CHEMICAL AND BEHAVIORAL REGULATION OF COLONY SOCIAL ORGANIZATION IN FIRE ANTS

by

HAOLIN ZENG

(Under the direction of Kenneth G. Ross)

ABSTRACT

The red imported fire ant (*Solenopsis invicta*) is a pesty yet hugely successful global invader. Since its introduction to the US almost a century ago, *S. invicta* has become a model organism for the study of social evolution by virtue of its ease of collection and rearing. Particularly, the social form polymorphism in *S. invicta* presented a unique research opportunity to understand how colony social structure is regulated. The two forms of *S. invicta* differ not only in queen number but also in a variety of natural history traits. The genetic basis of the social form polymorphism is an inversion-based supergene. In the multiple-queen social form, supergene-carrying workers induce the whole colony to accept additional queens, but only the ones carrying the supergene. Mechanistic details regarding the chemical communication of supergene status and factors governing queen genotype preference are needed to fulfill the genotype-to-phenotype map underlying multiple-queen social form. In this dissertation, I reviewed the functional properties of queen pheromones in ants. Next, using high throughput behavioral assays, I identified unsaturated cuticular hydrocarbons as the pheromonal signal through which supergene-carrying queens communicate their genotype status to workers. I then designed novel

experiments to reveal the critical factors through which supergene-carrying workers influence colony collective queen preference.

INDEX WORDS: Ant, Social Evolution, *Solenopsis invicta*, Supergene, Queen Pheromones, Unsaturated CHCs, Green-beard

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DEDICATION

For my parents, ZZP and LYY.

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CHAPTER 1

INTRODUCTION

In the latest estimate, a single family of insects amassed a cumulated weight exceeding that of the total of wild birds and mammals (Schultheiss et al. 2022). This highly successful group of animals is ants (order Hymenoptera, family Formicidae). The key to the ecological dominance of ants is shaped by their social habitat, or eusociality (Hölldobler and Wilson 1990). Living in a highly integrated society, members in an ant colony are divided into two general castes, the queen caste and the worker caste. The queen caste monopolizes reproductive rights while the workers, who are typically offspring of the queens, specialize in non-reproductive tasks like foraging, nest building, and brood care (Hölldobler and Wilson 1990). In other words, the queen's offspring have forgone their reproductive privileges and instead act as the extended phenotype of the queen (Dawkins 2016). Together, all members in an ant colony function effectively as a single superorganism (Wheeler 1910). Besides ants, eusociality has evolved independently in many groups of insects and has shown to be a successful strategy (Anderson 1984).

The quest to understand the evolution of reproductive altruism in eusocial insects has been a major puzzle since Darwin (Korb and Heinze 2016). A few theoretical frameworks to explain social evolution were brought forth by generations of biologists, but debates remain to date (Hamilton 1964; Boomsma 2009; Nowak et al. 2010; Boomsma et al. 2011). Having a systematic understanding of how ant societies are regulated both genetically and phenotypically would be crucial to inform experimental designs to test these evolutionary theories.

My research focuses on the red imported fire ant, *Solenopsis invicta*. Commonly seen in suburban lawns and pastures in the Southeast United States, this ant is considered one of the most successful and nasty global invaders among ants (Ascunce et al. 2011). Due to its abundance and ease of collection and rearing, *S. invicta* has since become a model species for the study of social evolution, natural history, and developmental biology of ants (Tschinkel 2013).

A particular feature regarding the colony social organization of *S. invicta* has contributed to their importance as research model organisms. Two distinct forms of colonies are displayed in *S. invicta* population, with one form housing a single reproductive queen and the other form housing multiple reproductive queens (Gotzek and Ross 2007; Huang and Wang 2014). Why would queens share their reproductive rights in a multiple-queen colony? How is the queen number regulated leading to the two types of social structure? Learning how this social form evolved may inform the evolution of sociality and understand the forces driving the success of ants.

Recent advances in sequencing technology have provided powerful tools and yielded much important insight into the genomic and transcriptomic levels. The genetic basis of this social form polymorphism has been mapped to a large non-recombining section on chromosome 16 formed by chromosomal inversions, termed *Sb* supergene (Wang et al. 2013; Helleu et al. 2022). This supergene manifests in multiple-queen colonies in a selfish greenbeard fashion (Ross and Keller 1998; Keller and Ross 1998). When supergene-carrying workers are present in the pool, even in a small minority, the colony will accept multiple queens, but only those carry the *Sb* supergene (Ross and Keller 2002; Gotzek and Ross 2008). However, major mechanistic details underlying the regulation of social structure are lacking. Specifically, what is the chemical composition of the genotype signal through which workers recognize the queen

genotype? How do *Sb*-carrying workers induce the colony to reach a consensus to tolerate *Sb* queens?

In this dissertation, I provided discoveries to help fulfill the missing pieces in the genotype-to-phenotype map underlying the multiple-queen social form. I first reviewed functional properties of queen pheromones demonstrated explicitly by experimental evidence in ants. Then I examined the composition of the pheromone that communicates the queen supergenotype status to workers. Lastly, I explored how supergene-carrying workers influence their non-supergene nestmates to accept Sb carrying queens.

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CHAPTER 2

FUNCTIONAL PROPERTIES OF ANT QUEEN PHEROMONES IN ANTS[1]

[1] H. Zeng. To be submitted to Behavioral Ecology and Sociobiology

Abstract

Ants are one of the most successful groups of animals by virtue of their social colony structure, where up to millions of individuals cooperate to survive, compete, and reproduce as a single superorganism. Members of ant colonies typically are categorized into a reproductive queen caste and a non-reproductive worker caste. Crucial to the superorganismal nature of ant colonies, the queen conveys her presence via a suite of queen pheromones, which also act as agents that manifest various effects on the colony. With decades of studies on queen pheromones, there is still a lack of in-depth knowledge connecting the identity of constituent chemicals, mode of action, and known functions in any single ant species. This review summarizes functional properties of ant queen pheromones demonstrated from experimental studies. These functional effects include attraction of workers, contribution to colony identity and cohesion, maintenance of reproductive dominance, and mediation of colony social organization. General characteristics of queen pheromones and directions for systematically identifying pheromonal compounds are discussed.

Introduction

The ecological success of ants (Hymenoptera: Formicidae) is attributable largely to their highly integrated social lifestyles, where numerous individual ants cooperate to survive, compete with conspecifics, and produce sexual forms (reproductive individuals), effectively functioning as a single organism, or a superorganism (Wheeler 1910; Schultheiss et al. 2022). Male ants contribute little or nothing to the maintenance and growth of the colony, confining their activities to competing for matings during reproductive seasons. Females typically are divided into two castes: members of the queen caste monopolize reproduction as the primary egg-layers, whereas

members of the worker caste perform routine, though essential tasks such as foraging, nest building, and brood care, acting as the extended phenotype of the queen (Wheeler 1910; Hölldobler and Wilson 1990; Smith et al. 2008b). The queen caste in most ant species is morphologically and physiologically distinct from the worker caste; in such cases, workers have completely lost their reproductive potential (Keller et al. 2014). These workers lack sperm storage organs (spermathecae) and, often, functional ovaries. In some species, subtle but consistent differences exist between the queen and worker castes. Workers in these species retain reproductive potential. If the colony loses its original queen, one or few workers may ascend to function as a dominant reproductive female, acting as a queen(s) (Powell and Tschinkel 1999; Penick et al. 2021). In a minority of ant species, the queen caste and worker caste are not morphologically or physiologically well differentiated (Peeters 1991).

Regardless of the degree of caste dimorphism, the dominant reproductive female, or the "queen," must communicate her presence and signal her reproductive status to other colony members for the colony to function properly (Keller and Nonacs 1993). Communication in ant colonies is primarily chemical in nature, which is likely related to their ancestral subterranean habits (Tschinkel 2015, 2021), although visual, vibrational, and tactile signals or cues are also deployed in some species (Hölldobler and Wilson 1990; Hölldobler 1995; Barbero et al. 2009; Golden and Hill 2016; Knaden 2019; Yilmaz and Spaethe 2022). Chemical compounds used for such intraspecific communication are termed pheromones (Karlson and Lüscher 1959). The potential durability and transmissibility of pheromones may have helped ants to evolve massive colony sizes and become the dominant invertebrates in most terrestrial ecosystems (Schultheiss et al. 2022).

A variety of pheromones are deployed in ant colonies, such as alarm pheromones that warn nestmates of invaders and disturbance, and trail pheromones that guide nestmates between travel destinations and nest locations during foraging or nest migration (Jackson and Ratnieks 2006; Grüter and Keller 2016; Vargo 2019). Studies of the functions of ant pheromones have not only deepened our understanding of the social biology of ants but also inspired such seemingly distinct efforts as designing network computation algorithms (Bonabeau et al. 2000).

Although the queen(s) does not actively participate in the colony's daily maintenance and brood-rearing tasks, her presence has dramatic effects on worker brood care activities, nestmate discrimination, developmental trajectories of larvae, colony coherence, reproductive output, and colony social structure, as reviewed here (Hölldobler and Wilson 1990). Most, if not all, of these effects are manifested through queen pheromones, a particular group of molecules produced by the queen and perceived by any colony members other than the focal queen (Kocher and Grozinger 2011). Thus, much like invisible wires that connect the queen to the rest of her colony and extend her influence, queen pheromones are key regulators of colony ontogeny and the emergent superorganismal features of ant societies.

While queen pheromones of ants have been broadly studied in many species for over seven decades, there is a lack of in-depth knowledge connecting the identity of constituent chemicals, mode of action, and complete functions in any single ant species. Due to the huge diversity in caste, reproductive, and social systems of ants, it is unrealistic and unnecessary to completely comprehend queen pheromones in multitudinous species. However, learning about them in a handful of representative species to a level like the honey bee would reveal essential information for broadening our understanding of the functional roles of queen pheromones in social Hymenoptera (Keeling et al. 2003; Bortolotti and Costa 2014; Princen et al. 2019).

In this review, I summarize our current knowledge of queen pheromones from experimental studies that expressly demonstrate the functional properties in ants (Fig. 2-1). I first go over the effects of queen pheromones on general worker behaviors. I then discuss how queen pheromones help to maintain the reproductive dominance of the queens as well as the overall reproductive division of labor in a colony. Lastly, I present the latest studies on how queen pheromones mediate the regulation of colony social structures, specifically regarding the number of queens in the colony.

Effects on general worker behaviors

Attraction toward and retrieval of the queens

Upon examining the queen in an ant colony, one notices that she is surrounded by workers that often lick her cuticle or otherwise groom her. The most immediate function of the queen pheromone is attraction, enabling workers to locate the queen, so as to care or rescue her. In a way, the colony is where the queen is. This notion is evident in the rock ant *Temnothorax rugatulus*, whose scooting workers collectively and democratically choose new nest sites, but when a colony was experimentally split into two nests under equal conditions, they almost always reunited with the queen (Doering and Pratt 2016).

Early investigations pointed to the existence of chemical pheromones by showing that queens were attractive to their workers and that this attraction capacity could be transferred in cuticular solvent extracts. Stumper (1956) demonstrated the species specificity of queen pheromones by applying extracts of *Pheidole* queens to corpses of *Lasius* queens, which then were adopted by *Pheidole* workers for a period of about 24 hours, clearly demonstrating that workers distinguish the identity of a queen at some level based on her cuticular chemicals. Workers generally will accept queens of a closely related species, given that the colony has been

queenless for an extensive period, as demonstrated in *Myrmica* (Brian 1986a, 1988a, b). This is likely due to the similarity of the chemical makeup of queen recognition signals among recently diverged taxa, a feature that ultimately may pave the road for the rise of social parasites (Lenoir et al. 2001).

When comparing worker responses to conspecific queens, many studies noted a general correlation between the intensity of attraction of a queen and her fecundity or weight (Sommer and Hölldobler 1995; Hannonen et al. 2002). Such a trend is pertinent to other queen pheromone effects in the discussion that follows, and is most parsimoniously explained by a higher level of pheromone production occurring in more fertile queens. This collective, accurate assessment of the fertility condition of an individual is fundamental to the signaling function of queen pheromones (Keller and Nonacs 1993).

The chemical basis of worker-attracting queen pheromones was further supported from studies showing the same effect from queen corpses or chemical extracts of queens. In weaver ants (*Oecophylla* spp.), Hölldobler and Wilson (1983) described the strong attraction of workers towards the queen, the effect persisting on a queen corpse for up to six months after the queen died. Additionally, weaver ant workers routinely present unfertilized trophic eggs to the queen, a behavior also induced by the queen corpse (Hölldobler and Wilson 1983). Studies in army ants and carpenter ants showed that workers were attracted to areas of filter paper previously occupied by a queen (Watkins and Cole 1966; Fowler and Roberts 1982). However, this type of experiment cannot separate the effect of an attractant pheromone produced by the queen from the effect of an aggregation pheromone produced by the workers themselves (Vander Meer Robert and Alonso 2019).

In the red imported fire ant, *Solenopsis invicta*, functional properties of ant queen pheromones have been studied systematically, thanks to their serious pest status and abundance in suburban areas in the American South, and the relative ease of collecting and rearing them in controlled conditions. Jouvenaz et al. (1974) reported that workers were attracted to areas on a piece of blotter paper previously occupied by live queens or dosed with hexane extract (200 newly mated queens/1 ml), showing the existence of chemical-based pheromones. Fire ant workers' responses toward the queen's presence are more dramatic and "obvious" when the colony is disrupted and the queen exposed, such as described in Glancey et al. (1983): when a queen is found outside of the nest, workers will (1) quickly be attracted to her, (2) cluster around her, (3) move brood items to or around her, (4) form a pheromonal trail that the queen can follow back to the nest, and/or (5) pull the queen towards the nest, should she not move voluntarily.

Glancey et al. (1984) devised a single score metric to measure worker responses towards synthetic compounds and natural pheromones based on these commonly observed behaviors. Unfortunately, it is hard to discern from their score metric which category of behavioral response was clearly displayed, as the score was obtained by weighted summation of a subjective rating of each of the five behavioral categories. More recent studies of *S. invicta* showed that workers exhibit the same series of stereotyped behaviors toward a paper dummy dosed with reproductive queen hexane extract as they do toward live queens or fresh queen corpses, i.e., collectively retrieving the treated dummy into the nest and keeping it there for eight hours or more (Trible and Ross 2016; Zeng et al. 2022). Therefore, such typical behaviors may be good general features to assay for active components of queen pheromones in ants, but caution should be exercised when designing bioassays and the corresponding scoring metrics.

As reviewed in the following sections, the effects of queen pheromones are often manifested through worker behaviors. Animal behavior is complex and subject to anthropogenic interpretation. It is not always straightforward to devise an assay scoring system that is most informative while rooted in a firm understanding of the natural history of the species and the natural context of the behavior. Assigning a single numeric score to measure complex behavioral traits in a social context is difficult but can be achieved based on a sufficiently large dataset and validation of the biological meaning of the score through back-testing. This was recently demonstrated by Wild et al. (2021) who tracked movements and interactions among individual honey bees in a hive. Based on the location and interaction network information, each bee was assigned a single numeric "network age", which accurately predicted task allocation, activity patterns, survival, and future behavior.

Alternatively, it is efficient to take measurements of distinct actions that are reliably quantifiable and use these directly as a score metric to assess worker response to a queen. One example was provided by Dietemann et al. (2003), who measured the number of antennation inspections by workers towards a glass slide applied with queen cuticular fractions of *Myrmecia gulosa*, as an approximation of the intensity of worker response to potential queen pheromones. Another way to design an informative bioassay is by observing and quantifying behaviors unique to the study species. For instance, workers of *Odontomachus brunneus* display submissive gestures when a queen or other dominant individual is nearby (Fig. 2-2) (Medeiros et al. 1992). Smith and colleagues tested candidate queen pheromone compounds by counting the frequency of such submissive gestures from workers toward a focal worker dosed with queen-specific CHCs (Smith et al. 2012b, 2013, 2015).

Effects on colony maintenance

In many ant species, a newly mated queen performs all necessary tasks to initiate the growth of the colony by herself. She prepares a nest site (e.g., digs a burrow in the soil) and raises the first cohort of workers from the eggs she then lays. But when worker adults emerge, the queen stops engaging in colony maintenance tasks and transitions to acting strictly as an egg layer (Cassill et al. 2002; Majidifar et al. 2022). The continued presence of the queen not only ensures the steady production of fertilized eggs and, subsequently, additional brood and adults, but a few studies suggest that the queen also boosts the level of worker activity in brood care and acts to maintain the cohesion of the colony. In other words, the queen (or queen pheromones) may function as a "catalyst" to boost colonial development and a "gravity center" to maintain colony cohesion, as shown in the studies below.

Berton et al. (1992) showed that overall worker activities were reduced if the queen was removed in *Cataglyphis cursor*. However, no brood items were added to the experimental units, and a small number of workers was used (ten workers in each assay), so the study lacked a natural social context. Vienne et al. (1998) recorded worker activities in queenright and queenless colonies of *Myrmica* sp. and *Manica* sp. Their results showed that workers from queenright groups antennate the brood more often and stay longer with the brood than queenless workers. As well, workers without a queen tend to leave the nest for foraging and guarding tasks, interacting more often with adult workers instead of the brood. These results suggest a decrease in brood care activities in the absence of a queen.

In a study of *Atta sexdens*, Della Lucia et al. (2003) removed the queen and observed worker behavior. One week after queen removal, minim workers, which usually act as fungal gardeners and nurses, were frequently found outside the nest in the foraging area. In the 30 days

after the queen removal, workers departed eight times more frequently from the colony than when the queen was present. Another study in *Atta sexdens*, by Sousa-Souto and Souza (2006), reported an increase in the daily worker mortality and a decrease in refuse accumulation, but no change in foraging efficiency when the queen was removed, although this was based on a comparison of only two colonies.

More recently, in *Temnothorax curvispinosus*, Keiser et al. (2018) compared the performance of queenright and queenless halves of mature colonies hosting or lacking fungal infections. Not only did the queenright subcolonies outperform their queenless counterparts in most measurements of colony efficiency such as the proportion of workers feeding newly discovered food or interacting with brood and the time required to discover misplaced brood, queenright subcolonies were more resistant to fungal pathogens, as shown by the fact that fewer of their workers died after artificial induction of the fungus.

These studies showed that a higher level of brood care, stronger cohesion, and better overall performance of the colony was induced by the presence of the queen. These shifts in worker behavior in the presence of the queen may be explained as secondary effects of the attractiveness of the queen causing, for instance, a more structured spatial distribution of the brood and, as a result, more efficient brood care. However, since all the studies discussed above compared worker behavior between conditions of presence or absence of a live queen, the pheromonal basis of these effects remain to be confirmed by experiments using queen corpses or chemical extracts.

Effects on nestmate discrimination

Each ant colony carries a set of cuticular chemical odor labels, which workers use to distinguish nestmate from non-nestmate conspecifics (Ozaki et al. 2005; Sturgis and Gordon

2012). Many studies indicated that the presence of one or more queens has a significant impact on the odor label of a colony, such that queenless colonies exhibit reduced territoriality, acting as if they have lost some component of their distinct colony identity. Compared to queenless workers, workers from queenright colonies are more subject to aggression by non-nestmate conspecific workers, as well as being more aggressive themselves towards such workers. In terms of the superorganism analogy, a queenless colony could be viewed as a "headless" body, until a new queen is adopted in species with sterile workers, or one or more emerge(s) from the worker pool to assume the dominant reproductive role in species with ovary-bearing workers(s).

