

Egg Policing and Fertility Signaling

Across Colony Development

in the Ant *Camponotus floridanus*

by

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A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved November 2012 by the
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December 2012

ABSTRACT

Of all the signals and cues that orchestrate the activities of a social insect colony, the reproductives' fertility pheromones are perhaps the most fundamental. These pheromones regulate reproductive division of labor, a defining characteristic of eusociality. Despite their critical role, reproductive fertility pheromones are not evenly expressed across the development of a social insect colony and may even be absent in the earliest colony stages. In the ant *Camponotus floridanus*, queens of incipient colonies do not produce the cuticular hydrocarbons that serve as fertility and egg-marking signals in this species. My dissertation investigates the consequences of the dramatic change in the quantity of these pheromones that occurs as the colony grows. *C. floridanus* workers from large, established colonies use egg surface hydrocarbons to discriminate among eggs. Eggs with surface hydrocarbons typical of eggs laid by established queens are nurtured, whereas eggs lacking these signals (i.e., eggs laid by workers and incipient queens) are destroyed. I characterized how workers from incipient colonies responded to eggs lacking queen fertility hydrocarbons. I found that established-queen-laid eggs, incipient-queen-laid eggs, and worker-laid eggs were not destroyed by workers at this colony stage. Destruction of worker-laid eggs is a form of policing, and theoretical models predict that policing should be strongest in incipient colonies. Since there was no evidence of policing by egg-eating in incipient *C. floridanus* colonies, I searched for evidence of another policing mechanism at this colony stage. Finding none, I discuss reasons why policing behavior may not be expressed in incipient colonies. I then considered the

mechanism that accounts for the change in workers' response to eggs. By manipulating ants' egg experience and testing their egg-policing decisions, I found that ants use a combination of learned and innate criteria to discriminate between targets of care and destruction. Finally, I investigated how the increasing strength of queen-fertility hydrocarbons affects nestmate recognition, which also relies on cuticular hydrocarbons. I found that queens with strong fertility hydrocarbons can be transferred between established colonies without aggression, but they cannot be introduced into incipient colonies. Queens from incipient colonies cannot be transferred into incipient or established colonies.

ACKNOWLEDGMENTS

This work has been improved by the contributions of many others. First and foremost, I am indebted to my advisor, Jürgen Liebig, whose gentle guidance has kept me from veering too far from the course and whose intellectual contributions have greatly enriched the final product.

I thank my committee, Jürgen Gadau, Stephen Pratt, Ron Rutowski, and Brian Smith, for sharing their time and wisdom with me.

It has been a privilege to share this time with my colleagues in the Liebig Lab, the Social Insect Research Group, and the School of Life Sciences. Special recognition goes to my ant brothers: Adrian Smith, who made it look easy, Clint Penick, who made it look awesome, and Kevin Haight, who valiantly resisted my assaults on his sanity for the better part of a decade. I also thank Taka Sasaki, Rebecca Clark, Emily Richter Schoerning and Lisa Taylor for their camaraderie.

Thanks to my parents, Doug and Shari Moore. Feeding and housing me for the past five months has been the least of their contributions to this work.

Finally, I owe unending gratitude to Chris George. Thank you for your companionship in this and every adventure.

During my tenure as a graduate student, I was generously and graciously funded by the National Science Foundation, ASU's Graduate College, ASU's School of Life Sciences, ASU's Graduate and Professional Students' Association, Danmarks Grundforskningsfond, and the International Union for the Study of Social Insects.

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Chapter 1

INTRODUCTION

Social behavior requires information. Animals obtain information from signals and cues produced by conspecifics (Bradbury & Vehrencamp 1998). Group-living animals respond to an array of signals and cues that reveal information about food, predators, group membership, social status, and reproductive activity (Dukas 1998). The eusocial insects represent extreme cases of social living, and they use correspondingly complex systems of signals and cues to inform their behavior. Of all the cues and signals that coordinate life in a social insect colony, perhaps the most fundamental are the queen pheromones that result in reproductive division of labor. It was recently discovered, however, that queen pheromones are not necessarily present in the early stages following a social insect colony's founding (Endler et al. 2006; Holman et al. 2010). The absence of queen pheromones in incipient colonies presents an invaluable opportunity to advance our understanding of both the ultimate and proximate mechanisms that contribute to the extreme cooperation observed in social insects.

Queen Pheromones, Signaling, and Policing

Reproductive division of labor means that only one or a few individuals in a colony produce offspring of their own while the rest forgo personal reproduction (Wilson 1971). Along with overlapping generations and cooperative brood care, it is a defining characteristic of eusociality. Although the ultimate explanations of

worker sterility are still debated (Foster et al. 2006; Ratnieks & Wenseleers 2008; Fletcher & Doebeli 2009; Nowak et al. 2010), much evidence indicates that queen pheromones are the proximate mechanism that inhibits worker reproduction (Peeters & Liebig 2009).

Queen pheromones may induce worker sterility in one of two ways. They may act as a mechanism of queen control, manipulating workers into sterility against their fitness interests, or they may serve as an honest signal to which workers respond adaptively (Keller & Nonacs 1993; Heinze & d'Ettorre 2009). Overall, theoretical arguments and empirical evidence provide more support for the honest signal or queen fertility hypothesis than the queen control hypothesis. A chemical that caused workers to act against their fitness interests, like any dishonest signal, would be evolutionarily unstable (Keller & Nonacs 1993). At this time, there is no strong evidence that queens manipulate workers through dishonest signals (Heinze & d'Ettorre 2009; Peeters & Liebig 2009). The signaling hypothesis is more parsimonious, and in the absence of compelling data in support of the queen-control hypothesis, I adopt the queen fertility perspective throughout my dissertation.

The active compounds of the queen's fertility signal have been positively identified in only a handful of species, but hydrocarbons are widely implicated. Hydrocarbons coat the cuticle of insects and the surface of their eggs. Originally involved in desiccation resistance (Hadley 1980), they have been secondarily co-opted for use as signals in many insect orders (Howard & Blomquist 1982, 2005). They are an amenable substrate for signal evolution because of their structural

diversity and because their synthesis integrates multiple biochemical pathways. To serve as the queen signal, hydrocarbons should reveal the queen's fertility status. The composition of cuticular hydrocarbons correlates with reproductive development in some non-eusocial insects, including flies, cockroaches, and beetles (Dillwith et al. 1983; Schal et al. 1991; Schal et al. 1994; Trabalon et al. 1994; Steiger et al. 2007). In social insects, hydrocarbons are known to correlate with fertility in more than 28 genera, including ants, bees, wasps, and termites (Liebig et al. 2009; Peeters & Liebig 2009; Weil et al. 2009). Direct evidence of a hydrocarbon as a fertility signal comes from *L. niger*. In this species, the relative abundance of the hydrocarbon 3-methylhentriacontane (3-MeC₃₁) is six times greater on queens than on workers (Holman et al. 2010). Exposing queenless workers to artificially synthesized 3-MeC₃₁ reduces ovarian development relative to workers exposed to pentane and C₃₁ controls (Holman et al. 2010).

The cuticular hydrocarbon profile of a female is often similar to that on the surface of her eggs (Monnin & Peeters 1997b; Schal et al. 1998; Endler et al. 2004; Endler et al. 2006; Smith et al. 2008; Holman et al. 2010). If the queen's fertility pheromone is indeed a hydrocarbon, then the pheromone is probably present on her eggs. Evidence that queen eggs bear the queen's fertility pheromone comes from the ant *Camponotus floridanus*, where it has been shown that queen-laid eggs inhibit worker reproduction (Endler et al. 2004). The hydrocarbon profile of queen-laid *C. floridanus* eggs resembles the queen's hydrocarbon profile and is distinct from the profile of *C. floridanus* workers and their eggs. This provides a mechanism by which the queen can spread her

pheromone throughout the colony (Endler et al. 2004). A mechanism to increase the range of a hydrocarbon pheromone is crucial because hydrocarbons of the lengths observed on insect cuticles are non-volatile or weakly volatile and direct contact with the queen occurs regularly only at small colony sizes (Kikuchi et al. 2008).

In addition to signaling the queen's presence and fertility, differences in the surface hydrocarbon composition of queen- and worker-laid eggs can affect the response of workers to eggs. In many social insect species, workers eat eggs laid by other workers but not those of the queen (Ratnieks & Visscher 1989; Kikuta & Tsuji 1999; Foster & Ratnieks 2001; d'Ettorre et al. 2004). Differences in the composition of surface hydrocarbons on queen- and worker-laid eggs is consistent with the hypothesis that workers use hydrocarbons to discriminate between queen- and worker-laid eggs. Direct support of this hypothesis comes from *C. floridanus*. Workers from large, established colonies (i.e., colonies with more than 1000 workers and more than 1 year old) destroy worker-laid eggs, but worker-laid eggs can be rescued from destruction if the fractionated hydrocarbon extract of a queen's cuticle is artificially applied to the egg surface (Endler et al. 2004). Similar results have been reported in the ant *Pachycondyla inversa*, where the artificial application of hydrocarbons rescues worker-laid eggs when the addition of hydrocarbons makes worker-laid eggs resemble queen-laid eggs (van Zweden et al. 2009).

