong evidence that the queen-specific cuticular hydrocarbons serve as both a releaser pheromone and a primer pheromone in this highly polydomous species. The mechanism for distribution of the queen fertility signal throughout the colony has yet to be confirmed but is likely to be the presence of the same fertility signal on the queen-laid eggs. The unique egg-clustering structures produced by the queen may facilitate transport of the eggs from one arboreal nest to another within the colony.

The challenges a polydomous colony faces when regulating reproductive division of labor is similar to foraging challenges faced by ant colonies where food source information much be communicated over long distances, and the signals need to be sufficiently persistent to be honest signals of a good food source. The signal vocabulary present in the regulation of worker reproduction in polydomous species is likely to be as rich and diverse as that found in the foraging and trail laying contexts. Our results support the idea that the regulatory vocabulary of ants is likely more diverse than just cuticular hydrocarbons. Our results alone suggest that further study of brood, stereoisomers, and volatiles may be warranted in spatially distributed ant colonies. This diversity of regulatory mechanisms further suggests the necessity to consider all aspects of the colony structure when studying the maintenance of reproductive division of labor.

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APPENDIX A

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