Obin and Vander Meer (1989) tested the effect of queens on nestmate discrimination in the monogyne (single queen per colony) form of *S. invicta*. As expected, adult worker ants from mature queenright colonies experienced immediate aggression from non-nestmates when confronted with them. In follow-up experiments, queenless adult workers (subject workers) were obtained by rearing monogyne workers from the brood to adult emergence in the absence of a queen for 26 weeks before the experiments. Subject workers received almost no aggression from original nestmate workers when reintroduced into their queenright natal colony. However, when subject workers were induced to tend a newly added foreign reproductive queen, even for only 15 minutes, they were attacked by their original nestmate workers when confronted with them. Interestingly, subject workers in these queen-added experimental units that did not contact the new queen received almost no aggression from original nestmate workers (Obin and Vander Meer 1989). Vander Meer and Alonso (2002) then showed that worker aggression towards non-nestmate workers and queens decreased after their mother queen was removed and effectively disappeared after about two weeks. Thus, in monogyne fire ants, a colony's unique chemical identity is attributable, at least in part, to its sole reproductive queen.

Similar phenomena were shown in other species. In *Cataglyphis niger*, workers kept in queenless conditions for five months did not show aggression towards and did not receive aggression from original nestmate workers from the parent queenright colony (Lahav et al. 1998). In *Camponotus* species, worker aggression towards non-nestmates largely disappeared in queenless colonies regardless of colony size and reappeared after an unrelated queen was adopted into the colony (Carlin and Hölldobler 1983, 1986, 1987). Additionally, queens with less developed ovaries or that were incompletely inseminated had a weaker effect on worker nestmate discrimination abilities (Carlin and Hölldobler 1987).

A counterexample to the trend that queen presence induces nestmate discrimination came from *Rhytidoponera confusa*, where nestmate discrimination was not affected by the absence of the queen (Crosland 1990). However, this result does not argue against queen pheromone effects on nestmate discrimination and, indeed, provides complementary evidence for a correlation between the degree of queen-worker dimorphism and the strength of the effect of the queen pheromones (Smith and Liebig 2017). In *R. confusa,* queen-worker dimorphism is not pronounced and workers are fully capable of reproduction. In the wild, queenless colonies are a common occurrence (Ward 1981, 1983). In other words, *R. confusa* colonies remain independently reproductive with or without the queen. Arguably, such a colony does not conform to a superorganism, because all the members can be the "germline" if needed and the distinct "soma" of the superorganism is not present. Given that the queen is not an indispensable component of the colony identity, it is reasonable that her absence did not impact worker nestmate recognition abilities (Carlin and Hölldobler 1991). In contrast, in taxa with pronounced caste differences, such as *Solenopsis* or *Camponotus*, queenless colonies are destined to die unless re-queened, as if the colony identity is linked to the queen identity. We can thus predict a

stronger effect on nestmate discrimination influenced by queen pheromones in species with pronounced caste differences.

It remains unclear, however, if such an effect is due solely to the transfer of queen-derived cuticular or other chemicals to the workers. It is unlikely that the queen can produce enough cuticular compounds and that their distribution is so efficient that every worker in a sprawling, populous colony (such as weaver ants in a tropical tree canopy) receives some portion, since the overall transfer of CHC from workers to queens has been shown to be much higher than from queens to workers (Lahav et al. 1998). In contrast, the queen pheromones may also stimulate workers to produce unique semiochemicals that signal queen presence, a possibility that remains to be tested.

Maintenance of reproductive dominance

Inhibition of larval sexual development

As the hallmark of eusociality, reproductive division of labor requires that members of the queen caste maintain their reproductive dominance, which is achieved via an essential and well-studied class of queen pheromones. The documented effects of such pheromones occur in almost all life stages of female colony members.

In many ant species, queen pheromones have been shown to inhibit sexualization (development as queens) of female larvae, thus biasing worker production over gyne (virgin winged queen) production. An early series of studies of such queen effects was conducted by Brian and colleagues in *Myrmica* species. The influence of the queen on brood development included improved survival, earlier pupation, and lower larval and pupal weights of both males and females, in apparent accord with the notion of the queen stimulating general colony function

and cohesion (Brian 1957, 1986b; Brian and Carr 1960). In more detailed observations, adult worker behaviors appeared to be susceptible to queen influence and to mediate the inhibitory effects. Winter hibernation is required for *Myrmica* larvae to retain totipotency, the ability to develop as gynes or as workers. After hibernation, small-sized (worker-destined) larvae received more care while large-sized (gyne-destined) larvae received less care from workers when the queen was present in the colony (Brian and Hibble 1963). As well, bite marks were found on the large larvae only in the queen's presence, and subsequently, many such larvae were killed. Without contacting the larvae, the queen was able to stimulate workers to bite these larvae, although the queens themselves also engaged in such attacks. As a result, the presence of the queen reduced the output of gynes while boosting worker production (Brian and Carr 1960; Brian 1973).

A chemical origin of these effects was inferred because workers engaged in larval biting only when able to make bodily contact with a live queen or fresh queen corpse. Moreover, dead inseminated queens were effective, while dead or living unmated queens were not (Brian 1970, 1973). Interestingly, corpse body sections held by insect pins arranged in the correct anatomical order (head, thorax, and then gaster) were effective, but any section alone or together in the wrong order were not (Brian 1973). This finding suggested that structural and topological features of the *Myrmica* queen played a role in the pheromonal effects.

In the Pharaoh's ant, *Monomorium pharaonis*, the presence of a fertile queen in a colony was shown to inhibit development of both male and female sexual brood (Petersen-Braun 1975, 1977). However, unmated queens, fertile queens made sterile by exposure to a juvenile hormone analogue, freshly killed queen corpses, and queen solvent extracts did not yield the effect (Berndt and Nitschmann 1979; Edwards 1987; Boonen and Billen 2017). Instead, Edwards (1987) found

that eggs were the means of dissemination, as the introduction of eggs could, by itself, induce the effect. Edwards (1991) subsequently showed that workers cannibalized sexual brood in the presence of fertile queens; such workers would not tolerate sexual larvae or pre-pupae from another nest, while always accepting worker brood from a foreign source. Most recently, a compound produced only by egg-laying queens of several *Monomorium* spp., the monocyclic diterpene neocembrene, was demonstrated to be a queen pheromone component, as its synthetic compounds elicited a weak "queen retinue" attraction as well as reduced production of both male and female sexuals (Edwards and Chambers 1984; Oliveira et al. 2020).

Vargo and colleagues showed for *S. invicta* that the presence of live queens or queen corpses inhibited rearing of new sexuals (Vargo and Fletcher 1986a). They created polygyne (multiple-queen) colonies with equal numbers of worker adults and brood but with varying numbers of reproductive queens, and colonies with more queens produced fewer sexual pupae than those with low queen numbers (Vargo and Fletcher 1986b). Additionally, the gaster of dealate queens (those that have shed their wings, a behavior associated with onset of egg-laying) held most of the inhibitory pheromone, being as effective as a whole dealate queen, whereas the isolated head and the thorax had almost no effect (Vargo 1988). The pheromone was non-volatile because direct contact with the queen corpse was required. Klobuchar and Deslippe (2002) further showed that adding dealated virgin or mated queens, fresh dealate queen corpses, or dealate queen saline extract to queenless colonies induced workers to kill sexual larvae. The pheromone producing this effect was stored in the poison-sac and was stable at room temperature.

Similarly, in the polygyne Argentine ant *Linepitheme humile*, live reproductive queens or parts of queen corpses prevented the production of new gynes in small colonies (Vargo and

Passera 1991). Winged gynes, corpses washed with pentane, or daily addition of eggs from reproductive queens did not reproduce the inhibitory effects, strongly suggesting the presence of a pheromone. Workers attacked and killed early-stage gyne larvae when the queen was introduced into the colony, although queens were also seen attacking these broods. Interestingly, male larvae, as well as late-stage gyne larvae, prepupae, and pupae were spared from attacks (Passera et al. 1995). In *Aphaenogaster* and *Novomessor*, gyne production was found to be inhibited by a mated queen, with direct worker contact with the queen required for the inhibition (Hölldobler and Carlin 1989; Boulay et al. 2007, 2009). In the Indian jumping ant, *Harpegnathos saltator*, workers bite queen-destined larvae only in the presence of an adult queen (Penick and Liebig, 2017). These presumed queen-destined larvae differ in their hydrocarbon profiles from worker-destined larvae, which were almost never bitten (Penick and Liebig 2017); the distinct hydrocarbons may comprise cues used by attacking workers to distinguish the two types of larvae.

The majority of experiments discussed above indicated that the queen pheromonal effect on caste development was engendered by the behavior of workers, either by their limiting the larval food supply, biting the larvae to suppress growth, or simply killing sexualized larvae. Adult workers probably sense the queen pheromones mainly through antennal chemoreceptors, which was supported by strong electrophysiological responses of worker antennae to queen extracts and candidate queen pheromones (D'Ettorre et al. 2004; Holman et al. 2010b; de Narbonne et al. 2016). Upon perceiving queen pheromones, workers respond by actively suppressing larval sexual development. However, whether larvae may also sense queen pheromones, and whether queen pheromones can directly affect larval physiology, remain to be studied.

Inhibition of reproductive development of adult females

Queen pheromones also are known to suppress physiological changes tied to reproductive development in adult females, including in workers with latent ovaries, alate (virgin) gynes, and mated nestmate queens, the latter circumstance being perhaps the best studied. The effects can be measured in diverse behavioral and physiological changes in the trajectory of reproductive development, including dealation (wing shedding), activation of ovarian development, weight gain, and time to onset of egg laying.

Dealation is the first observable indication of the onset of reproductive development in adult gynes in many ants. In a queenright *S. invicta* colony, alate gynes typically remain winged until a mating flight event, after which the newly mated gynes kick off their wings and dig a burrow in the soil to start their own colony (Tschinkel 2013). However, mating is not necessary to initiate the process. Winged virgin gynes start to dealate as soon as 12 hours after separation from fertile queens, with their alary muscles beginning to histolyze at the same time (Fletcher and Blum 1981, 1983a; Vargo and Laurel 1994). Ovarian development also commences around the time of dealation, and oviposition starts in another two to three days. By this time, the gyne begins to exhibit routine queen attractiveness and to produce the inhibitory pheromones (Vargo 1999) (Fig. 2-3).

The fecundity (weight) of a queen has been found to be correlated with her ability to suppress reproductive development in nestmate queens. In *S. invicta*, corpses of highly fecund queens (weights from 15 to 25 mg) suppressed dealation of winged gynes for up to eight days, while lighter queen corpses (average weight of 10 mg) suppressed dealation of winged gynes for only about one day, which was not different from the queenless controls (Fletcher and Blum 1983b; Willer and Fletcher 1986). The correlation between queen fecundity/weight and the

strength of such inhibitory effects is likely due to a link between weight and the level of pheromone production. Indeed, evidence for such a link came from the fact that queenless monogyne workers were able to consistently recognize and adopt as a new queen the more fertile of two presented that differed in weight (Fletcher and Blum 1983b). The inhibitory effect of reproductive queen pheromones acts on other nestmate reproductive queens as well: addition of live queens or queen corpses was found to reduce fecundity of other queens in polygyne colonies (Vargo and Laurel 1994).

At a physiological level, queen pheromones likely perceived via the antennal sensilla may cause downregulation of dopamine production, a consequence of which is suppression of production of juvenile hormones and inhibition of reproductive development (Robinson and Vargo 1997; Boulay et al. 2001). Juvenile hormones (JH) are critical regulators not only of reproduction, but of development and behavior, throughout the lifecycle of insects (Jindra et al. 2013). Topical treatment of alate gynes with JH or JH analogue induced dealation in *S. invicta* even in the presence of the queen, overriding the inhibitory effect of the queen pheromone (Vargo and Laurel 1994). Notably, JH treatment can yield opposing effects on reproductive development depending on the species and the size of the treatment doses used, suggesting a condition-depending cost of JH and calling for more studies on the endocrinological regulation of reproduction (Robinson and Vargo 1997; Cuvillier-Hot et al. 2004; Penick et al. 2011; Holman 2012).

Holman and colleagues studied cuticular hydrocarbons in *Lasius* to evaluate their possible role(s) as reproduction-suppressing queen pheromones. The methyl-branched molecule 3-methylhentriacontane (3-MeC31) was found to be highly abundant on the queen cuticle and on the eggs (Holman et al. 2010a, 2013). Its abundance was correlated with fecundity and, unlike

other CHCs, was reduced by immune challenge (Holman et al. 2010a, b). Either natural or synthetic 3-MeC31 effectively suppressed egg-laying by workers in three different *Lasius* species, making this the first compound to be identified as a queen inhibitory pheromone. Similar to *S. invicta*, *Lasius* workers chose replacement queens with relatively high amounts of 3-MeC31 to replace their experimentally removed queen while killing those with lower amounts (Holman et al. 2010a).

Studies in other ant species have shown similar inhibitory effects by live queens, queen corpses, or queen-laid eggs. In *Temnothorax* species, queen presence led to worker restraint in laying eggs (Dejean and Passera 1974; Brunner et al. 2011). In *Diacamma* species, the morphological queen caste is missing. Instead, a dominant mated worker, now termed a gamergate, functions as a queen (Fukumoto 1989). The gamergate signals her presence through direct contact with the workers, as only workers restricted from contacting the gamergate become aggressive and egg-laying (Tsuji et al. 1999). In *Oecophylla* weaver ants, the presence of queen corpses coincided with a lack of worker production of males for periods of up to six months (Hölldobler and Wilson 1983). In *Plagiolepis pygmaea*, direct contact with parts of queen corpses was associated with the absence of development of worker ovaries (Passera 1980). In *Camponotus floridanus*, exposure to queen-laid eggs inhibited worker egg-laying in queenless groups, while exposure to larvae and cocoons did not. Surface chemical profiles of eggs corresponded to the cuticular chemical profiles of the respective egg-laying queen or worker, providing support for the idea that inhibitory queen pheromones are spread among adult workers via eggs (Endler et al. 2006).

Another general effect of such inhibitory pheromones is a shortening of worker longevity when queens are present. Reproduction and longevity are typically a trade-off in animals, since

reproductive activities impose an energetic cost that otherwise could be spent in foraging, home building, territory defense, or any life-extending activities (De Loof 2011; Blacher et al. 2017). However, in social insects, reproduction and longevity are instead positively linked (Blacher et al. 2017), perhaps due to a reproductive division of labor where costly tasks burden the non-reproductive worker caste. In *Temnothorax* species, workers developed enlarged ovaries and extended lifespans in the absence of the queen (Kohlmeier et al. 2017; Negroni et al. 2021). Studies in *Atta* and *Acromyrmex* leafcutter ants showed that longevity of workers increases as they become more reproductively active after queen removal, probably by means of increased resilience to oxidative stress (Majoe et al. 2021). In *Harpegnathos saltator*, a ponerine ant with reduced caste differentiation in which workers are fully capable of reproduction (Peeters et al. 2000), removal of the queen (and her pheromones) prompted workers to engage in antennal duels, a ritualistic competition to re-establish hierarchy that occurs in many ant species (Powell and Tschinkel 1999; Penick et al. 2014). The winners of these antennal dueling competitions transition into gamergates (Sasaki et al. 2016). *Harpegnathos* gamergates had about five times the lifespan (average 1100 days) of normal workers (average 217 days) (Yan et al. 2022). In addition, gamergates had decreased brain and optic lobe volumes, typical of queens, as well as decreased venom production. They also behaved more like queens, remaining inside the nest and hiding from intruders rather than confronting them (Penick et al. 2021). Interestingly, all of these queen-like traits can revert to a worker-like state if a gamergate is is exposed to a strong source of queen pheromones (Penick et al. 2021).

Induction of worker policing

Given that workers in many ant species retain functional ovaries, they could produce males by laying unfertilized eggs, a consequence of the haplo-diploid genetic system of

hymenopteran insects. However, this poses a conflict of reproductive interest with the queen (Bourke and Franks 2019). On top of that, reproductively active workers impose a cost on colony productivity if they spend energy in reproduction rather than fulfilling their specialized worker tasks (Helanterä and Sundström 2007a). As a means of resolving such conflict, workers in social hymenopteran colonies may engage in policing behaviors to suppress the reproductive output of nestmate workers (Ratnieks and Wenseleers 2005). Typical acts of policing include direct aggression toward adults or destruction of their offspring (e.g., by egg cannibalism). Many studies have shown that when the queen is present, policing behaviors are more prominent, suggesting that these behaviors are under the influence of queen pheromones. To achieve effective policing, workers must correctly assess the fertility status of colony members, as well as recognize the origin of offspring through pheromones present on the egg surface or post-embryonic cuticle. In some cases, queens actively mark suspect individuals with pheromones to direct the policing, as discussed below.

Similar to the well-studied phenomenon in honeybees (refs?), workers of many ant species will cannibalize eggs laid by nestmate workers, which presumably lack queen pheromones that mark the queen-laid eggs. *Pachycondyla* (a ponerine genus) workers normally lay a few non-viable trophic eggs, which are offered immediately to the queen as food (Dietemann and Peeters 2000). However, if separated from the queen by a double mesh, workers start to lay viable eggs capable of embryonation and further development, suggesting that a non-volatile queen pheromone inhibits workers from reproducing (Dietemann and Peeters 2000), in line with the evidence discussed in the previous section. Worker-laid eggs, if detected, are eaten by nestmate workers at a higher frequency than queen-laid eggs (D'Ettorre et al. 2004). Such policing of worker-laid eggs was more prominent in queenright than queenless colonies, as well

as more common in polygyne colonies than monogyne colonies (D'Ettorre et al. 2004), alluding to a link between the intensity of queen pheromone and degree of policing. Additionally, the pheromonal cues by which workers distinguish egg origin were persistent and non-transferable, as workers could still distinguish worker-laid eggs and queen-laid eggs after 45 minutes of mutual contact between the eggs (D'Ettorre et al. 2006).

Workers in the genus *Formica* are highly fertile and can lay eggs even in the presence of the queen (Helanterä and Sundström 2005, 2007b). Egg-transfer experiments showed that workers distinguish nestmate eggs from non-nestmate eggs, and worker-laid eggs from queen-laid eggs, but the latter ability was displayed only when an adult queen was present (Helanterä and Sundström 2007a; Helanterä and Ratnieks 2009a, b; Chernenko et al. 2013). Analysis of the hydrocarbon profile of eggs showed robust and consistent differences among species, colonies, and even among matrilines within a colony, demonstrating a link between genetic variation and pheromonal variation (Helanterä et al. 2014; Helanterä and d'Ettorre 2015).