Egg eating is a form of worker policing. Worker policing is defined as any trait in workers that biases the production of males away from other workers and

in favor of the queen (Ratnieks 1988). In addition to egg eating, worker policing can occur when workers attack reproductively active nestmates, also known as “physical policing.” As in egg policing, physical policing is informed by differences in the hydrocarbon profile between reproductive and non-reproductive workers (Liebig et al. 2000b; Dietemann et al. 2003; Hartmann et al. 2003; Cuvillier-Hot et al. 2004; Dietemann et al. 2005b; Smith et al. 2008, 2009). The use of hydrocarbons to inform policing decisions is consistent with the hypothesis that hydrocarbons signal the queen’s fertility to workers; both functions require that hydrocarbons are honest indicators of reproductive activity.

Worker policing has recently been argued to be essential for the evolution of eusociality (Wenseleers et al. 2004a; Wenseleers et al. 2004b; Ratnieks & Wenseleers 2005; Ratnieks et al. 2006; Wenseleers & Ratnieks 2006a; Ratnieks & Wenseleers 2008; Ratnieks & Helanterä 2009). Still there is debate over the evolutionary origin of policing behavior. If policing is necessary for the evolution of eusociality, then what explains the adaptive value of policing? The hypotheses that receive the most attention are the relatedness hypothesis and the colony efficiency hypothesis. The relatedness hypothesis argues that policing evolves when workers are more related to the queen’s sons than the sons of workers, as when the colony has multiple queens or a single queen that is multiply mated (Ratnieks 1988; Wenseleers & Ratnieks 2006b). The colony efficiency hypothesis states that worker policing evolves when worker reproduction reduces colony productivity, thus lowering the inclusive fitness of workers (Cole 1986; Ratnieks 1988; Hammond & Keller 2004). Although these two hypotheses are often

contrasted against each other, they are not mutually exclusive, and the debate is akin to arguing whether the c or r of Hamilton's rule is more essential to the inequality. Nor are these hypotheses exhaustive. At least two other hypotheses could explain the evolution of worker policing. The adaptive reproduction schedule hypothesis predicts worker policing could evolve if workers reproduce too early in the colony's development, thus shunting resources toward reproduction that would be more fruitfully invested in colony growth (Ohtsuki & Tsuji 2009). The sex ratio hypothesis argues policing could be favored when gynes are more profitable to workers than males (Trivers & Hare 1976; Mehdiabadi et al. 2003). An important step toward understanding the evolution of policing behavior, and consequently, the evolution of eusociality, is understanding the forces that modulate the extent of policing behavior in extant species.

Queen Pheromones and Egg Policing in Camponotus floridanus

Much of what we already know about fertility signaling and policing comes from studies of the ant *C. floridanus*. *C. floridanus* queens and queen-laid eggs have greater abundance of shorter-chained hydrocarbons than workers and worker-laid eggs (Endler et al. 2004). There is direct evidence that workers use hydrocarbons to discriminate between queen-laid eggs, which are nurtured, and worker-laid eggs, which are destroyed (Endler et al. 2004). It is also known that queen-laid eggs inhibit worker reproduction (Endler et al. 2004). Physical policing has not been observed in this species (Endler et al. 2007). *C. floridanus* is the first species in which it was shown that queens and queen-laid eggs in

incipient colonies lack the distinct hydrocarbons that distinguish queens and their eggs in established colonies (Endler et al. 2006). This is consistent with the hypothesis that hydrocarbons are reliable indicators of reproductive activity; the daily egg-laying rate of an incipient queen is a small fraction of the egg-laying rates observed in established queens (Endler et al. 2006).

The dramatic change in queen fertility hydrocarbons in *C. floridanus* raises some tantalizing mysteries. Foremost among these is the question of how the egg policing behavior of workers changes with colony growth. In large established colonies workers destroy eggs of incipient queens at the same rate as worker-laid eggs (Endler et al. 2006). However, workers from small, young (incipient) colonies should not respond to eggs lacking queen hydrocarbons in the same manner as workers from established colonies, as this would mean destroying the eggs of their own queen. In Appendix A, I present results from a study that investigates how egg-policing behavior changes with colony growth. I find that workers from incipient colonies do not destroy worker-laid eggs, eggs laid by their own queen, or eggs laid by a foreign, established queen. Egg policing behavior emerges only with colony growth.

The results reported in Appendix A contrast with the theoretical prediction that worker policing behavior should be most strongly expressed while the colony is growing rapidly (Ohtsuki & Tsuji 2009). In Chapter 2, I gather evidence to test if another mechanism of policing is at work in incipient colonies. Finding none, I suggest that policing, though perhaps critical for the evolution of eusociality, is not necessarily expressed at every stage in derived social insect colonies. The

evolution of policing behavior can allow for the subsequent evolution of social systems in which the importance of policing is significantly diminished.

The plasticity in workers' response to eggs laid by workers and incipient queens reported in Appendix A was the first report of policing behavior changing with colony age (Bonckaert et al. 2011; Walter et al. 2011). It is unknown what mechanism accounts for this behavioral flexibility. In Chapter 3, I present evidence that workers use a combination of innate signals and learned cues to make context-dependent decisions about whether to nurture or destroy a conspecific's eggs.

Finally, the hydrocarbons of social insects serve as socially meaningful cues in contexts besides fertility signaling. Among the most important information encoded in the hydrocarbons of a social insect is colony membership, which allows workers to protect the colony's resources from outsiders. Because social behavior is predicated on recognition, understanding how multiple levels of identity information are processed simultaneously is critical for understanding the organization of a social insect colony. In Appendix B, I take advantage of the change in queen fertility hydrocarbons to test how information about fertility and colony membership is integrated in *C. floridanus*.

Chapter 2

REPRODUCTIVE RESTRAINT WITHOUT POLICING IN EARLY STAGES OF A SOCIAL INSECT COLONY

Punishment of social cheaters can suppress within-group competition and promote cooperation, as has been observed in animal species as diverse as humans (Fehr & Gächter 2002; Hauert et al. 2007), fish (Raihani et al. 2010), and insects (Ratnieks & Wenseleers 2005, 2008). According to current theory, social sanctions penalizing undesired reproductives were essential for the evolution of near-sterile workers in eusocial insect colonies (Wenseleers et al. 2004a; Wenseleers et al. 2004b; Ratnieks & Wenseleers 2005, 2008; Ratnieks & Helanterä 2009). These sanctions, known as “policing,” include any behavior that interferes with a nestmate’s reproductive success (Ratnieks 1988). Because policing behavior was crucial for the evolution of eusociality in ants, bees, and wasps, there has been much interest in explaining the origin of policing. Two non-mutually exclusive hypotheses have been advanced: the relatedness hypothesis and the colony efficiency hypothesis. In the rush to test these hypotheses concerning the evolution of policing behavior, the factors that modulate the expression of policing behavior in modern social insect species have been neglected (Ohtsuki & Tsuji 2009; Bonckaert et al. 2011). Failure to consider factors that may influence policing behavior in present-day species interferes with our ability to draw sound conclusions about the original evolution of policing.

The evolution of policing behavior can allow for the subsequent evolution of social systems in which the importance of policing is significantly diminished. Effective policing means that worker-produced offspring are unlikely to survive. Under these conditions, reproductive restraint (i.e., “self-policing”) can spread if the increase in workers’ inclusive fitness from self-policing outweighs the time and energy costs of attempted reproduction that are typically thwarted by policing by others (Ratnieks 1988). Self-policing, in turn, reduces selection for policing behavior. Policing behavior also promotes the evolution of larger colony sizes, which are associated with a suite of mutually-reinforcing traits such as loss of reproductive potential for workers and increasing morphological skew between queens and workers (Bourke 1999). These traits lower the profitability of worker reproduction and, potentially, the importance of policing in enforcing worker sterility.

One consequence of increased colony size is a prolonged phase of ergonomic growth in which new workers are added to the colony but no sexuals are produced (Oster & Wilson 1978). Ohtsuki and Tsuji (2009) created a model to explore how a colony’s developmental stage affects the expression of policing behavior. According to their model, producing workers instead of sexuals is in the self-interest of both queens and workers during the ergonomic growth phase. Because worker offspring are males and therefore sexuals, workers should refrain from reproducing at this stage. Ohtsuki and Tsuji predict that if worker reproduction occurs during ergonomic growth, it will result in a strong policing response. This prediction is upheld in recent studies of the ant *Temnothorax*

unifasciatus (Walter et al. 2011) and the wasp *Dolichovespula saxonica* (Bonckaert et al. 2011), where policing of reproductive workers was found to be stronger in earlier stages of colony development. However, Ohtsuki and Tsuji's insight is also compatible with an alternate evolutionary outcome that is not explicitly stated in their paper: if self-restraint is sufficient to prevent worker reproduction early in colony development, then policing behavior may not need to be expressed in this stage. This alternative may explain the behavior observed in the ant *Camponotus floridanus*.

C. floridanus is a monogynous species in which queens are single-mated (Gadau et al. 1996). In large *C. floridanus* colonies (1000-5000 workers), workers destroy worker-laid eggs (Endler et al. 2004; Endler et al. 2006). This behavior has been interpreted as policing. In a recent study, we show that workers from incipient colonies (60-80 workers) do not destroy worker-laid eggs (Moore & Liebig 2010a). This contradicts the explicit prediction of Ohtsuki and Tsuji (i.e., worker policing should be strongest in the earliest phases of colony growth), and is consistent with the implicit alternative (i.e., self-restraint is in worker's self-interest in early colony stages, therefore policing does not need to exist). Of course, absence of egg policing in incipient colonies does not preclude the existence of an alternative policing mechanism. The present study tests two alternate mechanisms that could enforce worker sterility in incipient *C. floridanus* colonies: egg policing by the queen and male larvae elimination.