In *Temnothorax* species, workers in queenright colonies produce mostly trophic eggs. After the removal of the queen, workers fight to establish hierarchy and dominant workers produce viable male-destined eggs in the absence of the queen (Heinze et al. 1997). When both queen-produced and worker-produced eggs were introduced to queenright colonies, worker-laid eggs were not preferentially targeted (Stroeymeyt et al. 2007). Instead, egg-laying workers from the queenless sub-colony were attacked when reunited with the queenright sub-colony. However, only few workers attacked the egg-layers and these aggressive workers became reproductively active themselves in the absence of the queen (Stroeymeyt et al. 2007).

In *Novomessor cockerelli* (previously *Aphaenogaster cockerelli*), workers also retain functional ovaries that can produce viable unfertilized eggs. Unlike *Formica*, worker eggs are

not policed in *N. cockerelli* colonies; importantly, the absence of discrimination in this context is associated with a lack of substantial differences in the surface chemical profiles of the two types of eggs (Smith et al. 2008a). Nonetheless, queenright colonies did not tolerate the presence of a supernumerary reproductive female, such that any workers whose ovaries become activated were attacked by nestmate workers as well as by the queen herself (Smith et al. 2011). Smith et al. (2009) suggested that worker reproductive status was signaled by cuticular hydrocarbons, particularly unbranched alkanes such as tri-, tetra-, penta-, and hexacosane. These compounds were produced by the queens as well as reproductive workers, but were absent in non-reproductive workers. Application of the compounds to the cuticle of live non-reproductive workers induced aggression from nestmate workers, but only when a queen was present, and the queen herself did not display aggression toward the chemically treated worker. Subsequently, Smith et al. (2012a) suggested that the queen directed aggression towards a reproductive worker by discharging compounds from her Dufour's gland, located in the gaster (abdomen,) onto the worker to mark it for aggression by nestmates. This notion was supported by the presence of a set of queen-specific chemicals from the queen's Dufour's gland on her gaster after she attacked a reproductive worker, and the observation that application of queen's Dufour's gland extract on an worker elicited lethal aggression from nestmate workers. Additionally, the queen herself was often attacked, and even killed, by workers when she behaved aggressively toward a reproductive worker, likely due to self-contamination by the gland content. This is similar to the situation in *Dinoponera quadriceps*, where high-ranking, reproductive gamergates also mark challengers with Dufour's gland secretion to induce and direct aggression by workers ranking low in the dominance hierarchy toward the challengers (Monnin et al. 2002).

Smith and colleagues also investigated queen pheromones in *Odontomachus brunneus*, a ponerine trap-jaw ant. Similar to *Harpegnathos*, when the original queen was removed from the colony workers competed in antennal dueling to become the new dominants (Powell and Tschinkel 1999). When the colony reunited with the original queen, workers policed the supernumerary reproductive individuals by biting and pulling them. A hydrocarbon (Z)-9-nonacosene ($Z9:C_{29}$) appeared to act as a fertility signal in this case, as 1) the relative abundance of $Z9:C_{29}$ was higher in reproductive than non-reproductive individuals; 2) isolated workers displayed the typical submissive gesture upon detecting a worker treated with $Z9:C_{29}$; 3) in queenright colonies, $Z9:C_{29}$-treated workers were policed by nestmate workers (Medeiros et al. 1992; Smith et al. 2012b, 2013). The role of $Z9:C_{29}$ is conserved across populations, but it evidently functions synergistically with another background chemical, as queens and $Z9:C_{29}$-treated workers from different populations than the queens elicited fewer submissive responses than those from the same population (Smith et al. 2013). Furthermore, $Z9:C_{29}$-treated glass slides failed to suppress egg laying in queenless workers, further suggesting that other compounds are required for the workers to recognize the queen caste/fertility signal in *O. brunneus* (Smith et al. 2015).

Induction of execution of reproductive adults

Since males are haploid in hymenopteran insects, in monogyne ant colonies with singly mated queens, workers are more closely related to their sister workers (r=0.75) than to their own daughters (r=0.5) (Queller and Strassmann 1998). The resulting greater fitness benefit of rearing sisters than rearing daughters was theorized to be a major factor driving the evolution of the "selfless" worker caste, and thus, eusociality in hymenopteran insects (Hamilton 1964; Gardner et al. 2011). Accordingly, kin selection theory suggested that a queenright monogyne colony

typically will not tolerate additional reproductives as this would dilute the relatedness of workers to the brood and subsequently decrease indirect fitness benefits to workers (Hamilton 1964). As a prerequisite to the destruction of supernumerary reproductive adults, queen pheromones signal the presence and identity of the queen to workers.

Solenopsis invicta workers imprint on the pheromonal signature of their mother queen, killing any other dealate (wingless reproductive) queens, including newly mated queens that attempt to enter the colony after a mating flight, as well as gynes reared in their natal colony (full sisters) who attempt to reproduce, presented to them in a variety of different laboratory experiments (Fletcher and Blum 1983b; Gotzek and Ross 2007). Only when a monogyne colony is rendered queenless for a week or more will it accept an unrelated queen, and the longer the colony stays queenless, the more accepting of a foreign queen it becomes (Fletcher 1986; Vander Meer and Alonso 2002). Additionally, when presented with multiple reproductive queens, such hopelessly queenless workers usually select the most physogastric one, which supposedly has a higher amount of some attraction pheromone (Fletcher and Blum 1983b).

In *Aphaenogaster senilis*, where colonies reproduce through fission, workers attack supernumerary gynes and only the oldest gyne ascends to become the sole reproductive queen (Chéron et al. 2009). In Argentine ants *Linepithema humile*, queenless colonies show lower aggression towards intruder queens, compared to queenright colonies, which usually kill intruder queens within 24 hours (Vásquez and Silverman 2008). Fecundity of the intruding queens did not affect adoption decisions, but queens with a more similar CHC profile to the nestmate queens are more likely to be accepted (Vásquez and Silverman 2008; Vásquez et al. 2008). Thus, there appears to be a tight but variable linkage between queen pheromones involved in nestmate recognition, fertility signaling, and regulation of reproduction in ants, and a major task for the

future is to determine whether in specific cases these comprise one or more unique semiochemical systems.

Regulation of colony social structure

Ant colonies can be composed of a single family headed by a single queen and her offspring workers (monogyny), or a group of families headed by multiple queens (polygyny). Phylogenetic analysis suggested that eusociality of ants evolved under the monogyne condition, while polygyne forms evolved independently in many ant taxa (Ross and Carpenter 1991; Huges et al 2018). In a general sense, the evolution of social structure, from monogyny to polygyny and the evolution of eusociality in ants, from solitary lifestyles to colony social lifestyle, raised similar problems on how individuals are willing to share to forgo reproductive rights. Thus, valuable insights can be gained from the understanding of how colony social structure is regulated.

Queens pheromones are also involved in the regulation of such variation in colony social structure, an important but overlooked class of function. Possibly, such function of queen pheromones is ubiquitous in diverse ant taxa (Hölldobler and Carlin 1985; Evison et al. 2012; Abril and Gómez 2019), but the only such case that has received careful study to date is the regulation of colony social form in *S. invicta*. Both monogyne and polygyne social forms are present in this species: the monogyne form houses a single reproductive queen in a colony while the polygyne form houses multiple reproductive queens, as many as a few hundred, in a colony. These social forms are distinct from each other not only in the number of queens but also in many natural history traits, such as nest density in the habitat, the average weights of both mature

alate gynes and reproductive queens, and worker size distributions (Keller and Ross 1995; Gotzek and Ross 2007; Tschinkel 2013; Huang and Wang 2014).

The genetic underpinning of this social form polymorphism in *S. invicta* and several congeners is an inversion-based selfish genetic element termed the *Social b* (*Sb*) supergene. The element spans a large portion of chromosome 16, estimated to be 11.4 Mb encompassing over 500 described genes (Helleu et al. 2022), and comprises three adjacent inversions that evidently spread throughout the group of socially polymorphic *Solenopsis* species closely related to *S.invicta* following a single origin of the complete modular unit (Yan et al. 2020; Stolle et al. 2022; Helleu et al. 2022). The supergene does not occur in the monogyne form, where all female colony members are homozygous for the alternate, wild-type haplotype (*SB*). Workers in a queenright monogyne colony tolerate only a single *SB/SB* reproductive queen, executing any additional queens of any supergene genotype, as discussed in the previous section (Fletcher and Blum 1983b). In polygyne colonies, however, all reproductive queens are carriers of the supergene. In the North American (invasive) range, the sole *Sb* haplotype variant has a strong recessive deleterious effect on fitness, so that all polygyne queens necessarily are heterozygous for the supergene (genotype *SB/Sb*), while *Sb/Sb* workers and gynes may occur at very low to negligible frequencies (Ross and Keller 2002; Gotzek and Ross 2009). The striking genotype composition of polygyne queens is enforced by workers in a green-beard fashion. Polygyne workers will accept additional queens as reproductive nestmates, but only those carrying the *Sb* supergene, while they are intolerant of and execute *SB/SB* queens, including most nestmate *SB/SB* gynes shortly after they emerge as adults (Ross and Keller 1998; Keller and Ross 1998).

The pheromonal basis of genotype signal was first demonstrated by the finding that polygyne workers rubbed against SB/SB queens were attacked by their nestmate workers (Keller

and Ross 1998). Subsequent studies of the cuticular chemicals of queens that varied in their *Sb* genotype and state of sexual maturity identified specific cuticular hydrocarbons uniquely present on the cuticle of *SB/Sb* queens, the abundance of which increased as the fertility of the queen increased (Eliyahu et al. 2011). Trible and Ross (2016) tested worker responses towards paper dummies treated with queen solvent extracts and confirmed the presence of a supergene pheromone: polygyne workers showed strong preferences toward polygyne queen extracts over monogyne queen extracts. In the latest effort to identify queen supergene pheromone components, Zeng et al. (2022) devised improved, high-throughput bioassays that evaluated polygyne worker preferences between differently treated dummies. Paper dummies dosed with effective queen supergene pheromones were quickly retrieved into the nest by workers, where they were retained for eight hours or more. These results confirmed that a blend of unsaturated CHCs from queen cuticular rinses function as a signal of the *Sb* genotype to workers. Additionally, Zeng et al. (2022) found that a synergistic queen caste signal residing in a fraction including polar cuticular compounds (the CHCs are apolar) was required for the workers to recognize queens as a distinct caste and assess their reproductive status, congruent with the findings of Dietemann et al. (2003) in *Myrmecia*. Recent efforts by Zeng and colleagues resulted in creation of a synthesized blend of 19 unsaturated CHC molecules that fully reproduced the natural pheromonal effects. Additional experiments are still needed to reveal the exact combination and the relative proportions of the blend constituents that are necessary and sufficient to elicit fully the characteristic discrimination behavior.

Discussion

Ant queen pheromones exhibit a variety of functional properties revealed by experimental analysis (summarized in Fig 2-1), involving the attraction of workers, contribution to colony

identity and cohesion, maintenance of reproductive dominance, and mediation of colony social organization. Several functions or properties described are hypothetical, because the chemical nature of the signal was not demonstrated experimentally. In other words, the presumed functions were associated with the presence of the queen without direct causal mechanisms or links to any isolated chemicals or even crude solvent extracts from queens. I include such studies to help lay out a functional framework for ant queen pheromones and to provide guidance to future studies.

Despite decades of research, very few compounds have been identified and explicitly shown to induce one or more of the discussed pheromonal effects. Several characteristics of queen pheromones are helpful for the identification of their specific components. To begin with, the candidate compounds are often uniquely present in the queen caste in species with pronounced caste dimorphism. Additionally, the amount of candidate compounds may be correlated with fertility status such that individuals with the highest fertility produce the highest levels of pheromones. Consequently, a more fertile queen should also exert a stronger pheromonal effect than a less fertile one (Ortius and Heinze 1999; Oi et al. 2015).

Like any biological phenomenon, exceptions to the evident general characteristics of queen pheromones exist. A group of piperidine molecules was hypothesized to be the queen pheromone constituent signaling queen reproductive status in *S. invicta* because these were the most abundant compounds on the cuticle of queens and their abundance was positively associated with fertility (Eliyahu et al. 2011). Contrary to expectations, Zeng et al. (2022) showed that these piperidine molecules did not contribute to the queen caste signal; instead, other polar compounds in trace quantities displayed the expected pheromonal effects. This finding suggests that the

relative abundance of a compound, even in the expected context, is not always a reliable indicator of its role as a queen pheromone.

Traditionally, chemical ecologists aspire to find a single molecule with extensive, if not complete, effects comprising the focal behavioral trait ascribed to the putative pheromone. Such reductionist thinking does not reflect the complex chemical make-up and multiple glandular and tissue origins of most insect pheromones clearly revealed by many studies. The same also applies to ant queen pheromones, especially considering the various functional properties (Dietemann et al. 2003; Smith et al. 2015; Zeng et al. 2022). Thus far, glandular and cellular sources shown to produce pheromonal components in ants include the oenocytes, poison sac, postpharyngeal gland, metapleural gland, and Dufour's gland (Vargo 1997; Vargo and Hulsey 2000; Yek and Mueller 2011; Kocher and Grozinger 2011). Multiple glandular sources of queen pheromones affecting a singular behavioral response have been demonstrated clearly in *S. invicta* (Vargo and Hulsey 2000), and we can expect the same for other ant species.

Most studies to date attempting to identify ant queen pheromones have focused on the CHCs. This seeming obsession with CHCs as queen pheromones is grounded in their critical signaling roles in the colony social life of ants, distinguishing life stages, caste, sex, nestmate, and task status (Martin and Drijfhout 2009). As well, CHCs are readily quantifiable and often identifiable by gas or liquid chromatography coupled with mass spectrometry (Oi et al. 2015), making them attractive objects of study. Despite that, in only a handful of cases have CHCs been experimentally confirmed to be active components of queen pheromones (Holman 2018). While studies on CHC queen pheromones remain important, it is high time that researchers look further at other classes of molecules potentially playing important roles as queen pheromones (Villalta et al. 2018). Once a holistic suite of queen pheromones is identified in even a few model ant

species, advances in the neurophysiology of odor reception and the genomic and genetic basis of reproductive division of labor will follow, providing opportunities to grasp more fully how queen pheromones work to regulate ant colony life.

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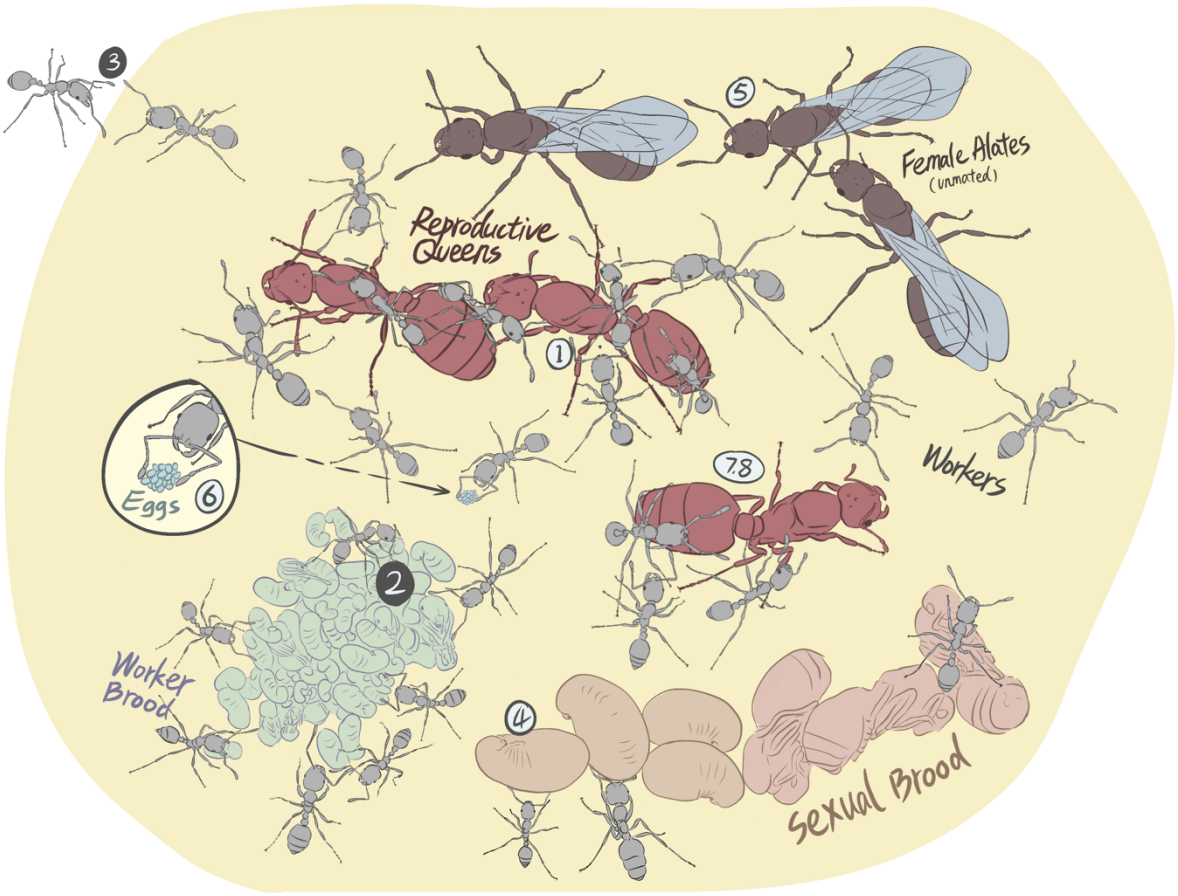


Figure 2-1 Summary of generalized functional properties of queen pheromones in ants, as represented in a polygyne *Solenopsis invicta* colony. These functions include: 1) attracting workers; 2) inducing colony maintenance (such as brood care); 3) inducing nestmate discrimination; 4) inhibiting larval sexual development; 5) inhibiting adult reproductive development; 6) inducing worker policing; 7) inducing execution of supernumerary queens in monogyne colonies; and 8) mediating the regulation of queen acceptance in polygyne colonies. White numbers in dark circles indicate that a chemical basis has not yet been demonstrated for this functional category.

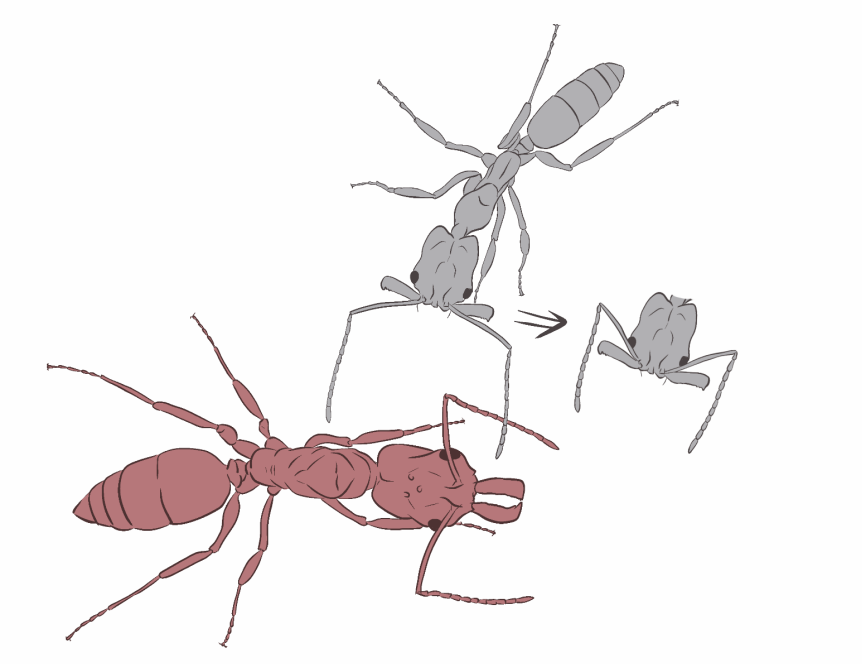


Figure 2-2 Workers (gray) of *Odontomachus* species display typical submissive gestures (crouching and retracting their antennae) upon detecting a queen (red) or queen pheromones.

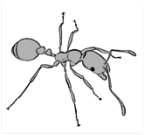
Types of female	 Worker	 Alate Gyne	 Polygyne Reproductive Queen (or Virgin Dealate)	 Monogyne Reproductive Queen
Mating Status	Unmated	Unmated	Unmated/Mated	Mated
Fertility Condition	☆☆☆☆☆	☆☆☆☆☆	★★★☆☆	★★★★★
Pheromone Quantity	☆☆☆☆☆	★☆☆☆☆	★★★☆☆	★★★★★
Effect Strength of Queen Pheromone	☆☆☆☆☆	☆☆☆☆☆	★★★☆☆	★★★★★

Figure 2-3 Characteristics of ant queen pheromones. Drawings are exemplified by *Solenopsis invicta*. Number of filled stars represents the relative rating in each measure.

CHAPTER 3

CHARACTERIZATION OF QUEEN SUPERGENE PHEROMONE IN THE RED IMPORTED FIRE ANT USING WORKER DISCRIMINATION ASSAYS[2]

[2] Zeng, H., Millar, J. G., Chen, L., Keller, L., & Ross, K. G. 2022. *Journal of Chemical Ecology*, *48*(2), 109-120; reprinted here with permission of the publisher

Abstract

Ants use chemical signals to communicate for various purposes related to colony function. Social organization in the red imported fire ant, *Solenopsis invicta*, is determined by the *Sb* supergene, with colonies of the monogyne (single-queen) form lacking the element and colonies of the polygyne (multiple-queen) form possessing it. Polygyne workers accept new reproductive queens in their nest, but only those carrying *Sb*; young winged queens lacking this genetic element are executed as they mature sexually in their natal nest or as they attempt to enter a foreign nest to initiate reproduction after mating and shedding their wings. It has been suggested that queen supergene genotype status is signaled to workers by unsaturated cuticular hydrocarbons, while queen reproductive status is signaled by piperidines (venom alkaloids). We used high-throughput behavioral assays to study worker acceptance of paper dummies dosed with fractions of extracts of polygyne queens, or of blends of synthetic counterparts of queen cuticular compounds. We show that the queen supergene pheromone comprises a blend of monoene and diene unsaturated hydrocarbons. Our assays also reveal that unsaturated hydrocarbons elicit discrimination by polygyne workers only when associated with additional compounds that signal queen fertility. This synergistic effect was obtained with a polar fraction of queen extracts, but not by the piperidine alkaloids, suggesting that the chemical(s) indicating queen reproductive status are compounds more polar than cuticular hydrocarbons but are not the piperidine alkaloids. Our results advance understanding of the role of chemical signaling that is central to the regulation of social organization in an important invasive pest and model ant species.

Key Words-*Solenosis invicta*, supergene, pheromone, cuticular hydrocarbons, piperidines, unsaturated hydrocarbons.

Introduction

Ants (Hymenoptera: Formicidae) are enormously successful animals in a variety of terrestrial ecosystems, judging from both the proportion of biomass they represent and their ecological diversity (Hölldobler and Wilson 1990). The ecological success of ants is directly linked to their social habits, with division of reproduction and task specialization among members of different castes being a hallmark of most species. Their often complex and highly interdependent social habits can yield numerous ecological advantages over solitary animals under particular environmental conditions, most notably high efficiency in carrying out potentially challenging or risky tasks such as brood care, resource acquisition, and nest defense (Hölldobler and Wilson 1990).

To coordinate activities and maintain the cohesiveness of the colony social unit, ants have evolved communication systems that ordinarily involve the production and perception of diverse chemical compounds (Hölldobler 1995). For instance, colony members have unique cuticular semiochemical profiles advertising their life stage, caste, and sex (Kocher and Grozinger 2011), which allow adult workers to distinguish among these groups and engage in relevant interactions with their nestmates. Additionally, workers utilize various volatile and non-volatile pheromones to communicate in real time the necessity to perform complex tasks such as exploiting ephemeral food resources, expelling would-be intruders, or quickly retrieving their queens and brood when the nest is disturbed (Vander Meer and Alonso 1998).

In most ant species, a distinct morphological queen caste is present, and these individuals are the sole or primary reproductive females in a colony (Crozier and Pamilo 1996). They communicate their presence to workers via pheromones. Pheromones also influence caste differentiation of the female brood, suppress the reproductive development of other adult queens,

and modulate various behaviors of workers that are involved in regulating colony queen numbers (Fletcher and Blum 1981, 1983; Nonacs and Keller 1993; Vargo and Laurel 1994; Kocher and Grozinger 2011). Thus, understanding the composition and functions of queen pheromones can provide crucial insights into the regulation of colony ontogeny and can inform hypotheses on the evolution of social organization.

The red imported fire ant, *Solenopsis invicta*, has been the subject of many studies of the characteristics and functions of putative pheromones produced by queens. Since its introduction into the United States almost a century ago, *S. invicta* has become widespread and well established throughout the American South and in other Pacific-rim countries (Ascunce et al. 2001; Wylie et al. 2020). This invasive pest species causes substantial economic and ecological damage linked to its enormous colony populations, massive nest structures, aggressive nature, and potent venom (Jemal and Hugh-Jones 1993; Tschinkel 2013). However, it also has become a model organism for studies of behavior, ecology, and social evolution, due in part to its abundance, ease of collection, and amenability to rearing in the laboratory, facilitated by a vast literature on its basic natural history and social biology.

Perhaps the most important biological feature of *S. invicta* contributing to its utility as a model organism is its polymorphism in colony social organization. This polymorphism is manifested as the co-occurrence of two distinct social forms. A colony may house a single reproductive queen (monogyne form) or multiple reproductive queens (polygyne form) (Fletcher et al. 1980; Huang and Wang 2014). Polygyne behavior is directly correlated with the presence of an inversion-based complex of tightly linked genes spanning half of chromosome 16, termed the *Social b* (*Sb*) supergene (Wang et al. 2013), and which is marked by the *b* allele of the gene *Gp-9* in U.S. populations (Ross 1997; Keller and Ross 1998; Krieger and Ross 2002). While the

Sb supergene is present in all reproductive queens and generally more than half of the workers in polygyne colonies, it is entirely absent among the inhabitants of monogyne colonies (Ross 1997; Ross and Keller 1998, 2002). This region of reduced recombination includes several hundred genes (Wang et al. 2013), many of which are thought to have essential functions in the synthesis and/or transport of cuticular semiochemicals, or in their perception (Wurm et al. 2011). At the behavioral level, colony queen number and identity are regulated by the adult workers. Temporarily queenless monogyne workers (which lack the *Sb* supergene) will accept only a single replacement queen that also lacks *Sb,* whereas polygyne colonies, all of which contain workers with the *Sb* supergene, accept multiple queens, but only those that bear *Sb* (Keller and Ross 1998; Ross and Keller 1998). Such "worker *Sb* discrimination" is mediated by chemical signals present on the queen's cuticle that presumably communicate the supergene status of individual queens (Keller and Ross 1998; Trible and Ross 2016).

To aid in identifying pheromones underlying worker *Sb* discrimination, Eliyahu et al. (2011) conducted detailed chemical analyses of whole-body extracts of fire ant females. They found that cuticular semiochemical profiles of *S. invicta* queens vary according to the social form, age, degree of sexual maturity (reproductive development), and supergene genotype of individuals. Significantly, the concentration of a particular group of abundant, queen-specific piperidine venom alkaloids (Vander Meer et al. 1980; Vargo 1999; Vargo and Hulsey 2000) was positively associated with reproductive maturity of queens regardless of their supergene genotype. One of these piperidines, *cis*-2-methyl-6-*n*-undecylpiperidine (solenopsin), is the most abundant chemical in extracts of sexually mature queens, with the level increasing as a queen becomes fully reproductive (Eliyahu et al. 2011), leading these authors to hypothesize that the compound conveys information on queen fertility status.

Eliyahu et al. (2011) further determined that unsaturated cuticular hydrocarbons (CHCs), which along with myriad other CHCs form a protective wax layer on the surface of the insect cuticle (Blomquist and Ginzel 2021), differ in their abundance in queens with or without the *Sb* supergene. Young winged queens bearing the supergene produced increasing levels of unsaturated CHCs as they matured sexually in their natal nests. The highest amounts were produced by the fully reproductive queens carrying the *Sb* supergene while queens lacking the *Sb* supergene failed to produce appreciable amounts of unsaturated CHCs, no matter what their age or stage of reproductive development. Importantly, parallel time courses in the reproductive ontogeny of queens, in their dynamic CHC profiles, and in escalation of polygyne worker aggression toward queens lacking *Sb* as they mature sexually led Eliyahu et al. (2011) to hypothesize that unsaturated CHCs convey information as to whether or not a queen bears the supergene.

Trible and Ross (2016) subsequently took an important step in investigating the queen pheromone that elicits worker *Sb* discrimination after developing high-throughput worker behavioral assays. They confirmed the presence of a supergene-marking pheromone by showing that cuticular extracts of queens, applied to paper dummies, induced appropriate worker responses of acceptance or rejection as effectively as live queens or fresh queen corpses. We here extend the work of Trible and Ross (2016) by incorporating improved assay procedures and scoring metrics that capture the various behavioral elements that collectively emerge as the process of queen acceptance by workers, and by testing various constituents of queen cuticular extracts. Our goal was to better characterize the critical components of queen cuticular pheromones that elicit the worker responses central to regulating colony social organization in *S. invicta*.

Methods and materials

Ant Collection and Rearing. Colonies of *S. invicta* were collected in Athens-Clarke, Oconee, and Oglethorpe Counties, Georgia, U.S., from 2016 to 2020. Soil mounds separated by at least 10 m from each other were excavated into 5-gallon talced buckets, then returned to the laboratory (Banks et al. 1981). After removal from the soil by means of a water dripping method, colonies were maintained in large plastic trays, the sides of which were treated with Fluon® anti-traction compound, in a rearing room under standard conditions; all colonies and assay fragments derived from them were provided with nests (plastic petri dishes with plaster bottoms; Ross 1988) and tubes of water, and they were fed every other day with a diet including freshly frozen or re-hydrated freeze-dried insects as well as high-sugar and high-protein artificial diets (Ross 1988; Ross and Keller 2002; Eliyahu et al. 2011). The social form of each colony was determined by carefully searching for reproductive queens. These determinations were confirmed by checking for the presence of *Sb* supergene genotypes diagnostic for the polygyne form; pooled DNA extracts of 10 – 20 workers per colony were used as template in a modified multiplex PCR procedure for scoring genotypes at the marker gene *Gp-9* (Valles and Porter 2003; Ross and Shoemaker, 2018).

Cuticular Extract from Monogyne Incipient-Reproductive Queens. To obtain the chemical extracts that provide workers with the information that the paper dummies (below) were reproductively active queens, we first removed the single reproductive queens (wingless queens with developed ovaries) from monogyne colonies also containing young pre-reproductive winged queens. Such removal induces the latter queens in the colony to shed their wings in a few days and initiate reproduction themselves by laying unfertilized eggs (Vargo 1992; Vargo and Laurel 1994; Mir et al. 2003). The cuticular extract from these wingless monogyne incipient-

reproductive (MIR) queens contains a chemical signal of queen reproductive activity that is attractive to queenless workers of either social form. We examined the queenless monogyne colonies daily after removing the original queen and collected healthy MIR queens that were not being attacked by workers. The MIR queens were then stored immediately in a -80°C freezer pending their use in an assay. MIR extract was obtained by placing three MIR queen corpses in 250 □l of hexane in 12 x 75 mm glass culture tubes. The tubes were then placed on an orbital shaker for 20 min. The queen corpses were removed, a paper dummy was placed in the MIR queen extract, and the dummy was dried down under a fume hood for ≥4 h, until there were no visible traces of hexane. The three-queen-equivalent (3QE) dosage in hexane extract added to each dummy corresponds to the dosage of treatment compounds used in all of the experiments described below. Our preliminary experiments suggested that 3QE was the optimal dosage for obtaining unambiguous, repeatable results across experiments.

Cuticular Extract and Extract Fractions from Polygyne Queens. Reproductive queens from polygyne colonies collected in Georgia were shipped on Dry Ice to The University of California, Riverside, where the chemical extraction and fractionation procedures were performed. Samples were thawed, then soaked in hexane for 10 min, yielding crude cuticular extract. Part of this crude extract was set aside to be used in Positive Reference assays (below), while the remainder was fractionated in multiple steps (see Fig. 2-1a, Fig. 2-2). The fractionation steps are described in detail in the Supplementary Online Information. Briefly, the crude extracts were first fractionated into nonpolar (saturated and unsaturated hydrocarbons) and polar compounds by liquid chromatography on silica gel and/or silica gel impregnated with 10% silver nitrate. The unsaturated CHC fraction was then further fractionated into monoenes, dienes, and trienes using a silver ion-loaded ion exchange column. The polar compounds were fractionated into

piperidines and other polar compounds; henceforth, we refer to the latter fraction generically as the “polar fraction”. Initial attempts to separate the piperidines as a class had variable outcomes; in some cases, elution of silica gel columns successively with ether and ethyl acetate removed the piperidines with other polar compounds, whereas in other cases, it was necessary to further elute the column with 4% Et_3N in ether to recover the piperidine fraction. Because of these inconsistencies, we developed an alternative fractionation method by first extracting the crude hexane extract with aqueous acid to remove the piperidines as their salts. The resulting aqueous solution was then made basic with NaOH, and the piperidines were back extracted into ether as their neutral forms. The remainder of the hexane extract was then fractionated into saturated and unsaturated hydrocarbons and more polar compounds as described above. Each treatment chemical fraction was concentrated to 25 □l per 3 QE and stored in a -80°C freezer before application to dummies or queen corpses.

Synthetic Cuticular Compounds. We synthesized eleven unsaturated CHCs present in reproductive polygyne queen cuticular profiles but effectively absent from those of reproductive monogyne queens, making them candidate components of a signal of supergene status (Eliyahu et al. 2011). Seven monoenes and four dienes were synthesized (Table 1). These CHCs were constituted into two solutions, in ratios matching those found in the queen crude extract cuticular profile. The first solution contained the five most abundant compounds, while the second contained all eleven. We also synthesized the most abundant piperidine, *cis*-2-methyl-6-undecylpiperidine (solenopsin A) which is a prominent component of the cuticular chemical profiles of all reproductive *S. invicta* queens but is effectively absent from pre-reproductive queens (and workers). According to Eliyahu et al. (2011), these piperidines may signal queen caste or fertility status. The (2*R*,6*S*)-2-methyl-6-undecyl-piperidine and (2*S*,6*R*)-2-methyl-6-

undecylpiperidine enantiomers (Fig. 2-3) were both synthesized. The two enantiomers may be required in a specific ratio to be functional, so we first tested solutions of the individual enantiomers, then a 1:1 racemic blend. The detailed methods used in estimating the relative natural abundance of each CHC on the queen cuticle, the approximate absolute amounts for dosage calculations, and the procedures for synthesizing the CHCs and piperidines are described in the online Supplementary Information.

Behavioral Assay Setup and Procedure. Our general assay methods were developed based on the findings of Trible and Ross (2016) that polygyne queen crude cuticular extracts contain chemicals comprising a putative pheromone (or blend of pheromones) that mediates worker *Sb* discrimination behavior. We tested specific fractions of crude hexane extracts of polygyne queens, with the aim of identifying the key fractions or even individual chemicals that are responsible for this specific pheromonal effect.

Each assay unit was set up by using a plastic spoon to transfer 3 g of adult polygyne workers (about 5000 ants) from the source colony into a clean plastic tray (40 x 25 x 5cm) coated with Fluon® on the inside walls (Trible and Ross, 2016; Fig. 3-1a). A temporary nest, a water tube, and food (a mix of peanut butter and baby food) were provided to each unit. The workers were held in the tray for 24 h to make them more responsive to queen signals by virtue of having been queenless for a short period. The unit was then presented with two paper dummies placed equidistant from the nest on the floor of the tray. The paper dummies were 4 x 10 x 2 mm pieces of Ahlstrom Grade 470 chromatography blotting paper (Schleicher & Schuell CSS-470c) marked with a few loops of thin colored copper wire assigned randomly for identification. The size and weight of these paper dummies approximate those of a live *S. invicta* queen. For each replicate, one of the dummies was dosed with MIR reproductive queen extract, acting to control for the

baseline attractiveness due to the queen caste (fertility) signal (Fig. 3-1a). The second, treatment dummy was dosed with a cuticular fraction or reconstructed blend, usually in addition to the MIR extract. Both dummies were dried down before being used in assays. If a treatment contains an appropriate mix of essential queen supergene pheromone components (presented at biologically appropriate relative abundances and dosages), assay workers are expected to display *Sb* discrimination by showing a significant preference for the treatment dummy over the MIR dummy. Such preference was evaluated utilizing a newly developed score metric, explained in detail in the next section.

For each experimental series, we first conducted "Positive Reference" assays for which dummies were dosed with crude hexane extract of polygyne reproductive queens (see movie in the Supplementary Online Information); this was done to obtain a baseline distribution of scores that reflected representative worker *Sb* discrimination behavior and to which treatment score values in the series were compared (Fig. 3-4). In this way, we accounted for variation in uncontrolled factors that might influence worker responses, including the colony of origin of MIR queens, the test colonies used, the time of year, and the length of time the colonies had been in culture.

We conducted the assays for each experimental series by dosing treatment dummies with designated cuticular fractions, solutions of synthetic compounds, or combinations thereof (Fig. 3-4, treatments A to O). We assessed the effectiveness of a treatment by comparing the scores for assays using that treatment with those of the matching Positive Reference assays. A treatment was considered effective if the treatment assay scores and the matched Positive Reference assay scores did not deviate significantly from one another, using paired Wilcoxon tests ($P > 0.05$). Particular treatment groups and their corresponding Positive Reference assay group were from

the same parallel set of colonies—in other words, a specific source polygyne colony for the assay workers was always paired with a specific source colony for the MIR queen extract (Fig. 3-1a). This experimental design holds constant among-treatment variation arising due to interactions between specific polygyne source colony-MIR source colony pairs. All statistics were analyzed using R 3.4.4 and RStudio 1.1.463 (RStudio Team, 2016; R Core Team, 2017).