Queen policing is widespread among social insects (Nakata & Tsuji 1996; Monnin & Peeters 1997b; Kikuta & Tsuji 1999; Saigo & Tsuchida 2004;

Wenseleers et al. 2005b; Wenseleers et al. 2005a; Smith et al. 2011; Walter et al. 2011). Queen policing is most often described in species with relatively small colony sizes (but see Smith et al. 2011), consistent with the idea that larger colonies cannot be effectively patrolled by the queen, and therefore require indirect mechanisms to limit worker reproduction (Bourke 1999; Kikuchi et al. 2008). *C. floridanus* colonies are large at maturity (>8000 worker), but the queen may be important in preventing worker reproduction at early stages of colony development, when the colony is small. Furthermore, there is evidence that queens are sensitive to more subtle cues than workers when making policing decisions (Smith et al. 2011), meaning queens may be able to discriminate between reproductive and non-reproductive workers or worker- and queen-laid eggs, even when workers are not (Moore & Liebig 2010a).

Elimination of male larvae has been reported in a number of social insect species (Passera & Aron 1996; Helms et al. 2000; Wharton et al. 2008) and implied in several others (Aron et al. 1995; Keller et al. 1996; Sundstrom et al. 1996). Most of these studies interpret male larvae elimination as evidence of queen/worker conflict over sex allocation (Trivers & Hare 1976; Ratnieks et al. 2006), but the elimination of male larvae can also be explained as a mechanism to adjust investment in sexual reproduction (Reuter & Keller 2001; Wharton et al. 2008) or as a mechanism to prevent worker reproduction (Ratnieks 1988). The potential for differential treatment of male larvae in *C. floridanus* was investigated by Nonacs and Carlin (1990) using a larval retrieval assay. They found workers retrieved male and female larvae at similar speeds, suggesting

workers do not discriminate between male and female larvae in the context of this emergency response behavior. The long-term fate of male larvae in queen-right *C. floridanus* colonies, however, has not been explored.

In addition to testing these two mechanisms of worker policing, we tested if workers from incipient colonies are capable of activating their ovaries and laying male-destined eggs. This study is the first test of the idea that policing may not be necessary to enforce worker sterility in incipient colonies.

METHODS

Study Species and Culturing Conditions.

We used *Camponotus floridanus* colonies collected as founding queens or incipient colonies from the Florida Keys, USA. The incipient colonies in this study were collected between August and November 2009 and November 2011. Established colonies, which were used as controls and sources of female larvae, were collected in 2007 and 2008. Queenless worker groups, which provided male larvae and worker-laid eggs, were orphaned from colonies collected between 2001 and 2007. Laboratory culturing conditions were as described in (Moore & Liebig 2010b).

Queen Policing

We tested for queen policing of worker-laid eggs when the incipient colonies contained 40 to 60 workers. Two groups were isolated from each of 12 incipient

colonies. One group consisted of five workers and the queen; the other consisted of six workers. The isolates were housed in experimental chambers with moistened plaster floors and provided both sugar-water and water. After one hour, we presented each group 15 worker-laid eggs. Isolates from the same colony received worker-laid eggs from the same queenless worker group. Different queenless worker groups were used as worker-laid egg sources for each colony. In the hour immediately following the introduction of the eggs, we observed the behavior of the queen, looking especially for interactions between the queen and the worker-laid eggs (e.g., antennation, carrying, eating). One hour is more than sufficient to see policing in groups of *C. floridanus* workers from established colonies (Endler et al. 2004). After 24 hours, we counted the number of eggs in each experimental arena.

A direct comparison of the number of eggs recovered from groups with and without the queen was not possible because the queen continued to lay eggs during the experiment. To differentiate between queen-laid and worker-laid eggs, only worker-laid eggs with distinct embryonic development were used. This allowed us to reliably distinguish introduced eggs from eggs laid by the queen. We verified the accuracy of the classifications by photographing each egg and having a person who was not familiar with the experiment determine if the egg was freshly laid (i.e., uniformly milky and translucent) or not freshly laid (i.e., with distinct opaque or transparent patches). The naïve person agreed with 98.4 percent of our classifications. We compared the number of eggs with distinctive embryonic development remaining in groups with queens to the total number of

eggs in groups without queens using a Wilcoxon matched-pairs signed-ranks test. As a secondary assurance that our egg counts were accurate, we determined the egg-laying rate of queens four to five days prior to the start of the queen-policing assay by isolating the queen and five workers in an experimental chamber and counting the number of eggs laid in 24 hours.

Elimination of Male Larvae

To determine if workers from incipient colonies rear male larvae to adulthood, we provided incipient colonies (50-90 workers, $N=8$) with 40 small (< 2.5 mm) male larvae. Before adding the larvae, we removed all existing eggs and larvae from the colony, allowing us to follow a distinct pulse of brood through development. To control for the effect of the manipulation, an additional eight incipient colonies were provided 40 small female larvae following the same procedure. Finally, to control for intrinsic mortality of male larvae, we created eight groups of 60 to 80 workers from male-rearing worker groups (orphaned >60 days) and provided them with 40 small male larvae. The sex of larvae for this experiment was not empirically confirmed, but inferred from source (Nonacs & Carlin 1990).

We checked the replicates for the presence of larvae three, seven, and 10 days after the start of the experiment. On day 14, the larvae were removed, counted, and returned to the experimental group to continue their development. For the next eight weeks, we checked the colonies that received male larvae once per week and recorded the presence of adult males. The percentage of larvae

surviving at Day 14 was analyzed using a Kruskal-Wallis test; the proportion of groups producing adult males was compared using a Chi-square test.

Worker Reproduction

To determine if workers from incipient colonies will activate their ovaries and lay eggs when separated from the queen, we isolated groups of 100 workers from incipient colonies ($N=13$). By this time, the incipient colonies were 112 to 360 workers in size (median=150). Isolates of 100 ($N=13$) and 500 workers ($N=13$) were taken from both the nest chamber and the foraging arena of established, queenright colonies as controls. We did not control for demographic factors such as worker age or caste distribution because the number of workers collected from incipient colonies was sufficiently high to ensure the sample was representative of the colony, and our goal was to determine if workers from incipient colonies could lay eggs. Mean worker mass was calculated from the total mass of the isolated workers at the start of the experiment. At the start of the experiment, the difference in the mean mass of workers from incipient colonies (mean+SD=8.387+0.918 mg, $N=13$), and from established colonies (100-worker isolates: mean+SD=8.597+1.208 mg, $N=13$; 500-worker isolates: mean+SD=8.568+0.645 mg, $N=13$), was not statistically significant (ANOVA_{2,36}, $F=0.277$, $P=0.760$). All isolates were checked twice per week for evidence of egg-laying. After six months, two to 20 minor workers from each isolate were dissected to evaluate their ovarian development. Fifteen to 20 haphazardly selected workers from 13 queenright, established colonies and from 10

queenright, incipient colonies were also dissected. Ovaries were considered “developed” if the ovarioles contained developing oocytes (oval bodies) visible under a dissecting microscope. We also noted workers that had a fully developed egg in their ovarioles. The number of worker isolates that did not produce eggs was compared by a Chi-square test; the proportion of workers with developed ovaries was compared between groups using a Kruskal-Wallis ANOVA followed by post-hoc analysis as described in Siegel and Castellan (1988).

RESULTS

Queen Policing

Contrary to what would be predicted by the queen-policing hypothesis, the presence of the queen did not decrease the number of worker-laid eggs recovered after 24 hours. In fact, the median number of worker-laid eggs recovered from groups including the queen was greater (median=14, range=6-15, $N=12$) than the median number of eggs recovered from groups without the queen (median=13, range=0-15, $N=12$), but the difference was not significant (Wilcoxon Matched-Pairs Signed-Ranks test, $T=11$, $P=0.11$; Fig. 2.1). In the hour after worker-laid eggs were introduced into the test group, the queen never antennated or interacted with the worker-laid eggs. The median number of eggs laid by queens during the experiment was 7 (range=0-9, $N=12$), which is greater than the egg-laying rate observed before the experiment (median=3.5, range=0-9, $N=12$).

Destruction of Male Larvae

We added male larvae to eight incipient colonies and eight groups of workers isolated from male-producing worker groups; female larvae were added to additional eight incipient colonies. At least some larvae survived to Day 14 in all replicates except in one group of workers isolated from a male-producing worker group. Of groups with larvae on Day 14, larval survival was greatest in worker groups receiving male larvae (median percent surviving=85, range=45-95, $N=7$), followed by incipient colonies receiving male larvae (median percent surviving=81.25, range=72.5-92.5, $N=8$) and incipient colonies receiving female larvae (median percent surviving=62.50, range=42.50, $N=8$); there was no significant difference among treatments (Kruskal-Wallis test, $\chi^2_2=3.280$, $P=0.194$; Fig. 2.2). Seven out of the eight incipient colonies given male larvae reared the male larvae to adulthood, as did four out of seven worker groups ($\chi^2_1=2.618$, $p=0.106$). These results do not support predictions of the male larva destruction hypothesis.