Score Metric. We developed a novel score metric to measure the degree of preference of workers towards the treatment dummy over the MIR dummy (or queen corpses used in another set of assays), if any, in each assay replicate. This metric was based on earlier experiments (Trible and Ross 2016) combined with extensive preliminary observations of how workers treat both fresh polygyne reproductive queen corpses and paper dummies dosed with crude queen cuticular extracts. The total score ranges from -2.5 to 3, with a higher positive value indicating a stronger preference for the treatment dummy and negative values indicating that the MIR dummy was preferred.

This total score is the sum of two measures, "Initial Preference" and "Retention." The Initial Preference measure assesses how quickly the dummies were retrieved into the nest (a behavior signaling acceptance of a queen or queen-like surrogate (Fig. 3-1b; see Trible and Ross [2016]) as well as the relative difference in retrieval times between the treatment ($T_{treatment}$) and MIR (T_{MIR}) dummies (or queen corpses) over the initial 120 min of the assay period. The Initial Preference measure ranges from -0.5 to 1 and is calculated as:

$$[120 - (2 * T_{treatment}) + T_{MIR}] / 240$$

If a dummy was not retrieved within the initial 120 min period, we set $T_X = 120$. The Initial Preference measure is highest if the treatment dummy is retrieved relatively quickly and the MIR dummy relatively slowly (Fig. 3-1c). As with the total score, a higher positive value indicates a

stronger preference for the treatment dummy and a lower negative score indicates a stronger preference for the MIR dummy; scores around zero indicate no preference.

The Retention measure considers the positions of the two dummies in the assay unit (inside or outside the nest) at 2 h and at 8 h after their introduction. The Retention measure was +1 if the treatment dummy was inside the nest. If neither dummy was inside the nest, the Retention measure was zero; if the MIR dummy was inside while the treatment dummy was outside, the Retention measure was -1. Therefore, the Retention measures, taken at 2 h as well as at 8h, range totally from -2 to 2. After 8 h, the ants usually started to remove even highly preferred dummies from the nest, likely because the added chemicals degraded, volatilized fully, or were absorbed or adsorbed onto workers, so assays were terminated at this point. Similar to the Initial Preference measure, higher positive, zero, and lower negative Retention measures indicated a stronger tendency to retain the treatment dummy, no preference, or a stronger tendency to retain the MIR dummy, respectively.

Assays of Queen Corpses Adulterated with CHC components. We hypothesized that if the unsaturated CHCs occurring on polygyne queens represent the *Sb* supergene signal, then addition of these chemicals directly onto queens lacking *Sb* would convert their social phenotype, in the sense that workers would treat them as if they possessed the supergene. Applying unsaturated CHCs in hexane solution directly onto live queens might stress or even kill them, so we tested this hypothesis using queen corpses. We used the standard dummy assay procedures but replaced paper dummies with freshly frozen MIR queen corpses, distinguished by colored copper wires (randomly assigned) tied around their petioles. The procedures for these assays were identical to those for the assays with filter paper dummies (3 QE dosages of treatment chemical in 25 □l hexane), except that doped monogyne queen corpses rather than dummies constituted the test

objects. The first two groups of corpse assays paralleled the "treatment vs control/MIR" design (Fig. 3-5, Assays Co-A and Co-B), while the third group directly compared unsaturated CHCs (as treatment) and saturated CHCs (as control) to control for the effect of adding any hydrocarbons (Assay Co-C).

Results

Validation of Standard (Dummy) Assay and Score Metric. Our assay accurately recapitulated typical worker responses towards live polygyne queens and live MIR queens: scores for workers given dummies treated with queen extract (Positive Reference assays, mean score 2.42) were statistically indistinguishable from scores for workers given live queens (mean score 2.59, N = 13, *Mann-Whitney test*, $P = 0.912$).

Queen Sb Supergene Signaled by Unsaturated CHCs. Our first set of experiments (Fig. 3-4, group 1) aimed to test the role of cuticular hydrocarbons in queen *Sb* signaling. The first experiment (Fig. 3-4, treatment A) revealed that the combination of MIR extract and cuticular hydrocarbons (alkanes + alkenes) elicited responses from workers towards treated dummies that were as high as the responses to dummies treated with crude hexane extract of polygyne reproductive queens (positive reference) (*Wilcoxon test*, $P = 0.15$).

We next separated saturated from unsaturated CHCs to test their relative efficacy. These experiments revealed that the combination of MIR extract and unsaturated CHCs from polygyne queens (Fig. 3-4, treatment B) elicited a full response, with no significant difference from the positive reference (*Wilcoxon test*, $P = 0.116$). In contrast, the MIR extract + saturated CHCs did not elicit retrieval of treated dummies, with a response significantly lower than the positive control (Fig. 3-4, treatment C, *Wilcoxon test*, $P < 0.001$).

When dummies were coated with unsaturated CHCs but no MIR extract there again was no discrimination response by workers (Fig. 3-4, treatment D, *Wilcoxon test*, $P < 0.005$), indicating that the MIR extract is necessary to elicit such responses. These data show that the MIR extract includes some signal of queen fertility or caste, which evidently fools workers into interacting with the dummies as if they were real queens; as such, it is required as a synergist of the unsaturated CHCs in signaling the presence of the *Sb* haplotype. Without it, workers simply disregard CHC-treated dummies.

Queen Sb Supergene Signal Requires both Monoenes and Dienes. To narrow the set of candidate chemicals comprising the queen supergene pheromone, we further fractionated the unsaturated CHCs into monoenes (one double bond) and dienes (two double bonds) for separate testing (Fig. 3-4, group 2). The combined application of MIR extract + monoenes + dienes from polygyne queens resulted in effective retrieval and retention behavior by the workers (Fig. 3-4, treatment E, *Wilcoxon test*, $P = 0.345$). In contrast, MIR extract + polygyne queen monoenes (Fig. 3-4, treatment F, *Wilcoxon test*, $P < 0.01$) or dienes (Fig. 3-4, treatment G, *Wilcoxon test*, $P < 0.001$) were not effective, indicating that both monoenes and dienes must be present to elicit full *Sb* discrimination behavior.

Queen Sb Supergene Signal Not Recreated Using Blends of Synthetic CHCs. We next generated synthetic blends of the most abundant unsaturated CHCs to test whether we could elicit worker *Sb* discrimination similar to that triggered by the natural fraction (Fig. 3-4, group 3). We constructed synthetic blends of unsaturated CHCs comprising either the five or the eleven most abundant unsaturated CHC compounds in proportions and concentrations matching those in the natural blend on a reproductive polygyne queen's cuticle (Table 1). Neither reconstructed blend succeeded in reproducing the effect of the dummies treated with crude hexane extract of

polygyne reproductive queens when applied on an MIR background (Fig. 2-4, treatments H, I, *Wilcoxon tests*, all $P < 0.005$), suggesting that additional components might be required.

Queen Caste Identity/Fertility Signaled by Non-Piperidine Polar Compounds. Because previous work suggested that *cis*-dialkylpiperidines, which are abundant in extracts of reproductive queens, could be the substances signaling queen fecundity (Eliyahu et al., 2011), we investigated whether piperidines had a similar synergistic effect to the MIR extracts when used together with the unsaturated CHC compounds. In these experiments (Fig. 3-4, group 4) we also investigated the role of the non-piperidine polar fraction by conducting three separate experiments where unsaturated CHC compounds were combined either with the piperidines + polar fraction (treatment J), the polar fraction without the piperidines (treatment K), or the piperidines without the polar fraction (treatment L). These experiments revealed that the presence of the polar fraction with unsaturated CHCs elicited a response of workers towards dummies that was as strong as the response to dummies coated with crude hexane extract of polygyne reproductive queens (the positive reference) (treatments J and K, *Wilcoxon tests*, $P = 0.589$ and 0.113).

However, the piperidines apparently do not play a functional role in the pheromonal blend because the pairing of piperidines with unsaturated CHCs was much less effective than the Positive Reference (treatment L, *Wilcoxon tests*, $P = 0.003$). Moreover, there was no significant difference in scores when piperidines were added or not to extracts with the non-piperidine polar fraction + unsaturated CHCs (scores of treatment J vs. K, *Mann-Whitney test*, $P = 0.492$). Additionally, tests of the effects of synthetic versions of the racemic and enantiomeric forms of the major piperidine in extracts of reproductive queens (*cis*-2-methyl-6-undecylpiperidine, solenopsin A) validated that they were not effective at eliciting retrieval behaviors when

combined with the natural unsaturated CHC fraction (Fig. 3-4, group 5, treatment M, N, O, *Wilcoxon tests*, all $P < 0.02$).

Queen Social Phenotype Converted by Applying Unsaturated CHCs. In a final experiment, we conducted tests with queen corpses to investigate whether we could also obtain full *Sb* discrimination responses from workers by the addition of the polygyne queen unsaturated CHC fraction to freeze-killed monogyne queens. Workers retrieved monogyne queen corpses treated with unsaturated CHCs significantly more often ($N = 94.1\%$ of the time) than untreated corpses ($N = 41.9\%$, *Chi-square tests*, $P < 0.001$) or corpses treated with saturated CHCs ($N = 50\%$, Chi-square tests, $P < 0.001$). Also, there was no significant difference in retrieval proportions between untreated corpses and corpses treated with polygyne queen saturated CHCs (*Chi-square tests*, $P = 0.515$), further supporting the view that saturated CHCs play no role in worker *Sb* discrimination. Similarly, Initial Preference measures revealed that workers retrieved corpses treated with unsaturated CHCs significantly more quickly than untreated corpses (Fig. 3-5, Treatment Co-A; *Wilcoxon test*, $N = 16$, $P = 0.003$). In contrast there was no significant difference between corpses treated with saturated CHCs and untreated corpses (Fig. 3-5, Treatment Co-B; *Wilcoxon test*, $N = 16$, $P > 0.05$). Accordingly, workers also retrieved corpses treated with unsaturated CHCs significantly more quickly than corpses treated with saturated CHCs (Fig. 3-5, Treatment Co-C; *Wilcoxon test*, $N = 18$, $P < 0.001$).

Discussion

The presence of the *Sb* supergene in mature/reproductive polygyne queens was previously hypothesized to be signaled by the presence of one or more of the unsaturated CHCs found on the cuticles of such queens but effectively absent from monogyne queens, which lack this

genomic element (Eliyahu et al. 2011). We found that the *Sb* supergene signal indeed comprises unsaturated CHCs, based on a series of experiments progressively reducing the complexity of the CHC chemicals tested and, conversely, by combining relevant fractions to recover worker *Sb* discrimination. We further validated the effectiveness of unsaturated CHCs by applying them to monogyne queen corpses, which successfully converted their sociochemical phenotype from that of a queen lacking the supergene to that of one bearing it, as evidenced by the positive responses of polygyne workers to the treated corpses.

It is not unexpected that the *Sb* supergene signal resides in the CHCs; these molecules have the necessary properties to make them suitable to convey such information—they are collectively abundant and relatively stable on the cuticle, and are variable in their molecular structure and relative abundance (Blomquist and Ginzel 2021). Indeed, they are widely used as pheromones in ants and many other social insects (Gibbs and Pomonis 1995; Van Oystaeyen et al. 2004; Martin and Drijfhout 2009; Blomquist and Bagnères 2010). Additionally, we found that the unsaturated CHCs comprising the *Sb* signals likely consist of a complex blend that includes numerous different hydrocarbon molecules, because neither the monoene nor the diene fractions alone, nor our synthetic CHC blend composed of up to eleven CHC molecules, elicited the full effect of the natural pheromone.

Our findings help link previously reported genomic and gene expression data to fire ant social behaviors. Genes involved in both the production and, presumably, perception of CHCs differ in number and level of expression between the homologous regions of the alternate social chromosomes, Chr16 *Sb* and Chr16 *SB*. On the production side, Fontana et al. (2020) found duplications of genes likely involved in CHC synthesis in the *Sb* supergene that do not occur in the homologous *SB* region of Chr16, including genes encoding fatty acid synthases, desaturases,

elongases, reductases, and cytochrome P450s. The products of these genes are critically involved in various steps of the biosynthesis of unsaturated CHCs (Helmkampf et al. 2015; Holze et al. 2020). Some of the same genes likely involved in unsaturated CHC synthesis were found to be upregulated in *Sb* supergene-carrying (polygyne) queens compared to monogyne queens lacking the element (Nipitwattanaphon et al. 2013), as expected if unsaturated CHCs serve important roles as semiochemicals in the former.

On the perception side, many genes of the insect odorant-binding protein (OBP) family, some members of which have been shown to transport pheromones to odorant receptors in insect chemosensilla (Pelosi et al. 2018), have *Sb*-specific mutations or are duplicated in the *Sb* supergene, and some duplicates potentially have gained new functions via structural or regulatory diversification (Pracana et al. 2017; Dang et al. 2019). A classic example is the OBP gene *Gp-9*, commonly used to diagnose the presence of the *Sb* supergene because of the complete linkage disequilibrium between the two elements in the U.S. (Ross 1997). The *b* allele of *Gp-9*, which occurs in the *Sb* haplotype, has a considerable number of substitutional differences from the wild type *B* allele, which occurs in the *SB* haplotype, and several of these produce amino acid changes that may structurally change the ligand-binding cavity or C-terminal tail of the protein, possibly rendering a functional change to the protein that affects worker chemoperception (see also Crozier 2002; Krieger and Ross2005; Gotzek and Ross 2007). Besides *Gp-9*, many other OBP genes have been shown to be duplicated in the *Sb* supergene (Pracana et al. 2017). At the gene expression level, fourteen OBPs were reported to be differentially expressed between monogyne and polygyne workers (Pracana et al. 2017). Most recently, Dang et al. (2019) showed that an *Sb*-specific, duplicated OBP gene is expressed in the antennae of polygyne workers (as well as elsewhere in their bodies) and proposed that this

unique OBP contributes to the detection of *Sb* signals. Arsenault et al. (2020) further showed that the expression of this OBP was 12-fold higher in *Sb*-carrying pre-reproductive queens than in other queens, pointing to the evolution of gene regulatory mechanisms acting specifically on OBP genes. Additionally, any differences in chemoreception function between *Sb* and non-*Sb* workers may be attributed to variation in their odorant receptors (OR); these are products of another large family of genes that interact with OBPs and their volatile ligands at the sensory periphery (Brand et al. 2018), and so are also critical elements in insect chemoreception (Yan et al. 2020). Cohanim et al. (2018) suggested that many OR genes in the *Sb* supergene were positively selected to evolve novel primary amino acid sequences compared to the wild-type variants and showed that two OR genes are completely deleted in the *Sb* supergene. Collectively, these genomic and transcriptomic data suggest that *Sb* supergene-carrying workers differ from non-*Sb* workers in their abilities to recognize the specific *Sb* signals we characterized, but neurophysiological and/or genetic editing/knockdown studies are needed to confirm these ideas (also Crozier 2002; Dang et al. 2019).

An important finding of our study was that, although unsaturated CHCs are necessary as *Sb* signals to induce worker *Sb* discrimination, they are not by themselves sufficient. Instead, they must be presented along with a reproductive queen caste (or fertility) signal, such as occurs in our crude MIR extract, in order to generate such worker discrimination (Fig. 3-4, treatment B vs D). Previously, a specific component of the piperidines, a class of compounds that are major components of fire ant queen venom, was hypothesized to constitute this queen signal, because it was absent from workers and its abundance in queens was associated with their reproductive state, regardless of supergene status (Eliyahu et al. 2011). However, our results do not support this hypothesis. When the piperidine fraction of polygyne queen extract was paired with

unsaturated CHCs, the blend failed to produce full worker *Sb* discrimination behavior. Synthetic versions of the major queen piperidine also failed to produce such discrimination in several experiments (Fig. 3-4, treatments M, N, O), providing support for the initial finding, although factors such as inappropriate dosages or ratios may also have contributed to the failure of the synthetic versions. In contrast, our results indicate that these fertility signals reside in other non-hydrocarbon cuticular molecules in the polar fraction which, in combination with the unsaturated CHCs, elicited a full *Sb* discrimination response by workers. Potential queen caste signal candidates include fatty acids, esters, or other moderately polar compounds residing on the fire ant queen cuticle, although these molecules appear to be present at comparatively low levels (Fig. 3-2, bottom trace; Eliyahu et al. 2011).

This study helps lay the groundwork for future progress in identifying both the queen caste pheromone and the key molecules that signal queen *Sb* status in *S. invicta*, arguably two of the most fundamental semiochemical signals in the normal functioning of colonies of this ant. In particular, we have confirmed that compounds more polar that hydrocarbons comprise the former signal, and have confirmed that a blend of monoenes and dienes is involved in the latter signal. In so doing, we have advanced the goal of revealing the myriad complex links from genotype to collective social phenotype in an important social insect species. From an applied perspective, such progress may facilitate the future development of novel control strategies based on disruption of the chemical communication system that underpins worker regulation of colony queen number.

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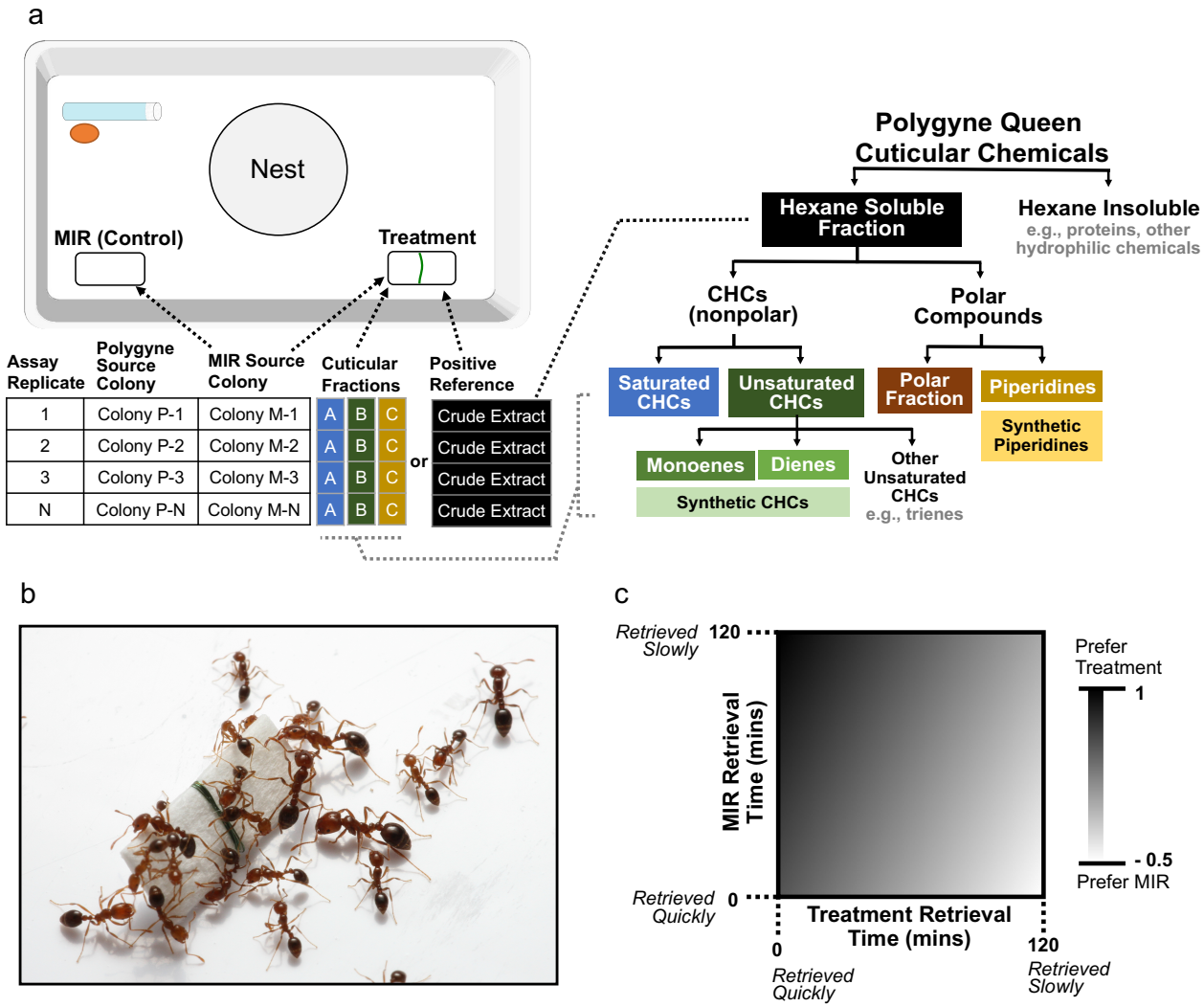


Figure 3-1 a Left half depicts the parallel assay design showing a cohort of dummy-assays comprising a single experimental series. Each polygyne colony was paired with a specific monogyne colony that was the source of the MIR queen extract. P = polygyne, M = monogyne, N = number of replicate assays. On the right is a diagram of queen cuticular chemical components. Colored text boxes indicate fractions or synthetic compounds used in behavioral assays. **b** Polygyne workers retrieving to the nest a paper dummy dosed with crude polygyne queen extract. The dummy typically would be retained in the nest for up to 8 hr or longer. **c** Relationship of the times of retrieval of treatment and MIR dummies (or corpses) to the

calculated “Initial Preference” measure for a single assay replicate. High Initial Preference measures result from rapid retrieval of the treatment dummy to the nest relative to the retrieval time for the MIR dummy (or corpses).

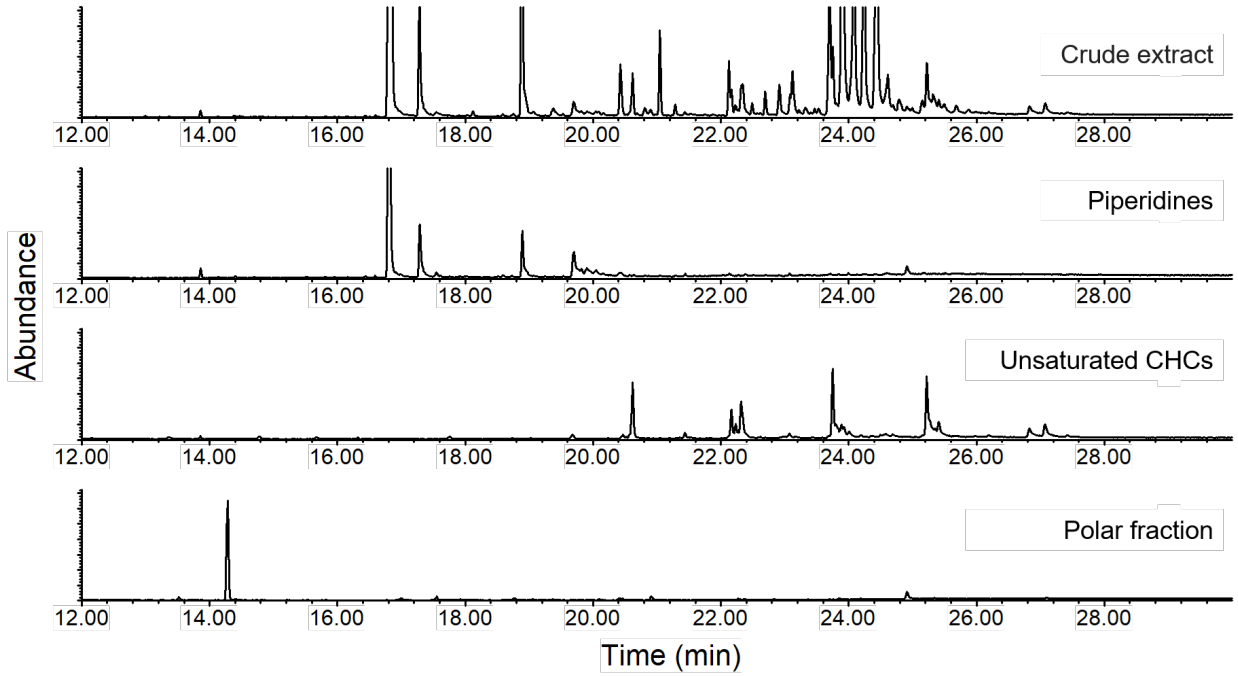


Figure 3-2 Stacked plot of GC-MS traces of crude polygyne reproductive queen extract and of select fractions of this extract. All fractions were injected at the same concentration (in queen equivalents). The single large peak in the polar fraction is a contaminant from the solvent. The large peaks visible in the crude extract that are not present in any of the fractions are from the saturated hydrocarbons.

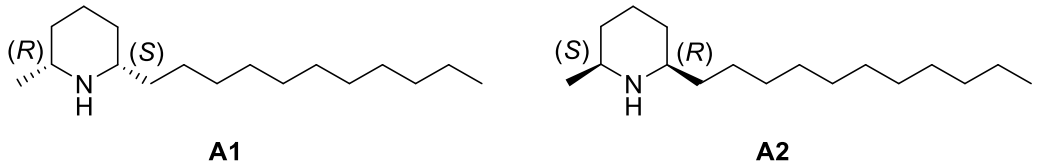
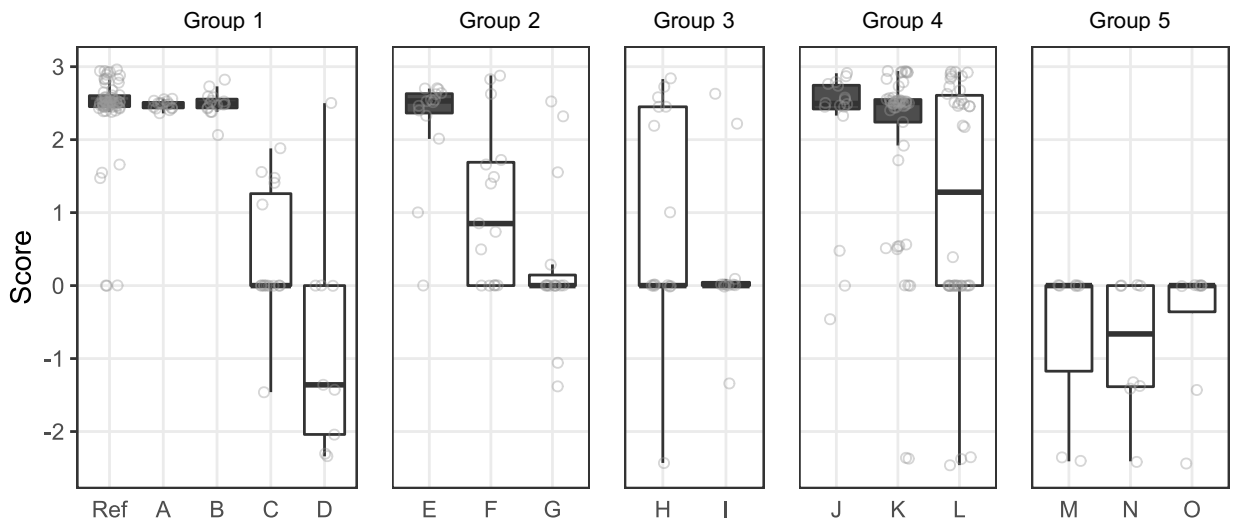


Figure 3-3 Structures of the two enantiomers of 2-methyl-6-undecylpiperidine (solenopsin A). A1: (2*R*,6*S*)-2-methyl-6-undecylpiperidine; A2: (2*S*,6*R*)-2-methyl-6-undecylpiperidine.



Group	Assay ID	Treatment Content	Sample Size	Mean Assay Score	*P* value – Wilcoxon	Effective
	Ref	Polygyne queen crude extract	66	2.42	NA	Yes
1	A	MIR ext. + Sat. CHCs + Unsat. CHCs	15	2.48	0.150	Yes
	B	MIR ext. + Unsat. CHCs	15	2.49	0.116	Yes
	C	MIR ext. + Sat. CHCs	15	0.40	<0.001	No
	D	Unsat. CHCs	15	-0.77	0.002	No
2	E	MIR ext. + Monoenes + Dienes	15	2.25	0.345	Yes
	F	MIR ext. + Monoenes	15	1.11	0.001	No
	G	MIR ext. +Dienes	15	0.28	<0.0001	No
3	H	MIR ext.+ 11 CHCs	15	0.92	0.003	No
	I	MIR ext. + 5 CHCs	11	0.33	0.003	No
4	J	Unsat. CHCs + Polar + Piperidines	16	2.12	0.589	Yes
	K	Unsat. CHCs + Polar	49	2.00	0.113	Yes
	L	Unsat. CHCs + Piperidines	38	1.14	0.003	No
5	M	Unsat. CHCs + Solenopsin racemic	7	-0.68	0.018	No
	N	Unsat. CHCs + Solenopsin A1	8	-0.82	0.011	No
	O	Unsat. CHCs + Solenopsin A2	8	-0.49	0.004	No

Figure 3-4 Boxplots showing distributions of scores for experiments in five dummy-assay treatment groups. Whiskers show the upper and lower quartiles, horizontal lines the median values, and small circles the individual score values. Filled boxes indicate that a treatment was effective in eliciting worker *Sb* discrimination. Group 1 tests different fractions of CHCs; group 2 tests subfractions of unsaturated CHCs; group 3 tests synthetic CHCs; group 4 tests different polar molecule fractions (as queen fertility signals); group 5 tests synthetic piperidines. Sample size is the number of replicate assays conducted for each treatment.

Assay	Control	Treatment	N
Co-A	MIR corpse	MIR corpse **+ unsaturated CHCs**	16
Co-B	MIR corpse	MIR corpse **+ saturated CHCs**	16
Co-C	MIR corpse **+ saturated CHCs**	MIR corpse **+ unsaturated CHCs**	18

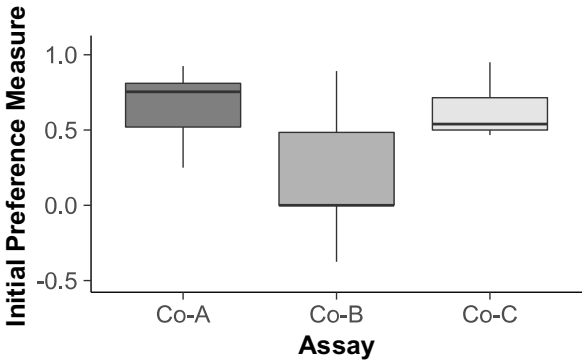


Figure 3-5 Extracts applied to control and treatment queen corpses used in three different assays to test the effectiveness of unsaturated CHCs as signals conveying *Sb* supergene presence in queens (top). Boxplots (bottom) show the distributions of Initial Preference scores from these assays.

Table 3-1 Synthetic unsaturated CHCs tested in dummy assays. The amount of a specific compound in each blend is in nanograms per three queen-equivalents, with the percentage of the total in parentheses.

Compound Name	Compound Abbreviation	Compound Type	Amounts in Blend	
			Blend of Five CHCs	Blend of Eleven CHCs
(Z)-5-tricosene	5Z-C23	Monoene	.	25.5 (5%)
(Z)-5-pentacosene	5Z-C25	Monoene	.	19.8 (4%)
(Z)-9-pentacosene	9Z-C25	Monoene	.	18.3 (3%)
(Z)-9-heptacosene	9Z-C27	Monoene	73.2 (17%)	73.2 (14%)
(Z)-7-nonacosene	7Z-C29	Monoene	.	9.9 (2%)
(Z)-9-nonacosene	9Z-C29	Monoene	141.0 (32%)	141.0 (26%)
(Z)-9-hentriacontene	9Z-C31	Monoene	33.0 (8%)	33.0 (6%)
(6Z,9Z)-6,9-pentacosadiene	6Z,9Z-C25	Diene	.	16.2 (3%)
(6Z,9Z)-6,9-heptacosadiene	6Z,9Z-C27	Diene	.	11.7 (2%)
(6Z,9Z)-6,9-nonacosadiene	6Z,9Z-C29	Diene	42.6 (10%)	42.6 (8%)
(6Z,9Z)-6,9-hentriacontadiene	6Z.9Z-C31	Diene	147.0 (34%)	147.0 (27%)

CHAPTER 4

EXPERIMENTAL DISSECTION OF BEHAVIORAL INDUCTION OF A MAJOR SOCIAL TRANSITION IN FIRE ANT[3]

[3] H. Zeng, K. Ross, T. Sasaki. To be submitted to the Journal of Experimental Biology

Abstract

The polygyne social form of the red imported fire ant (*Solenopsis invicta*) is associated with the *Sb* supergene, a large Mendelian genetic element formed by multiple chromosomal inversions. When workers carry this *Sb* supergene, the colony accepts multiple reproductive queens, if such queens also carry it. In contrast, when workers lack the *Sb* supergene, such as is the case in monogyne colonies, the colony tolerates only a single reproductive queen. Previous work showed that colonies display polygyne behavior with as few as 10% to 20% of workers carrying the *Sb* supergene. However, it remains unknown how such a low proportion of *Sb* workers affects the collective social phenotype of the whole colony. To identify essential factors in the collective acceptance of *Sb* queens, we conducted a series of behavioral assays using mixed polygyne-monogyne-worker units with the proportion of *Sb* workers manipulated to be around the threshold level of 20%. We changed the type and amount of interaction between the two worker types in the assays to learn what conditions favor acceptance of multiple polygyne queens. Our results indicate that *Sb*-carrying (polygyne) workers directly reduce aggression by monogyne workers towards the queen when they are able to roam freely among the monogyne workers, an effect potentially mediated via volatile pheromones. As well, *Sb* workers appear to indirectly influence queen preference behavior of the monogyne workers without contacting the queens. As the intimacy of interactions between worker types increased, acceptance of polygyne queens also increased, suggesting that compounds on the *Sb* workers' cuticle play a critical role in the process.

Introduction

The evolution of animal social behavior has puzzled biologists since Darwin (1859). It remains a difficult task to construct a complete roadmap from functional genetic factors to the resulting physiological and behavioral traits comprising collective social behaviors, especially those in advanced eusocial insect colonies, which are highly complex and typically involve the interactions of hundreds to thousands of sterile workers. One intriguing route, conceived by Hamilton (1964) and subsequently popularized by Dawkins (1989), invokes the "greenbeard effect" as a hypothetical driver of social evolution. A gene with a greenbeard effect manifests itself with readily recognizable traits, which function to guide other greenbeard gene carriers to help/benefit the carrier. Examples of greenbeard genes in nature are rare, presumably because, on the one hand, they can quickly become fixed in a population while, on the other hand, they are susceptible to being driven to extinction by cheaters (Gardner and West 2010).

Most identified greenbeard genes are found in microbes (Madgwick et al. 2019). The first and only greenbeard gene identified in animals with advanced social structure is an odorant-binding protein gene, *Gp-9*, in the red imported fire ant (*Solenopsis invicta*) (Keller and Ross 1998). This species is a major invasive pest, currently with a vast cosmopolitan distribution in warm-temperate to tropical regions (Ascunce et al. 2011; Wylie et al. 2020). The gene *Gp-9* is directly associated with regulation of colony social organization, specifically, the number of queens in a colony. A fire ant colony either houses a single reproductive queen (monogyne form) or a dozen to hundreds of reproductive queens (polygyne form) (Fletcher et al. 1980). Beyond queen number, these two social forms are distinct from one another in many natural history traits, including but not limited to the mode of colony founding and expansion, degree of territoriality, worker size and density, and queen weight (Huang and Wang 2014).

The gene *Gp-9* resides in an inversion-based complex of tightly linked genes spanning half of chromosome 16 and containing around 600 genes; this region is termed the *social b* (*Sb*) supergene (Wang et al. 2013). It is perhaps not surprising that *Gp-9* is linked to numerous genes since the greenbeard locus needs to encode both the greenbeard signal and the receptor machinery as well as release the appropriate behavior (Gardner and West 2010; West and Gardner 2010). The *Sb* supergene segment is inherited effectively as a single Mendelian element due to suppressed recombination from two causes: the physical misalignment with the homologous region on the wildtype *SB* chromosome inhibits effective crossing-over between *SB* and *Sb*, and the recessive lethality of the *Sb* supergene in invasive populations prevents recombinants between *Sb* chromosomes from being transmitted across generations (Ross et al. 1997; Wang et al. 2013; Yan et al. 2020).

The monogyne form of *S. invicta* lacks the *Sb* supergene altogether, that is, it is monomorphic for the presumed ancestral chromosomal configuration that lacks inversions (termed *SB*, for which the *B* allele of *Gp-9* is a marker). In contrast, the polygyne form features the *Sb* supergene in reproductive queens (all of which bear the *Bb* heterokaryotype) and, consequently, in a proportion of the workers as well (65% on average, estimated from Ross and Keller 2002; Goodisman et al. 2007; Trible and Ross 2016; Fig. 4-1). The greenbeard nature of the *Sb* supergene features both facultative helping by the greenbeard carrier and facultative harming of non-beard individuals: polygyne workers readily accept multiple *SB*/*Sb* queens while executing *SB*/*SB* queens once they reach sexual maturity, including those produced in their own colonies (Ross and Keller 1998; Keller and Ross 1998). The greenbeard effect is evident from the fact that *SB*/*Sb* workers are over-represented among those attacking *SB*/*SB* queens and those attracted to *SB*/*Sb* queens during queen rescue (Ross and Keller 1998; Trible and Ross 2016). In

contrast, monogyne colonies, which are composed only of *SB*/*SB* workers, are intolerant of any supernumerary queens, thus maintaining the single-queen colony structure typifying this form.

While it is well established that the presence of *SB*/*Sb* workers induces the emergent colony-level trait of polygyny, which is a product of the collective decisions of up to several hundred thousand adult worker ants, details of the mechanism(s) by which such social conversion occurs are unclear. What is clear is the fact that formerly monogyne colonies, once converted by the introduction of *SB/Sb* workers, remain stably polygyne, as assessed by their acceptance or rejection of introduced supernumerary *SB/Sb* queens over extended time periods (Ross and Keller 2002; Gotzek and Ross 2008). Recent studies have identified unsaturated cuticular hydrocarbons as components of a supergene pheromone produced by queens that conveys information about her supergene genotype to the workers (Zeng et al. 2022). Polygyne workers evidently discriminate in favor of *SB*/*Sb* queens and against *SB*/*SB* queens based on these unsaturated CHCs. However, the acceptance or execution of a queen is a collective colony behavior that requires some consensus decision to be reachrd among all workers, meaning that both *SB*/*SB* and *SB*/*Sb* workers must accept and nurture *SB*/*Sb* queens. We emphasize consensus here because a single rogue worker is capable of mortally wounding a queen even were she perfectly acceptable to all other workers. Thus, *SB*/*SB* workers in polygyne colonies behave in accordance with the polygyne behavioral phenotype characterizing their *SB*/*Sb* nestmates, differing strongly in this respect from their *SB*/*SB* counterparts in monogyne colonies. In other words, the presence of *Sb*-bearing workers in some way alters the behavioral phenotype of *SB/SB* nestmates, as part of an emergent supergene greenbeard effect. This latter conclusion rests partly on the fact that extensive gene flow occurs between the social forms across 15 of their 16

chromosomes, thus homogenizing the bulk of the genome in the two forms outside of the supergene (Shoemaker and Ross 1996; Ross et al. 1999; Yan et al. 2020).