Worker Reproduction

After six months, the proportion of ants with developed ovaries was highest in worker isolates from incipient colonies (median=0.81, range=0.60-1.00, $N=13$), followed by 100-worker isolates from established colonies (median=0.64, range=0.06-0.93, $N=13$), 500-worker isolates from established colonies (median=0.60, range=0.11-1.00, $N=13$), incipient queenright colonies (median=0.31, range=0.10-0.65, $N=10$) and established, queenright colonies

(median=0.20, range=0.00-0.47; Kruskal-Wallis test, $\chi^2_4=31.609$, $P<0.001$; Fig. 2.3). There was no statistically significant difference among the three types of worker isolates or between the two types of queenright colonies. In nine of 13 worker isolates from incipient colonies at least one worker had a fully developed egg in her ovaries, compared with five out of 13 100-worker isolates from established colonies, five out of 13 500-worker isolates from established colonies, and zero out of 12 samples from established, queenright colonies. These data were not collected for the incipient, queenright colonies, but we observed that the more developed oocytes in workers from this condition were more irregularly shaped and globular than the oocytes we observed in animals from worker groups (Fig. 2.4). After six months, eggs were observed in one isolate from an incipient colony, two 500-worker isolates from established colonies, and in none of the 100-worker isolates from established colonies. There was no difference among the treatments in the number of isolates that did not produce eggs after 6 months ($\chi^2_2=0.167$, $P=0.920$).

DISCUSSION

Policing behavior may have been critical for the evolution of eusociality in the social Hymenoptera, but that does not mean policing is always essential to limit worker reproduction in extant social insect colonies. In this paper, we test the idea that policing is less important in the earliest stages of colony growth, when life-history trade-offs select in favor of reproductive self-restraint by

workers. First, we tested for evidence of egg policing by the queen but found no evidence that the queen destroys worker-laid eggs in incipient colonies; the percentage of worker-laid eggs recovered after 24 hours was the same for experimental groups including a queen and experimental groups without a queen (Fig. 2.1). Furthermore, we never saw queen behavior directed at worker-laid eggs during our hour-long observations immediately following the introduction of worker-laid eggs.

We also tested the survival of worker-produced male-larvae in incipient colonies and found no evidence of male larvae elimination. In fact, more incipient colonies than male-producing worker groups reared at least one male to adulthood, although the difference was not significant. Interestingly, our results indicate that the survival of male larvae in incipient colonies may be slightly higher than the survival of female larvae, but again, the difference was not significant.

Finally, we show workers from colonies as small as 112 workers are capable of activating their ovaries and producing eggs, which excludes the possibility of a developmental or physiological mechanism (e.g., workers too small or underfed) inhibiting worker reproduction. In both orphaned worker groups and queenright colonies, workers from incipient colonies were more likely to have visible oocytes than workers from established colonies, but in neither case was the difference significant. Variables that were not controlled in the current experiment (e.g., worker age) could account for the difference. Our simple, dichotomous analysis of ovarian development does not capture quantitative or

qualitative differences in ovarian development that may exist between the different worker classes (Fig. 2.4). Nevertheless, our methodology was sufficient to conclude that workers from incipient colonies are physiologically capable of laying eggs.

Previous studies suggest two common forms of policing, egg destruction and physical aggression toward reproductive workers, do not enforce worker reproductive acquiescence in incipient *C. floridanus* colonies. The absence of egg policing in incipient colonies, first reported in (Moore & Liebig 2010b), is also supported by the results of our queen-policing assay (Fig. 2.1). Policing by physical aggression in *C. floridanus* has been investigated in large colonies (Endler et al. 2007). In large colonies, there is no evidence of aggression toward workers with activated ovaries. There was also no differentiation between reproductive and non-reproductive workers in their cuticular hydrocarbons, which are widely used as cues to identify reproductive individuals (Dietemann et al. 2005b; Peeters & Liebig 2009; Smith et al. 2009; Liebig 2010). For these reasons, we think it is unlikely that physical policing exists in incipient colonies.

While we cannot exclude the existence of another mechanism that ensures worker sterility in incipient colonies, we have examined several likely mechanisms of policing and found no evidence to indicate that any of them is operating in incipient colonies. Our results are consistent with the hypothesis that policing behavior may not be necessary in incipient colonies if the costs of worker reproduction are sufficiently high that it is in the self-interest of workers to refrain from reproducing (Ohtsuki & Tsuji 2009).

The absence of policing behavior in incipient *C. floridanus* colonies corresponds with the absence of the information necessary to make policing decisions. Much evidence indicates that reproductive workers and/or their eggs are identified by the blend of hydrocarbons on their surface (Monnin & Peeters 1997b, a; Liebig et al. 2000a; d'Ettorre et al. 2004; Endler et al. 2004; Dietemann et al. 2005b; Hartmann et al. 2005; Brunner et al. 2009; Smith et al. 2009). As mentioned above, the cuticular hydrocarbons of reproductive *C. floridanus* workers are indistinguishable from those of non-reproductive workers (Endler et al. 2007). Eggs laid by incipient queens lack the distinctive hydrocarbons that characterize the eggs of queens from large colonies and are similar to worker-laid eggs (Endler et al. 2006; Moore & Liebig 2010a).

If worker sterility is not behaviorally enforced in incipient *C. floridanus* colonies, this implies a reliable and effective stimulus that elicits reproductive self-restraint. It is not yet known what this stimulus is. Group size by itself is unlikely to be the cue because worker groups with fewer than 250 workers produce eggs (Endler et al. 2004) even though egg-eating behavior is not fully expressed in colonies of this size (Moore & Liebig 2010a). Information about queen presence is likely to be important. In large colonies of *C. floridanus*, queen-laid eggs have been shown to suppress worker reproduction (Endler et al. 2004), but queen-laid eggs in incipient colonies may not have the same inhibitory effect since they lack the distinctive surface hydrocarbons found on eggs laid by queens of large colonies (Endler et al. 2006). Direct contact with the queen herself may occur with sufficient frequency to serve as the stimulus in small colonies

(Dietemann et al. 2005a), as has been shown in the ant *Diacamma* sp. (Kikuchi et al. 2008), but this does not resolve the question of how the queen is recognized by workers, since queens of small colonies do not have the distinct cuticular hydrocarbon profile that distinguishes queens in large colonies (Endler et al. 2006).

Our results caution that predictions about policing behavior need to consider the natural history of a species as well as general evolutionary theories. The importance of this practice was recently underscored in a paper by Bonckaert et al. (2011). Bonckaert and colleagues revisited a seminal study of the wasp *Dolichovespula saxonica* (Foster & Ratnieks 2000), in which it was reported that workers policed other workers' eggs in the presence of a multiply-mated queen but not in the presence of a singly-mated queen. This provided strong support for the relatedness hypothesis of worker policing. The results from Bonckaert et al. suggest that the pattern of policing originally interpreted as facultative worker policing in the wasp was actually caused by differences in colony age, not within-colony relatedness (Bonckaert et al. 2011).

A recent study of the ant *Temnothorax unifasciatus* considered both colony stage and within-colony relatedness (Walter et al. 2011). Walter et al. manipulated colony size and worker relatedness and recorded the effect of their manipulations on policing effectiveness. They found policing effectiveness is greatest at small colony sizes, making their experiment the best evidence of Ohtsuki and Tsuji's (2009) prediction. They also found policing effectiveness was higher in colonies with lower relatedness, consistent with the relatedness

hypothesis (Ratnieks 1988). However, *T. unifasciatus* is a monogynous, monandrous species (Walter et al. 2011), meaning there is no variation in intracolony relatedness in natural populations of this ant (Heinze et al. 1997), which questions whether this is an adaptive response to changes in within-colony relatedness.

A similar criticism could be raised about our own study system, *C. floridanus*. As seen in the current study, workers are not effective reproducers. After six months, egg-laying was observed in only three of the 39 orphaned worker groups. In an earlier study, the proportion of egg-laying worker groups was higher, but even then, no eggs were seen before 60 days and after 180 days egg-laying had been observed in only half of the worker groups (Endler et al. 2004). To what extent does the threat of worker reproduction explain the persistence of egg-destruction behavior in *C. floridanus*? Perhaps the destruction of worker eggs in established *C. floridanus* colonies is the expression of a more general behavior, such as nest hygiene, that happens to have the auxiliary effect of preventing the invasion of a cheater phenotype. At the moment, we cannot distinguish between these two explanations of egg destruction in *C. floridanus*.

Worker policing in the evolutionary past allowed ant colonies to achieve states where workers limit their own reproduction or are physically sterile. In these societies, policing behavior may be lost or diminished. Our data remind us that current-day social insect societies are different from the species in which policing first evolved, and the factors that determine the expression of policing

behavior in extant social insects are likely to be different than the factors that explain the original evolution of this behavior.

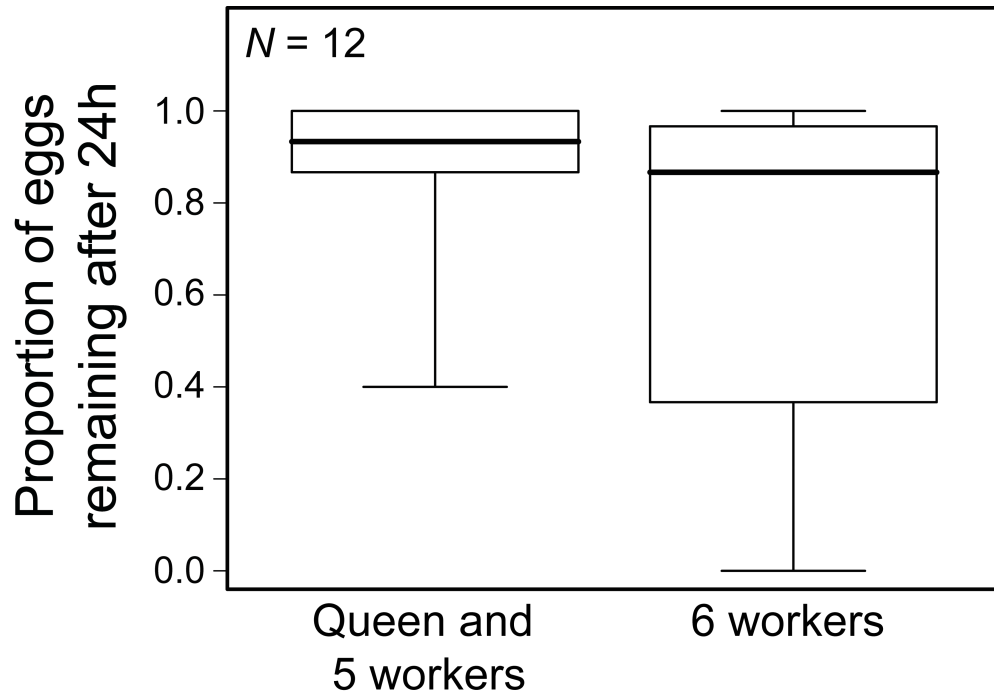


Figure 2.1. The number of worker-laid eggs recovered after 24 hours from groups consisting of a queen and five workers or six workers without a queen. Bars, boxes, and whiskers indicate median, quartiles, and range, respectively. (Wilcoxon matched-pairs signed-ranks test, $T=11$, $N=12$, $P=0.105$)

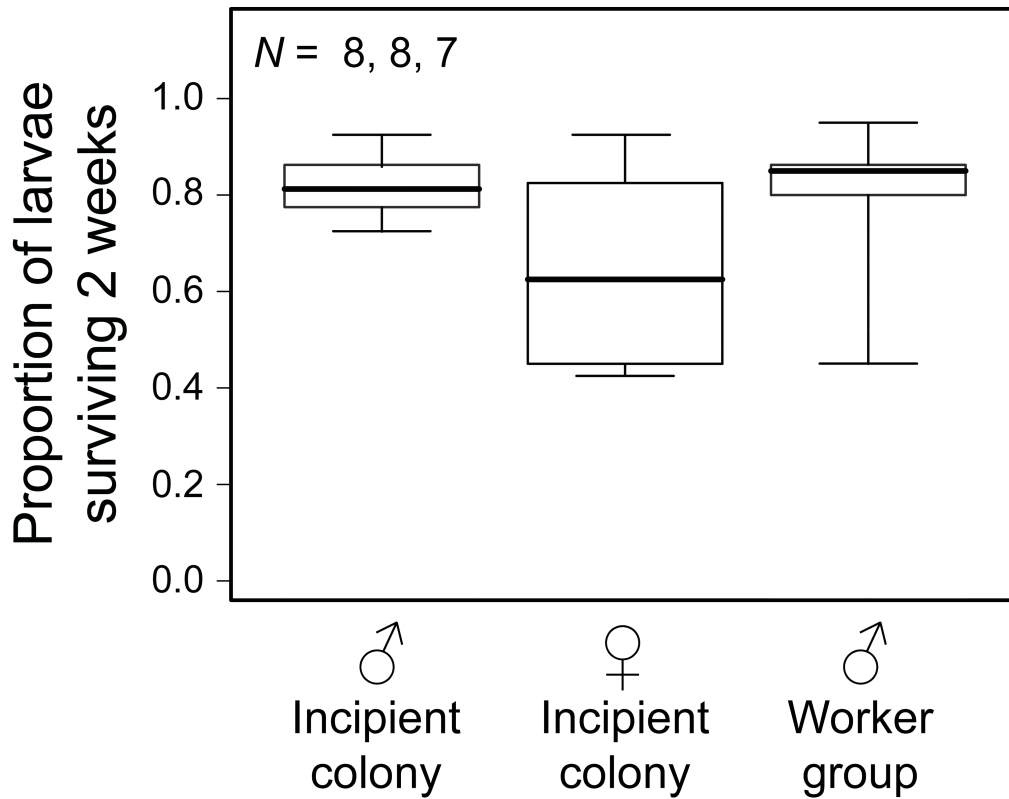


Figure 2.2. The number of larvae surviving two weeks after being introduced into either an incipient colony or a male-producing worker group. The sex of the added larvae, indicated by astronomical symbols, was inferred from larvae source. Bars, boxes, and whiskers represent median, quartiles, and range, respectively. (Kruskal-Wallis test, $\chi^2_2=3.280$, $P=0.194$).

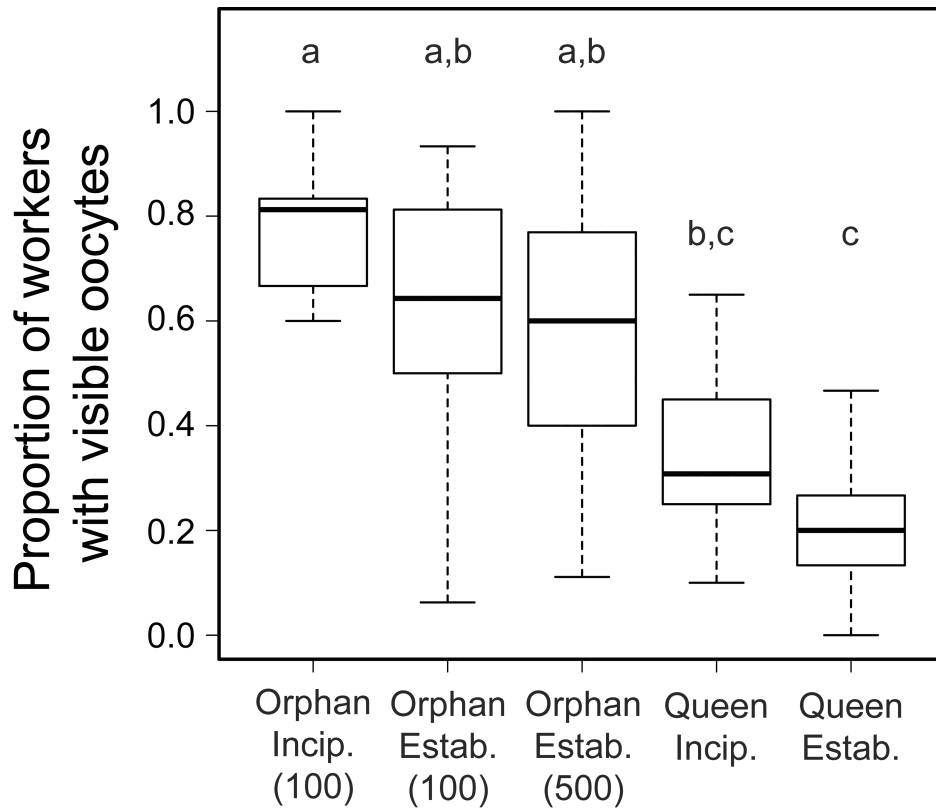


Figure 2.3. The percent of workers with visible oocytes in their ovaries at time of dissection. Workers were collected from groups that had been separated from their queen for six months (“Orphan”) or from queenright colonies (“Queen”). Source colonies were either incipient (“Incip.”) or established (“Estab.”). Orphaned groups originally contained either 100 or 500 workers, shown in parentheses. Lowercase letters indicate significant differences between groups. $N=13$ for all groups except incipient queenright colonies ($N=10$). Bars, boxes, and whiskers represent median, quartiles, and ranges, respectively. (Kruskal-Wallis test, $\chi^2_4=31.609$, $P<0.001$)

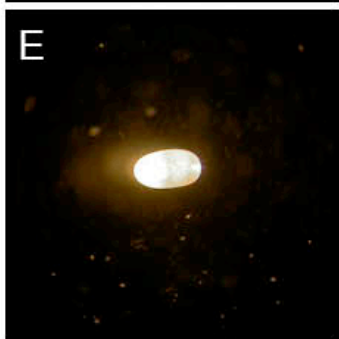
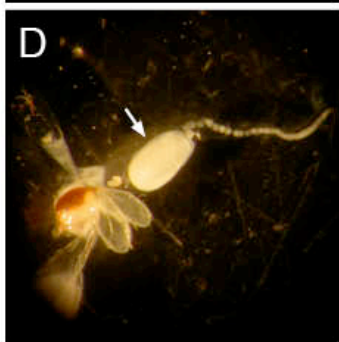
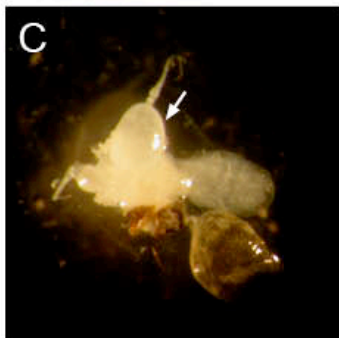
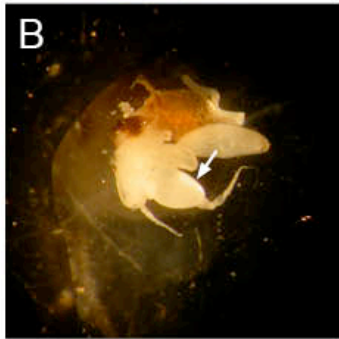


Figure 2.4. Representative photographs of the most developed ovaries observed in workers from queenright, incipient colonies (A-C) and worker groups orphaned from established colonies (D). An image of a worker-laid egg (E) is included for reference. Arrows indicate the largest oocyte in the image. Oocytes in workers from queenright, incipient colonies were more globular than oocytes in workers from orphaned worker groups, which were more oblong. The scale bar indicates 1mm; all photos are shown at the same scale.