Two previous studies yielded important background data for investigations of how *Bb* workers induce the transition from monogyne to polygyne social organization. Ross and colleagues manipulated the ratio of *SB/Sb* to *SB/SB* workers in colony fragments and tested if these fragments behaved as polygyne societies by introducing multiple new *SB/Sb* queens (Ross and Keller 2002; Gotzek and Ross 2008). Surprisingly, their results showed that a mere 10-20% of *SB/Sb* workers can induce a switch to polygyny in a previously monogyne colony; that is, a minority of the adult worker gatekeepers in a colony bearing the *Sb* supergene suffices to dictate the colony social structure.

In an effort to dissect this behavioral conversion, we conducted a series of informative behavioral assays designed to illuminate the critical behaviors by which *Bb*-bearing workers convert the social phenotype of *SB/SB* workers. We test two broad but not mutually exclusive hypotheses concerning the role of individual *SB*/*Sb* workers in mediating such conversion. First, *SB*/*Sb* workers deploy behavioral or physical intervention to stop *SB/SB* workers from physically contacting and harming introduced queens. Second, *SB*/*Sb* workers chemically induce *SB/SB* workers to become more tolerant of such queens. By varying the type and amount of interaction between worker types in what were originally monogyne colony fragments then testing their willingness to accept multiple polygyne queens (convert to polygyny), we hoped to gain some understanding of the proximate factors involved in such a transition in colony social organization.

Material and Methods

Ants

Fire ant colonies of both social forms were collected in Athens-Clarke, Oconee, and Wilkes Counties, Georgia, USA., from 2019 to 2021, by excavating soil nests into 5-gallon buckets. All collected nests were situated at least 10 m from any other nest in the field. The ants were separated from the soil by means of a drip-flotation method (Banks et al. 1981), then maintained in a controlled-environment rearing room under standard conditions (Ross and Keller 2002). The ants were housed in plastic trays (63.5 x 50 x 7 or 63.5 x 50 x 11.5 cm) treated with Fluon® anti-traction insect barrier on the inside walls and containing one or more nests (plastic Petri dishes with plaster bottoms; Ross 1988). The colonies were continuously provided water in cotton-stoppered glass tubes and food in the form of rehydrated freeze-dried insects as well as high-sugar and high-protein artificial diets (Ross 1988; Ross and Keller 2002). The social form of each colony was initially determined by searching for more than one dealate reproductive queen (diagnostic for polygyny). These determinations were confirmed by checking for the presence of the *Sb* supergene among workers; pooled DNA extracts of 10 – 20 workers per colony were used as templates in a modified multiplex PCR procedure for scoring allele presence/absence at the marker gene *Gp-9* (Valles and Porter 2003; Ross and Shoemaker 2018).

Screening Test Monogyne Colonies

Under normal circumstances, workers in queenright monogyne colonies immediately execute any foreign queens introduced into the colony (Fletcher and Blum 1983; Ross and Keller 1998; Vander Meer and Alonso 2002). Therefore, to render a baseline level of receptiveness of foreign queens, colony fragments (assay units) composed of monogyne workers must be held queenless

for some period. If this period is too short (e.g., 24 hours), no introduced polygyne queens will be accepted, whereas if this period is too long (e.g., 10 days) introduced polygyne queens will be accepted in virtually every instance. No relevant information on the effects of various treatments can be obtained from the assay if workers invariably reject or accept all introduced polygyne queens. Our previous experience suggests that 48 hours is the optimal period to render monogyne workers queenless because most, but not all, fragments treated this way will reject introduced polygyne queens.

There are two implications of these previous findings: (i) monogyne colonies vary in their propensity to accept polygyne queens (that is, there are significant colony-level effects on this trait), and (ii) 48 hours queenless evidently is near the threshold period required for most colonies to become receptive to introduced foreign queens, meaning the sensitivity of the assays should be optimal with regard to detecting treatment effects on worker receptiveness when the test units are held queenless for this period of time.

The colony-level variation in monogyne worker receptiveness to introduced polygyne queens at 48 hours queenless necessitated a series of Control assays to validate that every monogyne colony used as a source of an assay fragment indeed rejected polygyne queens after being queenless for this period. For these Control assays, 400 monogyne workers were transferred into a cleaned plastic tray (40 x 25 x 5 cm) coated with Fluon®, in which a temporary nest, water tube, and food were provided. Two polygyne queens were introduced into the units after 48 hours. If the queens were alive and in the nest 48 hours after their introduction, we consider them to have been accepted. In contrast, if the queens were not accepted, they were usually killed and torn apart by workers. Among the total 45 monogyne source colonies, 16

accepted one or both queens in the Control assays, leaving 29 of them as suitable sources of test monogyne assay fragments.

Experimental Units

Each assay unit was created as a queenless fragment of a monogyne colony's adult workers to which was added adult polygyne workers. The treatments differed in the manner and timing of the addition of the polygyne workers. The basic setup follows that described above for the control assays, with the following embellishments (Fig. 4-2). In each experimental unit (one replicate), 300 monogyne workers from a single monogyne colony and 100 polygyne workers from three different polygyne colonies were combined. The units likely averaged around 18% *SB/Sb* workers, somewhat above the typical threshold proportion (8-15%) for experimental social form conversion to occur (Goodisman et al. 2007; Gotzek and Ross 2008; Trible and Ross 2016). Our objective in creating such a worker genotype composition was that *Sb*-bearing workers would be common enough to induce colony conversion but not so common as to hinder observation of any interactions between them and monogyne workers that might be critical to inducing this conversion.

The polygyne workers were added to each assay unit 24 hours after the initial set-up of the queenless monogyne workers; thus, monogyne workers were queenless for a total of 48 hours, and polygyne workers for 24 hours, before two unrelated polygyne dealate queens were added to the mixed fragment. These queens were introduced to learn if the originally monogyne fragment underwent social conversion to polygyny, as was indicated by acceptance and retention in the nest of both queens for at least 48 hours. The polygyne workers added to a unit at 24 hours initially were confined within a 120 ml specimen cup with a plaster bottom in order to facilitate

their acceptance by the resident monogyne workers, which ordinarily display well-developed nestmate discrimination (Ross and Keller 1998). A layer of fine metal mesh screen (0.635 mm openings) was installed on the lid of the containment cup to allow the resident monogyne and added polygyne workers to contact each other only minimally, by mutual antennation and, possibly, contact of the maxillary and labial palps. For most treatments (except for Double-Screen, below), workers of the two types interacted only in this way until the polygyne workers were fully evacuated from the cup into the unit, shortly before the introduction of the polygyne queens. Following the release, workers of the two types were free to engage in intimate grooming, biting, or other mutual physical interactions.

By manipulating the type and length of interaction between the monogyne and polygyne workers in each treatment, we hoped to identify factors, potentially including grooming and biting, that mediate the collective decision-making process leading to social conversion. Six treatments were designed as detailed below (Table 2-1).

Videotaping of all interactions taking place in the assay unit tray, with a Sony Handycam FDR-AX100 camera at 4K resolution, commenced upon the introduction of the two polygyne queens and lasted at least two hours. Our preliminary observations showed that all meaningful interactions affecting the fate of the queens took place within this two-hour window; units behaving as if they were polygyne escorted queens into the nest and kept them there, whereas units behaving as if they were monogyne attacked the queens in the foraging arena and prevented them from entering the nest (supplementary video).

2-Hour and 10-Minute Treatments (brief intimate interactions)

These first two treatments were designed to determine if only a short period of intimate physical interaction between adult workers of the two types (following a long period of antennal contact) is sufficient to alter the social form phenotype of the originally monogyne colonies. In these two assays, the polygyne workers were released from their screened cups either two hours or ten minutes before introducing the two polygyne queens. Thus, the two types of workers interact freely before and during queen introduction, and polygyne workers also were able to interact directly with the queens once they were introduced.

Given that workers in these two treatments were freely interacting at the time of queen introduction, we found it advantageous to select only the largest workers from monogyne colonies and the smallest workers from polygyne colonies for inclusion in the assay units, so that the focal workers' social form of origin could be distinguished in the video recordings. To ensure that any effect of worker size was not confounded with the effect of worker social origin, we repeated six replicate assays from the 2-Hour treatment with the size composition reversed (the largest workers came from polygyne colonies and the smallest workers came from monogyne colonies). Queen acceptance rates did not differ between the two assay series featuring the contrasting worker size compositions ($p > 0.5$, Fisher's exact test), from which we concluded that worker body size did not influence the expression of social form behavioral phenotype in these assay units.

No-Release and Double-Screen Treatments (limited worker interaction; no polygyne worker/queen contact)

We next devised two treatments that increasingly limited the interaction between the two worker types in order to learn how this affected social conversion. In the "No-Release"

experiments, the polygyne workers were kept inside their containment cups for the entire period of the assay, never experiencing full-body, intimate physical interactions with monogyne workers. The "Double-Screen" experiment further reduced contact between the worker types. The barrier, in this case, was a double layer of the same fine-mesh screen used previously, with a gap of a few millimeters between the layers. This gap prevented any physical contact, even antennation, between the two worker types over the entire experiment. Note also that polygyne workers were unable to directly contact the introduced polygyne queens in these two treatments because the workers remained confined in their cups.

Frozen Worker and Large-Mesh Treatments (full worker interaction; no polygyne worker/queen contact)

Our preliminary results suggested that the 2-Hour and the 10-Minute treatments yielded high rates of queen acceptance, while the No-Release and Double-Screen treatments yielded very low rates. These two groups of treatments differ by two main factors: 1) whether the worker types interacted freely, with intimate, full-body contact, for even a brief period, and 2) whether the polygyne workers were able to directly contact the introduced polygyne queens.

We designed two additional assay treatments to distinguish which of these two factors plays a more important role in effecting social conversion. The "Frozen" treatment was identical to the Two-Hour treatment, except the polygyne workers that had been held in the cups were snap-frozen in a -80°C freezer then immediately dumped out of the cup into the assay unit tray two hours before introducing the queens. Monogyne workers thus were able to have full-body contact with these fresh polygyne worker corpses for about two hours before encountering the

introduced queens, while the option of the polygyne workers freely interacting (i.e., behaving) with either the monogyne workers or the introduced queens was removed.

The "Large-Mesh" treatment allowed intimate, full-bodied interaction between workers of the two types while also preventing polygyne workers from contacting the queens. The setup of the Large-Mesh experiment again mirrored the setup of the 2-Hour treatment, except at the crucial two-hour juncture before queen introduction we replaced the cup lid with a different lid on which was installed a screen with a larger mesh size (0.9 mm openings). This mesh allows minor and media workers to cross between the cup and assay unit tray but prevents major workers from crossing. To ensure that monogyne workers could roam freely between the unit tray and the cup while polygyne workers were restricted to the cup, we again reversed the size composition from that used in the 2-Hour treatment, selecting small monogyne workers and large polygyne workers at the time of set-up for each replicate.

Supplemental Treatments

We designed two additional treatments to account for effects unrelated to the *Sb* supergene but potentially contributing to the acceptance of *SB/Sb* queens. The first treatment takes into account a possible association between increased semiochemical diversity and tolerance of non-nestmate conspecifics evident in some ants. The addition of S*B/Sb* workers to a monogyne fragment broadens the cuticular chemical diversity to which the monogyne workers are exposed (Eliyahu et al. 2011), potentially habituating them to such diversity (expanding their recognition/discrimination template) and thereby inducing a greater tolerance of unfamiliar ants, including unrelated queens and, possibly, even queens bearing the *Sb* supergene. To examine this possibility, we exposed the focal workers to foreign monogyne workers in a set of "Mix-M"

treatment assays. The assay procedure again paralleled that of the 2-Hour treatment, except the polygyne workers in the cup were substituted by 100 monogyne workers held separately in three cups (33 or 34 workers from each of three colonies).

The second supplemental treatment considered the possible role of aggressive interactions among the assayed ants in favoring the acceptance of *Sb*-carrying queens. In both the 2-Hour and the 10-Min assays, a period of aggressive interactions between monogyne and polygyne workers typically occurred when the latter were first released from their cups into the assay trays. Such interactions may result in the release of alarm pheromone that disrupts mutual queen-worker chemical signaling and thereby spuriously leads to acceptance of the unrelated polygyne queens. To examine this possibility, we conducted a set of "Aggression" treatment assays, again following the basic procedures in the 2-Hour treatment assays. In this case, the polygyne *S. invicta* workers in the cup were substituted by 100 workers of *Tapinoma sessile*, a common household ant in the southern U.S., the release of which stimulated intense fighting between workers of the two species. Two polygyne dealate queens were introduced into the assay units, as usual, two hours after the release of the *T. sessile* workers.

Video-Based Behavioral Analysis

Preliminary results from our experiments suggested that the *SB/Sb* queen acceptance rate was higher in treatment conditions allowing polygyne workers to interact directly with the queens. We further observed that monogyne worker aggression (i.e. biting and stinging) towards *SB/Sb* queens rapidly subsided in the 2-Hour and 10-Minutes experiments but almost always intensified in the Control, Double-Screen, and No-Release experiments, eventually leading to the death of the attacked queens. Additionally, worker fire ants are known to collectively retrieve

their queens, fresh queen corpses, or dummies applied with queen extracts, if found outside the nest (Zeng et al. 2022). Pheromonal communication among the workers is likely deployed during these processes. Therefore, we hypothesize that polygyne workers, especially *Sb*-carrying workers, release specific "acceptance/retrieval" signals upon perceiving a *SB/Sb* queen outside the nest, which may function to call off aggression by other workers directed towards the queens as well as to induce queen retrieval by these workers.

Because there is no way to measure the intensity of pheromonal signals, we use the number of polygyne workers interacting with the queen as a surrogate measure for potential pheromonal intensity. We tracked each queen from its introduction and recorded all queen-worker interactions in the ensuing 10 minutes. During each tracking period, we recorded the number of polygyne workers that antennated the focal queen in the first five seconds of every 30-second interval. The recorded data were used to compute the 'Polygyne Worker Presence' score for each queen, i.e., the average frequency of polygyne workers directly interacting with the queen. Within each tracking period, we also recorded the number of monogyne workers that interacted with the queens to obtain the 'Monogyne Worker Presence' score in the same way. We also calculated the 'P Worker Ratio' by dividing the number of polygyne workers by the total number of workers at each time point.

To test whether assay outcomes were related to the presence of *Sb*-carrying workers, we focused on eight monogyne test colonies for which the outcome between the 10-Minute and 2-Hour experiments differed (that is, these colonies accepted queens in one experiment while rejecting queens in the other). Such pairwise comparisons minimized the influence of any variation in the *Sb* frequencies among the workers comprising our assay units due to sampling effects and natural variation in the polygyne source colonies. Each monogyne test colony was

paired with the same polygyne source colony across treatments, the proportion of *Sb* workers among the polygyne workers in the assay units ideally would be quite similar between the units used in the two tests. Effectively these two experiments can be viewed as a coherent experiment for this analysis. Contradictory outcomes are thus unexpected in view of the very high rates of acceptance for both treatments. In this pairwise comparison, a higher Polygyne Worker Presence would equate to a higher number of *SB/Sb* workers. We predicted a higher Polygyne Worker Presence and P Worker Ratio in units that accepted the queens than in the ones that rejected the queens.

Results

In the 2-Hour and 10-minute experiments, we converted monogyne workers into polygyne behaving by mixing adult workers from two types of colonies in a short time frame. Almost 90% of the units accepted the two polygyne queens in the 2-Hour treatment, and almost 80%% of the units accepted the queens in the 10-Minute treatment (Fisher's exact test comparing treatments vs. Control, both $p < 0.001$; Fig. 4-3). These results validated previous studies showing that the presence of *SB/Sb* workers, even in the minority (predicted to be about 18%), can influence the collective behavioral phenotype of the colony worker force to accept multiple queens.

Eight monogyne test colonies produced contrasting outcomes between the 10-Minute and 2-Hour experiments, but not always in the same direction. Six of these colonies accepted the queens in the 2-Hour experiments while rejecting the queens in the10-Minute experiments. The opposite result occurred for the other two monogyne test colonies. To learn whether this natural variation might inform our developing views to aid in designing further experiments, we

performed extensive behavioral analyses based on the video recordings. We found that assay units accepting the queens had a higher 'P Worker Presence' than the units rejecting queens (Fig. 4-4)—average P Worker Presence for the queen accepting units was higher in 19 out of the 20 sampling time intervals (binomial test, $p < 0.001$). M Worker Presence was also higher in the same comparison (binomial test, $p < 0.001$). The acceptance outcomes, however, were not associated with P Worker Ratio (binomial test, $p < 0.001$).

We designed Large-Mesh, Frozen, and No-Release experiments to learn if polygyne workers must interact with *SB/Sb* queens to influence the behavior of SB/SB workers. In the Large-Mesh experiment, we permitted free interaction between the worker types but prevented contact between *SB/Sb* workers and the *SB/Sb* queens. The Large-Mesh experiment yielded a rate of acceptance of 33.33% (Fisher's exact test vs. Control, $p < 0.01$). In the Frozen experiment, the focal monogyne workers interacted with freshly frozen polygyne workers after the on-screen acclimation period. This experiment yielded a 37.50% acceptance rate (Fisher's exact test vs. Control, $p < 0.01$). In the No-Release experiment, where the monogyne workers only interacted with polygyne workers through the metal screen and the polygyne workers were kept inside the cup, the queen acceptance rates were lower at 20% (Fisher's exact test vs. Control, $p < 0.01$).

Finally, in the Double-Screen treatment, where the two worker types had no physical contact, only 2 of the 28 units accepted the queens, which was not significantly different from the control experiment (Fisher's exact test, $p = 0.24$).

Discussion

Polygyny in the fire ant, *Solenopsis invicta*, is controlled by a selfish genetic element, the *Sb* supergene. When a colony had *Sb* supergene-carrying members in the worker pool, even

at a low estimated proportion of about 15%, the colony would display polygyne-like social behavior to accept numerous *Sb*-carrying queens (Ross and Keller, 2002; Gotzek and Ross, 2008). This study aimed to discern key factors by which *SB/Sb* workers influence *SB/SB* workers to accept *SB/Sb* queens. We exposed 300 monogyne workers to 100 polygyne workers under experimental regimes that yielded varying degrees of contact and interaction in order to tease out the conditions under which workers of the *SB/SB* genotypes are converted to behave as polygyne. The overall results clearly suggested an association between *SB/Sb* queen acceptance and the amount of mutual contact between the two worker types.