Chapter 3

RECOGNITION OF QUEEN-LAID EGGS BY WORKERS OF THE ANT *CAMPONOTUS FLORIDANUS* INVOLVES INNATE SIGNALS AND LEARNED CUES

Recognition often involves innate and learned components. Innate templates are useful when the characteristics of an important stimulus are consistent across generations (Kindermann et al. 2009; Dixson et al. 2012). Learning is beneficial when cues are unique to a time or place (Wyatt 2010). In many cases, both innate and learned components contribute to successful recognition. For example, an innately meaningful signal, such as a fish's alarm pheromone, can act as an unconditioned stimulus that allows for the learning of a biologically relevant cue, such as predator odor, via classical conditioning (Magurran 1989). An innate template can serve as a scaffold for learned cues, as in the case of facial recognition in humans and wasps (Tanaka & Farah 1993; Sheehan & Tibbetts 2011). Learning can provide flexibility when an innately preferred stimulus is not present in the environment, as observed in butterflies that learn new hosts when their preferred host is unavailable (Weiss 1997).

One important recognition context in species that care for dependent kin is the recognition of appropriate targets of care (Roldán & Soler 2011). Species at risk of misdirected parental care, such as colonial breeders and those exploited by brood parasites, can often discriminate between their own offspring and unrelated young. Workers in eusocial insect colonies rear kin and not offspring, but

directing brood care toward the most profitable targets is a challenge social insect workers share with avian and mammalian parents (Kilner & Langmore 2011). In eusocial ants, bees, and wasps, it is often in the fitness interests of the colony's workers to rear queen-laid eggs over worker-laid eggs (Cole 1986; Ratnieks 1988; Pamilo 1991; Bourke & Franks 1995; Hammond & Keller 2004; Wenseleers & Ratnieks 2006b; Ohtsuki & Tsuji 2009). Workers in many eusocial species discriminate between queen- and worker-laid eggs (Ratnieks & Visscher 1989; Kikuta & Tsuji 1999; Foster & Ratnieks 2001; Foster et al. 2002; d'Ettorre et al. 2004; Endler et al. 2004). Queen-laid eggs are nurtured, whereas worker-laid eggs are destroyed.

Recognition of queen-laid eggs by social insect workers cannot be explained by innate signals alone (Helanterä et al. 2007). In the carpenter ant *Camponotus floridanus*, the queen-specific characteristics that workers use to distinguish queen-laid eggs from worker-laid eggs do not exist in the earliest stages of colony growth. In large (>1000 workers), established (>1 year old) colonies of the carpenter ant *Camponotus floridanus*, queen-laid eggs are coated with a blend of hydrocarbons that are not found on the surface of worker-laid eggs (Endler et al. 2004). Worker-laid eggs are usually destroyed by workers, but they can be rescued from destruction if hydrocarbons that resemble those found on queen-laid eggs are artificially added to their surface (Endler et al. 2004). In incipient colonies (<100 workers), however, queen-laid eggs lack the distinctive queen-specific hydrocarbons and are indistinguishable from worker-laid eggs. Workers from established colonies destroy eggs laid by an incipient queen

(Endler et al. 2006), but workers from incipient colonies do not (Moore & Liebig 2010a). Workers from incipient colonies also do not destroy worker-laid eggs or eggs laid by queens from established colonies (Moore & Liebig 2010a). This pattern of brood discrimination is incompatible with a simple recognition system in which eggs bearing queen hydrocarbons are accepted and eggs lacking them are destroyed. Until now, there has been no investigation of the mechanism that accounts for the flexibility in brood discrimination behavior.

We hypothesize that learned and innate responses to eggs combine to produce the pattern of brood discrimination behavior observed in *C. floridanus*. To separate the contributions of innate and acquired components to egg recognition, we conducted a series of three experiments in which we manipulated workers' egg experience and then tested their egg discrimination behavior. The first experiment tested if workers' experience with eggs affects their egg acceptance behavior. Adult workers were removed from an established colony and exposed to queen-laid eggs, worker-laid eggs, or no eggs; their response to queen- and worker-laid eggs was tested after several weeks of exposure (Fig. 3.1a). If egg experience influences egg acceptance, then workers exposed to eggs from different sources will exhibit differences in egg acceptance behavior. The second experiment tested if egg experience could establish egg acceptance in workers without adult experience with eggs. This experiment was similar to the first except that we isolated the experimental workers as pupae and thus they had no adult experience with queen-laid eggs (Fig. 3.1b). If egg experience establishes egg acceptance behavior, then workers with no prior adult experience with eggs

will respond differently to eggs than workers with adult experience with eggs. The third experiment was designed to determine if the experience-dependent changes in egg acceptance behavior observed in the first two experiments occurred because ants were updating their neural template to reflect the profiles of the eggs they experienced or because they were adjusting the permissiveness of their acceptance threshold (Fig. 3.2). Adult workers were removed from an established colony and exposed to queen-laid eggs, worker-laid eggs, or the queen-laid eggs of a different species, *Camponotus tortuganus* (Fig. 3.1c). We then tested the response of manipulated workers to all three egg types. If workers are acquiring a new recognition template as a result of their egg experience, only eggs that are similar to those that the workers have experienced previously will be accepted. If workers are relaxing their acceptance threshold, then eggs dissimilar from the ones they had experience will also be accepted.

METHODS

Study Species and Culturing Conditions

Camponotus floridanus colonies were collected as foundations from the Florida Keys, USA, between the years of 2001 and 2011 and maintained in the lab as described in Moore and Liebig (2010b). Source colonies for *C. tortuganus* eggs were collected and reared in the same manner. Worker-laid eggs came from queenless worker groups orphaned more than 60 days before the start of the

experiment. Throughout the methods and results, “queen-laid eggs” refers to eggs laid by the queen of a large, established colony unless otherwise specified.

Experiment 1: Does Egg Experience Affect Egg Acceptance?

To test if egg experience influences egg discrimination, we manipulated the egg experience of worker ants in queenless worker groups and then tested their response to queen-laid and worker-laid eggs after two, five, and nine weeks of exposure. We created a group of 350 workers from each of 45 established colonies (>1000 workers) that were two to seven years old. Groups were randomly assigned to receive queen eggs (N=15), worker eggs (N=15), or no eggs (N=15; Fig. 3.1a). Groups receiving eggs were provided with approximately 15 eggs three times per week. The eggs were a mixture of eggs from at least three different sources. The groups receiving no eggs received larvae to keep pace with the development of larvae in the groups receiving eggs. Experimental groups were maintained in plastic boxes (8×10×20 cm) with a dental plaster floor and fed *ad libitum* with water, sugar-water, Bhatkar diet (Bhatkar & Whitcomb 1970), and mealworms.

On the day the groups were created, the workers’ response to queen- and worker-laid eggs was tested using a standard egg discrimination assay. From each experimental group, we created two test groups of 20 workers each. Each group was placed in a plastic box (8×10×10 cm) with a moistened, dental-plaster floor and provided test tubes with sugar water and water. After a 30-minute acclimation period, 10 worker-laid or queen-laid eggs were introduced to the group. Twenty-

four hours later, the number of surviving eggs was counted. In nature, isolated groups of workers and brood comparable in size to our test groups are routinely found outside the nest in cavities such as hollow coconuts, and so removing ants from their colony or worker group overnight is not too artificial.

Colonies that did not show the typical pattern of egg discrimination (i.e., more than five queen-laid eggs survive and fewer than five worker eggs survive) in week 0 (N=16) were not used and a new group was created until there were 15 groups in each treatment. Excluding these groups was appropriate because we were not interested in characterizing the response of workers to eggs, which has been thoroughly described in other studies (Endler et al. 2004, 2006; Moore & Liebig 2010a). The purpose of the current study was to test if egg experience may be responsible for the change in workers' response to eggs. Accordingly, we used only colonies that exhibited a strong and unambiguous response to eggs in week 0.

The egg discrimination assay was repeated two, five, and nine weeks after the establishment of the groups.

Experiment 2: Does Egg Experience Establish Egg Acceptance in Naïve Workers?

Our first manipulation tested if egg experience modifies the egg acceptance behavior of workers that already showed the discrimination pattern typical of workers from established colonies. The purpose of our second experiment was to determine if egg experience could establish egg discrimination

behavior in workers without adult experience with eggs (“naïve workers”). Late-stage pupae were collected from 24 large, established colonies and placed in plastic Petri dishes (9×1.5 cm) with a dental plaster floor and a double-mesh lid along with 20 marked, non-naïve workers. The Petri dish was kept in the parent colony, and the double-mesh lid allowed the exchange of volatiles between the experimental group and the parent nest. Two days later, any pupae that had not eclosed were removed from the Petri dish, thus creating a cohort of naïve workers that eclosed within 48 hours of one another. Five days later, the marked, non-naïve workers were removed from the Petri dish, the five- to seven-day-old naïve workers were paint-marked and returned to the Petri dish, and 20 unmarked, non-naïve workers were transferred from the parent nest into the Petri dish.

Experimental groups were randomly assigned to one of three treatments: queen eggs ($N=8$), worker eggs ($N=7$), or no eggs ($N=8$; Fig. 3.1b). Groups assigned to the egg treatments got 15 queen- or worker-laid eggs two times per week. The eggs were always a mixture of eggs from at least three different sources. All groups received food (Bhatkar diet and half of a chopped mealworm) twice per week, and the unmarked, non-naïve workers were exchanged approximately every two weeks.