Monogyne workers in 2-Hour and 10-Minute experiments were converted to polygyne behavior after being mixed with polygyne workers for 24 hours, i.e., accepting two polygyne queens (Table 1). These findings are consistent with previous studies (Ross and Keller, 2002; Gotzek and Ross, 2008), but in those studies, queens were cross-fostered into whole colonies of the alternative social form, and workers of alternate genotypes were mixed for long periods (at least weeks, up to months). Here, we achieved the same results using small colony fragments and a short window of interaction period between the two worker types, showing that the influence of *Sb*-carrying workers on the queen preference of *SB/SB* workers is fairly swift.

When a queen is found outside of her nest, workers invariably form a trail between the nest and the queen to guide her return (Glancey et al. 1983). If the queen hesitates to move, workers may even grab the queen, typically by her head, and pull her towards the nest. Such behavior was observed frequently in the 2-Hour and 10-Minute, which almost always led to the acceptance of the queens. We suspected that in these trail-guided retrievals of queens, trail pheromones and possibly other "queen retrieving" pheromones were released by *SB/Sb* workers (Wilson 1959; Barlin et al. 1976). If so, a higher number of *SB/Sb* workers around the introduced

queen would stimulate additional release of "queen retrieving" pheromones, which would increase the chance of queen acceptance. This hypothesis was supported. When there were contrasting outcomes in the 2-Hour/10-Minute assays for the same monogyne test colonies and polygyne source colonies, Polygyne Worker Presence was almost always higher in queen-accepting units (Fig 4-2). Notably, the Monogyne Worker Presence was also higher in the same instances, which can also be explained by the higher attraction of workers due to the "queen retrieving" pheromones. Assay units from the other monogyne test colonies in the 10-Minute and 2-Hour experiments all accepted both queens. The queens in these assay units were seldom attacked and usually quickly retrieved into the nests, probably because the actual proportions of *Sb*-carrying workers in these units at the time of queen introduction were all high enough to induce the acceptance.

In addition to the direct and potentially pheromonal influence from *Sb* (or polygyne) workers, our results strongly suggested an indirect influence through cuticular contacts. A significant level of queen acceptance occurred in treatments where polygyne workers were prohibited from contacting the *SB/Sb* queens, but monogyne workers had cuticular contact with polygyne workers (Large-Mesh, Frozen, and No-Release experiments). Particularly, in the Frozen experiments, even if the polygyne workers were dead at the time of queen introduction, they still had the influencing power. In other words, polygyne workers could not have actively signaled the monogyne workers to accept the queens at the time of queen introduction. As additional support, when monogyne workers were prevented from having any physical contact with polygyne workers in the Double-Screen experiment, they did not accept *Sb* queens.

From these results, we speculate that certain cuticular compounds of *SB/Sb* workers affect perceptions towards *SB/Sb* queens. These compounds, when transferred onto the antennae

of the monogyne workers through antennation, caused monogyne workers to become more receptive towards *SB*/*Sb* queens, or more specifically, the queen supergene pheromones composed of unsaturated CHCs (Zeng *et al.* 2022). The candidate compounds might be found in molecules involved in pheromone perception such as odorant binding proteins, especially those uniquely present on *Sb*-carrying workers (Krieger 2005; Gotzek and Ross 2007). A future study could test whether polygyne worker cuticular extracts will affect the queen preference of monogyne *SB/SB* workers.

In the "Mix-M-Workers" experiments, focal monogyne workers were mixed with unrelated monogyne workers, instead of polygyne workers. Surprisingly, 4 out of 8 units accepted two queens. We suspect that the mixture of workers from other colonies increased genetic and cuticular profile diversity among the worker pool, which then increased the tolerance of unrelated foreign queens (Reeve 1989; Fürst et al. 2012). However, this result does not argue against the critical role of *Sb*-carrying workers in the determination of polygyne social form because the units that accepted the queens almost always eventually reduced the queen number to one. In other words, although mixing monogyne workers from several colonies increased the queen acceptance threshold, it did not fundamentally change their monogyne nature of tolerating a single queen.

Overall, our study helps us to understand how the presence of *Sb*-carrying workers, even as a minor proportion of the worker population, can induce the collective queen preference and determine colony social structure in *S. invicta*. *Sb*-carrying workers exert influence on nestmate *SB/SB* both directly, potentially via pheromonal signals, and indirectly, potentially via the transfer of cuticular compounds that influence the perception of *SB/Sb* queens. Put plainly,

Sb-carrying workers simultaneously persuade the group and alter individual decisions of their new nestmates.

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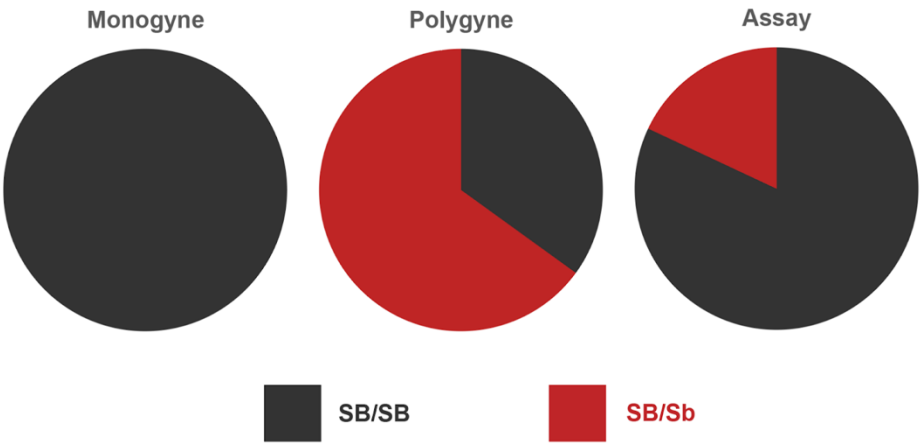

Figure 4-1 Average worker genotype composition in natural monogyne colonies, polygyne colonies, and assay units of this study.

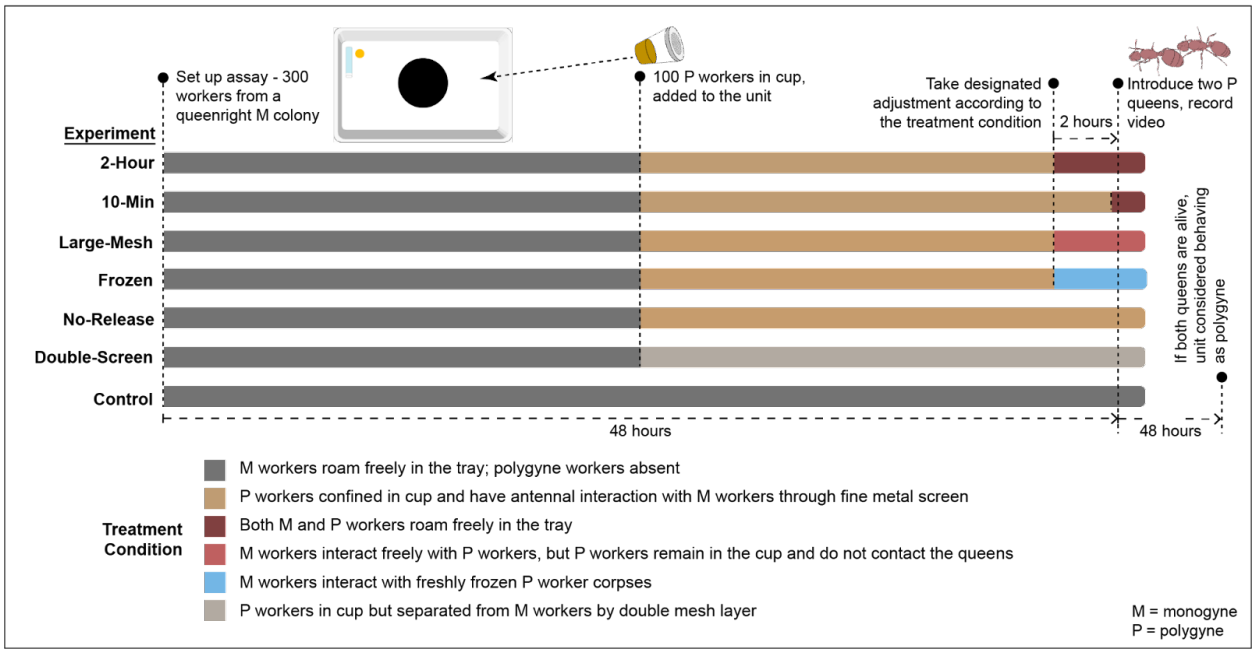

Figure 4-2. Design of the experiment. Colored bars represent different treatment variables affecting the nature of adult ant interactions, with the length proportional to duration.

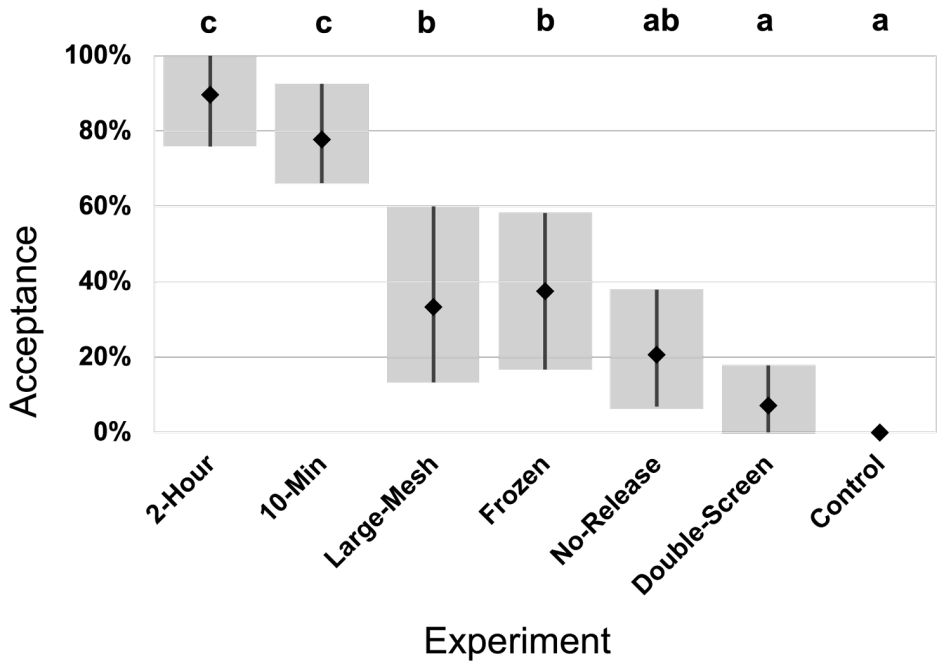

Figure 4-3 Summary of overall results from different experiments. Black squares denote the proportion of units accepting two introduced polygyne queens. Gray shaded bars represent estimated ranges from 5000 bootstrap samples.

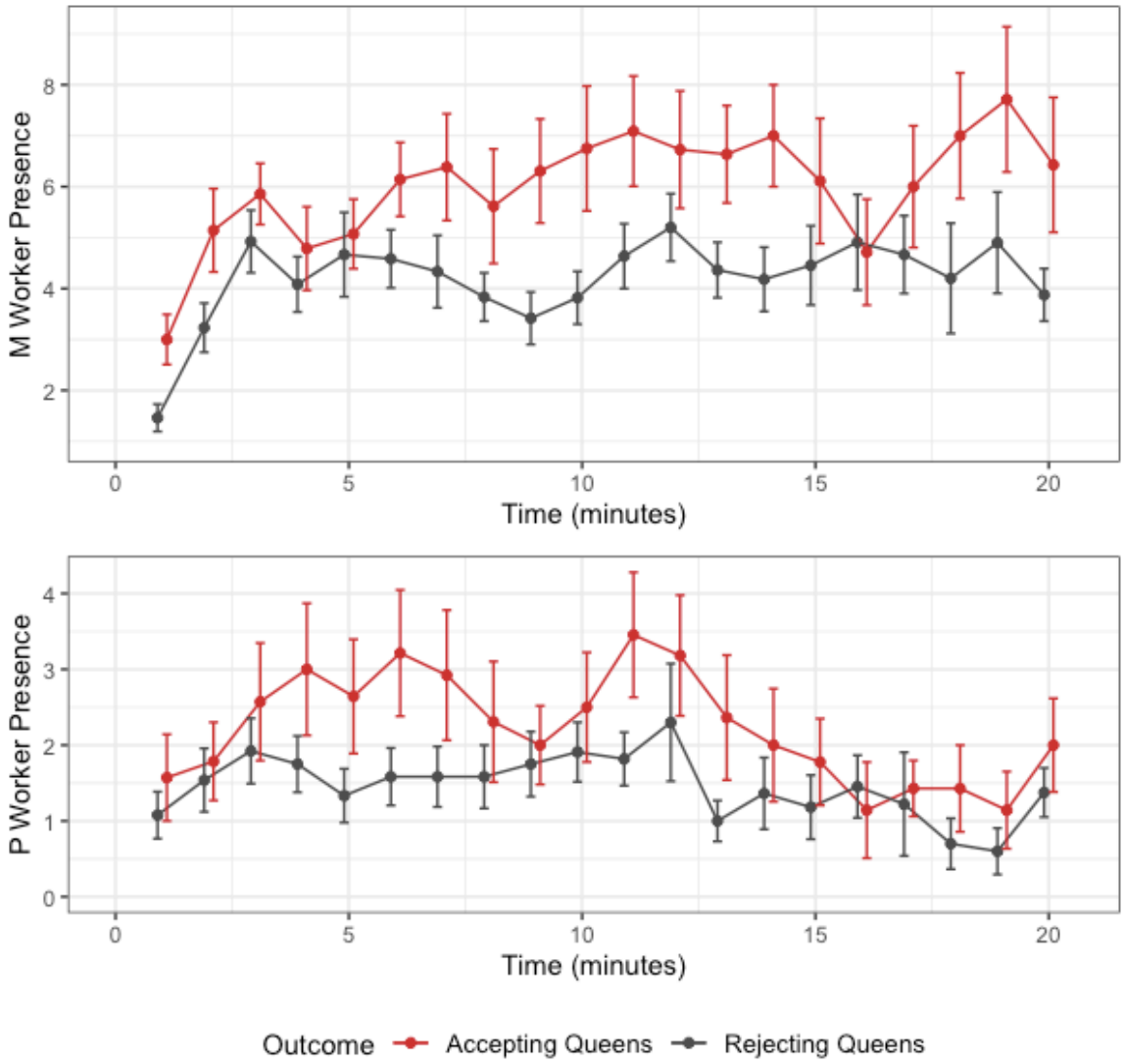

Figure 4-4 Comparisons of worker presence between queen accepting units and queen rejecting units of 2-Hour and 10-Minute experiments, from seven monogyne test colonies who yield contrasting results in the two experiments. Solid points are means of measures from all trackable queens, with error bars showing the stand error.

Table 4-1 Overview of treatment conditions and outcomes

Treatment	M-P Workers Interact on Screen	M-P Workers Freely Interact	M Workers Contact Full bodies of P Workers	P Workers Contact Queen	Rate of acceptance	Number of units accepted both queens	Number of replicates
2-Hour	Yes	Yes	Yes	Yes	89.66%	26	29
10-Minute	Yes	Yes	Yes	Yes	77.78%	21	27
Large-Mesh	Yes	Yes	Yes	No	33.33%	5	15
Frozen	Yes	No	Yes	No	37.5 %	9	24
No-Release	Yes	No	No	No	20.69 %	6	29
Double-Screen	No	No	No	No	7.14 %	2	28
Control	NA	NA	No	NA	0%	0	29

CHAPTER 5

CONCLUSION

An archetypical ant colony is monogynous, headed by a single reproductive queen and variable numbers of workers, who do not participate in reproductive activities (Hughes et al. 2008). Meanwhile, polygyne colonies evolved independently in many species, increasing trait diversities with respect to reproductive strategy, territoriality, morphologies, and so forth (Hölldobler and Wilson 1977; Gotzek and Ross 2007). *Solenopsis invicta* displays both of these social forms, with the polygyne form engendered by an inversion-based Y-like *Sb* supergene (Wang et al. 2013; Huang and Wang 2014). Extending from the genetic basis towards a complete genotype-to-phenotype map, my program aims to uncover the chemical and behavioral aspects of regulatory machinery that engender the polygyne social forms in *S. invicta*.

In Chapter Two, I reviewed experimental studies that demonstrated the functional properties of queen pheromones in ants. I pointed out that ant queen pheromones harbor a suit of functions from attracting workers, inducing colony development and cohesion, and inhibiting reproductive development and colony sexual output, to mediating colony social structure. The multifariousness of queen pheromone functions is supported by the multi-components and the multiple-glandular nature of the pheromone blend. Despite decades of research, no queen pheromones have been systematically identified in a single ant species to a degree similar to that of the honey bees. To promote a more effective search on queen pheromones, I argued against the ostensible obsession with cuticular hydrocarbons and advocated for attention towards other molecule types with the ever-advancing analytical chemical tools.

In Chapter Three, I deployed high-throughput behavioral assays to identify the chemical composition of queen supergene pheromones, based on the previous finding that workers distinguish queen supergenotype via queen cuticular chemicals. I developed a score metric to assess worker response toward testing cuticular fractions, including how quickly workers retrieve the treated dummy and how long the dummy is retained in the nest. I showed that the queen supergene pheromones composed of two synergistic signals, with the supergene genotype (presence of *Sb* supergene) signaled by a group of unsaturated cuticular hydrocarbons, and the queen caste identity signaled by non-piperidine polar molecules (Zeng et al. 2022). In the latest efforts, my colleagues and I successfully replicated the supergene pheromones with a fully synthetic blend of 19 hydrocarbon compounds. Further experiments could narrow down from this blend to identify the exact composition of the supergene pheromone, which will signify the identification of a new type of social insect pheromone to mediate colony social structures.

In Chapter Four, I devised novel experiments to tease out the critical factors by which *Sb-bearing* workers induce the colony-level queen preference. I exposed monogyne workers to polygyne workers with varying amounts of interaction, though in a short time frame of 24 hours. Monogyne non-Sb workers could be converted to polygyne behaving in such a short window. The results further suggested that their influence on non-*Sb* nestmates was two-fold: directly through pheromonal signals and indirectly through the transfer of odor-reception-related cuticular compounds.

The rapid development of sequencing technologies and bioinformatic tools has continued to identify candidate genetic elements underlying social traits across taxa (Gadau et al. 2012; Linksvayer et al. 2013; Rehan and Toth 2015). In the case of *S. invicta*, the *Sb* supergene was formed via three separate inversion events, and through introgressions, resulting in one large

non-recombining region (Helleu et al. 2022). Despite substantial knowledge of its evolutionary history, specific functional roles of individual genes within the supergene are largely missing. My research helps to lay out two directions toward identifications of functional genes. First, the identification of supergene pheromones will help to locate genes associated with the production of pheromonal compounds. Second, the potential identification of odor-reception-related cuticular compounds specific to *Sb*-bearing workers will help to locate genes associated with the perception of supergene pheromones. We will then have a clear overview of the genotype-to-phenotype map underlying the colony social structure in an ecologically and economically important ant.

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