The workers were maintained in the Petri dish until they reached 60 days of age, the age at which workers exhibit strong egg discrimination (see below). At this point, the naïve workers were divided into two groups of three to ten, depending on the number of naïve workers that survived. The egg discrimination of each group was tested as described above. Colonies used for this experiment

were collected between 2006 and 2008 and were three to six years old at the time of testing.

Experiment 3: Are Ants Updating Their Recognition Template or Adjusting Their Acceptance Threshold?

The previous two experiments included only two kinds of eggs: worker-laid eggs and queen-laid eggs. Because of this, we could not determine if increased acceptance of worker-laid eggs in groups that had experienced worker-laid eggs occurred because ants were learning the worker-egg profile, because experience with worker-laid eggs reduces egg discrimination behavior, or because experience with queen-laid eggs intensifies egg discrimination (Fig. 3.2). To test these alternatives, we performed a third experiment in which groups were trained with *C. floridanus* queen-laid eggs ($N=8$), *C. floridanus* worker-laid eggs ($N=8$), or queen-laid eggs ($N=8$) from a sympatric species, *C. tortuganus* (Fig. 3.1c). *C. tortuganus* eggs are chemically distinct from both worker- and queen-laid eggs of *C. floridanus* (Fig. 3.3). Groups of 80 minor workers were collected from inside the nest chamber of 24 colonies and maintained as described in the first experiment. All groups received 20 eggs three times a week. As above, the eggs came from at least three different sources. After two weeks, the egg discrimination behavior of each group was assayed as described above, except workers were tested in their response to *C. tortuganus* queen-laid eggs as well as *C. floridanus* queen- and worker-laid eggs.

The Response of Workers from Incipient Colonies to C. tortuganus Eggs

To further test if changes in egg acceptance are due to ants updating recognition templates or adjusting their acceptance thresholds, we tested the response of workers from incipient colonies to queen-laid *C. tortuganus* eggs. Workers from incipient colonies do not destroy worker-laid eggs, which are chemically similar to eggs laid by incipient queens, or established-queen-laid eggs, which are chemically distinct from eggs laid by incipient queens. If this pattern of acceptance arises because workers from incipient colonies have a permissive acceptance threshold, then workers from incipient colonies might accept *C. tortuganus* eggs. If acceptance arises because ants accept established-queen-laid eggs and eggs with familiar profiles, then *C. tortuganus* eggs would not be accepted. The assay was performed on colonies collected in 2009 when the colonies had between 40 and 75 workers (median=52, $N=15$). Thirty workers were removed from each colony and split into three groups of 10 and tested with *C.-tortuganus*-queen-laid eggs, *C.-floridanus*-worker-laid eggs, or eggs laid by their own queen following the egg discrimination assay described in Experiment 1.

Worker Age and Egg Discrimination

To determine at which age workers display egg discrimination behavior, cohorts of workers were created following the procedures explained in Experiment 2 from each of 38 large, established colonies. When the workers were painted on Day 7, they were removed from the experimental Petri dish and

returned to the parent nest. Painted workers were collected 15 ($N=9$), 30 ($N=10$), 45 ($N=10$), or 60 ($N=9$) days post-eclosion. Workers' response to worker-laid eggs was assayed using the egg discrimination assay described in Experiment 1. In most cases ($N=28$), there were enough workers' surviving at the time of testing to use 10 workers in the egg discrimination assays, but fewer workers (3 to 9, median=7.5) were used when no more workers were available ($N=10$). Colonies used in this study were collected between 2001 and 2008 and between two and 10 years old at the time of testing.

Worker Age and Egg Discrimination in Incipient Colonies

Temporal polyethism, or age-based changes in the propensity of workers to perform a particular task, is an important and widespread mechanism for allocating workers to the various tasks within a social insect colony (Wilson 1976; Seeley 1982; Tsuchida 1991). To test if the absence of egg discrimination in incipient *C. floridanus* colonies is a consequence of worker age in those colonies, adult workers in incipient colonies ($N=13$) were marked with paint when the colony contained between 29 and 54 workers. Thirty days later, 10 painted workers (i.e., workers > 30 days old) and 10 unpainted workers (i.e., workers < 30 days old) were collected from the incipient colonies and their response to worker-laid eggs was assayed using the egg discrimination assay described in Experiment 1.

Statistical Analyses

Egg survival was analyzed using Generalized Linear Mixed Models (Baayan 2008) with binomial error structure and logit link function. For Experiment 1, the fixed effects included workers' egg experience (queen, worker or none), the source of the test egg (queen or worker), and the week of the experiment (0, 2, 5, and 9) and all interactions as fixed factors. Week of experiment was Z-transformed before building the model (Schielzeth 2010). We began with a random effects structure that allowed for random intercepts for test group nested in training group and random slopes for training groups across weeks but we simplified the model to include just the intercept term after a likelihood ratio test showed no significant difference between the models. For Experiments 2 and 3, workers' egg experience and the source of the test egg, and their interaction were fixed factors; test group nested in colony of origin was included as a random effect. For the analysis of workers from incipient colonies to *C. tortuganus* queen eggs, test egg source was included as a fixed effect and test group nested in colony of origin was included as a random effect. In the model corresponding to the number of worker eggs recovered from workers from incipient colonies greater and less than 30 days old, the age class of the workers was included as a fixed effect and test group nested in colony of origin was included as a random effect.

The models were fitted in R (R Development Core Team 2009) using the function 'lmer' of the R package lme4 (Bates & Maechler 2010). After loading

the models, we checked for collinearity by examining the $\text{GVIF}^{1/2\text{df}}$ s of the general linear models lacking the random effect using the ‘vif’ function of the R package ‘car’ (Fox & Wiesberg 2011). Models were tested against a null model comprising only the random effects structure using a likelihood ratio test. After determining the significance of the full model at $\alpha=0.05$, we assessed the significance of the interaction term by comparing the full model to a model without the interaction term using a likelihood ratio test.

RESULTS

Experiment 1: Does Egg Experience Affect Egg Acceptance?

Experience with eggs does affect egg discrimination behavior (Likelihood ratio test, full model vs. null model: $\chi^2_{11}=378.1$, $P<0.001$). Specifically, the survival of eggs presented to workers from queenless worker groups was significantly influenced by the interaction of the workers’ egg experience, the source of the test egg, and the week of the experiment ($\chi^2_3=9.306$, $P=0.025$; Table 3.1). Survival of worker-laid eggs was low across all three treatments at the start of the experiment (4.0, 4.0, and 2.0 percent for queen-egg, worker-egg, and no-egg treatments, respectively, Fig. 3.4). After nine weeks, survival of worker-laid eggs increased across all three treatments, but the increase was much more dramatic in groups that were trained with worker-laid eggs (76.7 percent), compared to groups that received queen-laid eggs (24.0 percent) or no eggs (16.7 percent). In contrast, survival of queen-laid eggs was high at the onset of the

experiment (94.0, 88.7, and 78.7 percent for queen-egg, worker-egg, and no-egg treatments, respectively) and remained high after nine weeks for all three groups (75.3, 88.0, and 79.3 percent for queen-egg, worker-egg, and no-egg treatments, respectively).

Experiment 2: Does Egg Experience Establish Egg Acceptance in Naïve Workers?

Egg experience had no detectable effect on egg discrimination behavior on workers with no previous experience with eggs (Likelihood ratio test, full model vs. null model: $\chi^2_5=6.9043$, $P=0.228$). The survival of eggs presented to naïve workers from queenless worker groups was high across all treatments (93.6 to 100 percent) with the exception of worker-laid eggs given to workers that experienced queen-laid eggs (65 percent; Table 3.2; Fig. 3.5).

Experiment 3: Are Ants Updating Their Recognition Template or Adjusting Their Acceptance Threshold?

Eggs had the highest probability of survival when presented to workers that had been trained on that egg type (Likelihood ratio test, full model vs. null model: $\chi^2_8= 63.08$, $P < 0.001$; Fig. 3.6). The survival of eggs presented to workers from queenless worker groups was influenced by the interaction of the workers' egg experience and the test egg source (Likelihood ratio test, $\chi^2_4= 30.68$, $P < 0.001$; Table 3.3). *C. floridanus* worker eggs had higher probability of survival when presented to workers that had experienced *C. floridanus* worker eggs (93.8

percent) than workers trained with *C. floridanus* queen eggs (66.3 percent) or *C. tortuganus* queen eggs (33.8 percent). Similarly, survival of *C. tortuganus* queen eggs was highest in groups that had experienced *C. tortuganus* queen eggs (80.0 percent) followed by groups with *C.-floridanus*-queen-egg experience (35.0 percent) and *C.-floridanus*-worker-egg experience (17.5 percent). Survival of *C.-floridanus*-queen-laid eggs was high across treatments (98.8, 96.3, and 92.5 percent for *C.-floridanus*-queen-laid-egg experience, *C. floridanus*-worker-laid-egg experience, and *C.-tortuganus*-queen-laid-egg experience, respectively).

The Response of Workers from Incipient Colonies to C. tortuganus eggs

Egg survival among eggs presented to *C. floridanus* workers from incipient colonies was highest among eggs laid by the queen of the test colony (97.3 percent), followed by *C. floridanus* worker-laid eggs (78 percent) then eggs laid *C. tortuganus* queens (12 percent). The model including test egg source was significantly better predictor of egg survival than a null model (Likelihood ratio test, $\chi^2_2=52.763$, $P<0.001$; Table 3.4)

Worker Age and Egg Discrimination

Egg discrimination was fully expressed in groups of workers that were 60 days of age; no worker-laid eggs were recovered from these groups. The percent of worker-laid eggs surviving was 38.9, 29.0, and 27.0 for groups tested at 15, 30, and 45 days, respectively.

Worker Age and Egg Discrimination in Incipient Colonies

Egg discrimination was weak in incipient colonies regardless of worker age. Survival of worker-laid eggs was slightly higher when presented to workers less than 30 days of age (88.5 percent) than when presented to workers greater than 30 days of age (76.9 percent), but the model was not significant compared to the null model (Likelihood ratio test, $\chi^2_1 = 2.12$, $P = 0.144$; Table 3.5).

DISCUSSION

Recognition of biologically significant stimuli often involves both learned and innate components. Our results show that both learned and innate components contribute to the recognition and discrimination of eggs in the ant *Camponotus floridanus*. We manipulated the egg experience of *C. floridanus* workers and found egg experience affected egg acceptance. In the first experiment, worker-laid eggs had a greater chance of survival when they were presented to workers that had experienced worker-laid eggs than when they were presented to workers that experienced queen-laid eggs or no eggs (Fig. 3.4). In the second experiment, survival of worker-laid eggs was high across treatments but lowest among workers that had experienced queen-laid eggs (Fig. 3.5). In the third experiment, survival of *C. floridanus*-queen-laid eggs, *C. floridanus*-worker-laid eggs, and *C. tortuganus*-queen-laid eggs was highest when presented to workers that had experienced the same egg type (Fig. 3.6). Survival of queen-laid eggs with queen-

specific hydrocarbons was high regardless of workers' previous egg experience, suggesting that recognition and acceptance of queen-laid eggs is independent of egg experience. Altogether, our results indicate that *C. floridanus* workers accept eggs laid by established queens and eggs with which they are familiar.

Experience can affect egg discrimination behavior in two, non-mutually exclusive ways (Fig. 3.2). One way experience can affect egg discrimination is by adjusting the permissiveness of the acceptance threshold. Exposure to eggs carrying queen-specific hydrocarbons could intensify discrimination behavior by decreasing the permissiveness of the acceptance threshold, or experience with eggs lacking queen-specific hydrocarbons could reduce egg discrimination behavior by increasing the permissiveness of the acceptance threshold (Helanterä et al. 2007). The second way experience can affect egg discrimination is by changing the recognition template. Our results suggest that experience-dependent changes in egg acceptance arise because workers update their recognition template to reflect recent egg experience. In Experiment 3, workers trained on *C.-floridanus*-worker-laid eggs accepted more *C.-floridanus*-worker-laid eggs than *C.-tortuganus*-queen-laid eggs (Fig. 3.6). Similarly, workers trained on *C.-tortuganus*-queen-laid eggs accepted more *C.-tortuganus*-queen-laid eggs than *C.-floridanus*-worker-laid eggs. These results strongly suggest that workers are updating their recognition template to reflect the eggs they have experienced and not just adjusting their acceptance threshold. The hypothesis that ants are updating their recognition template is further supported by the response of workers from incipient colonies to *C.-tortuganus*-queen-laid eggs. Workers from incipient

colonies destroyed *C.-tortuganus*-queen-laid eggs, indicating that *C. floridanus* workers from incipient colonies are not indiscriminately tolerant of all eggs but only those that smell similar to the ones they have experienced (i.e., *C. floridanus* incipient-queen-laid eggs and worker-laid eggs) or that have queen-specific hydrocarbons (Moore & Liebig 2010a). The ability to learn a chemical profile and discriminate against unfamiliar profiles is well documented in social insects because it is the mechanism used to distinguish nestmates from non-nestmates (Howard & Blomquist 2005; Wyatt 2010). In ants, termites, and wasps, the chemicals used for nestmate recognition are hydrocarbons (van Zweden & d'Ettorre 2010), the same class of compounds *C. floridanus* workers use to distinguish between queen- and worker-laid eggs (Endler et al. 2004). Egg recognition could rely on a learning mechanism similar to the one that allows for nestmate discrimination.

Our results also strongly suggest the involvement of an innate component in egg recognition. Eggs laid by established queens were always tolerated by the ants in our experiment, even when ants had no adult experience with queen-laid eggs (Fig. 3.5). The same is true for workers from incipient colonies, which tolerate queen-laid eggs with queen-specific hydrocarbons despite having no experience with such eggs (Moore & Liebig 2010a).

We find that worker age does not account for the difference in egg discrimination behavior between workers from incipient and established colonies. The survival of worker-laid eggs did not differ when presented to workers from incipient colonies greater than or less than 30 days of age.

Learned and Innate Contributions to Recognition: Ants, Eggs, and Beyond

The use of both learned cues and innate signals allows *C. floridanus* workers to adjust their egg-eating behavior during colony growth while providing positive identification of the queen's eggs for most of the colony's lifespan.

Learning allows the recognition system to be flexible; workers that eclose in incipient colonies can learn the profile of the incipient queen-laid eggs in their environment and care for those eggs, even though workers that eclose later in the colony's life would destroy those same eggs. An innately meaningful signal identifying queen-laid eggs provides protection from recognition errors; the profile of established queen-laid eggs, which will be the eggs encountered by most workers, does not have to be learned to be recognized. The *C.-floridanus*-egg-recognition system is similar to other cases in which animals have been selected to respond to a prevailing, preferred stimulus, but can respond to other stimuli when their preferred stimulus is not available. Such a system is observed in Lepidopteran pollinators that have an innate preference for the most profitable flower type in their environment, but are capable of learning alternate flowers when their preferred flower is not available (Weiss 1997; Riffell et al. 2008).

Vertebrates often use learned cues to discriminate among of targets of brood care. American coots, *Fulica americana*, learn cues from the first-hatched chicks in their brood and use these cues to discriminate against intraspecific brood parasites, which hatch later (Shizuka & Lyon 2010). Bats, sheep, seals, and terns,

among others, rely on learned cues to identify their offspring (Buckley & Buckley 1972; Balcombe 1990; Charrier et al. 2002; Searby & Jouventin 2003).

There are surprisingly few examples of insects using learning in the context of recognizing targets for brood care. Offspring recognition, most probably via learning, has been reported in wasps (Lorenzi & Filippone 2000). A study by van Zweden et al. (2009) hints that learning might be important for egg discrimination by workers in *Pachycondyla inversa*, another ant species.

Offspring recognition, potentially via learning, is known in non-insect arthropods, including spiders (Evans 1998) and crayfish (Mathews 2011). Our study provides direct evidence that insects use learning to recognize and discriminate between potential targets of brood care. Learning is thought to be near-universal in insects, and insects use learning to perform many major life functions (Dukas 2008). We suspect insect examples of offspring recognition via learning are more rare in the scientific literature than they are in nature.

Perhaps more exceptional than the use of learned cues in the context of brood discrimination is the use of an innate signal. A signal is useful for brood discrimination only when the distinguishing characteristics of profitable care targets are consistent across caretakers. This is not the case when caregivers need to distinguish between related and unrelated young (Tibbetts & Dale 2007), but it does apply when caretakers are selected to recognize a brood item's class membership, such as when ant workers are selected to recognize queen-laid eggs. It could also apply when caregivers are selected to distinguish between young of their species and allospecific young, as in the case of species parasitized by

allospecific parasites. In these species, a signal that indicates the species identity of the offspring could evolve.

The acceptance of *C. tortuganus* queen eggs by *C. floridanus* workers raises a deeper question about innate and learned contributions to recognition: how do *C. floridanus* workers recognize an object is an egg? The egg-surface hydrocarbons of *C.-tortuganus-queen-laid* eggs are unlike those *C. floridanus* workers would encounter in their nest (Fig. 3.3), and *C. tortuganus* eggs are noticeably more oblong (DM personal observation). Despite these differences, ants that experienced *C.-tortuganus-queen-laid* eggs treated them like *C.-floridanus-queen-laid* eggs after only two weeks. Why were these eggs treated like eggs, and not food, brood, trash, or nesting material? At this time, we do not know what characteristics govern the recognition of an egg as an egg, though we suspect an innate recognition mechanism. In general, we know little about how animals recognize broad classes of objects in their environment.

Fertility Signaling

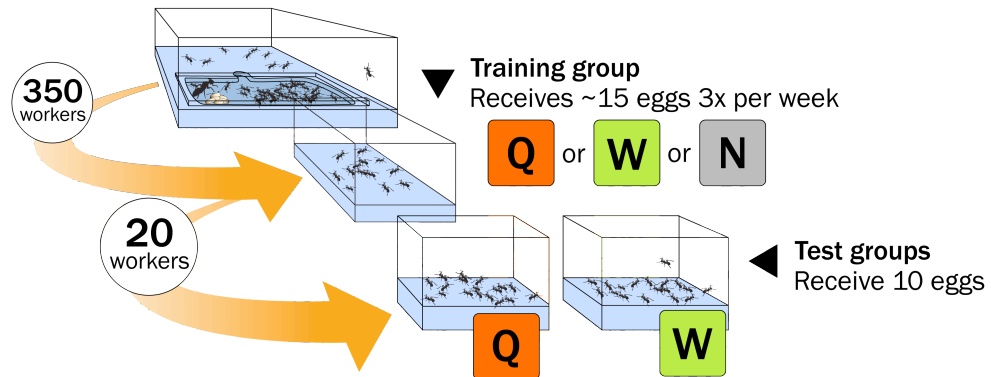
Our results also provide indirect support for the idea that queen-specific hydrocarbons on the surface of *C. floridanus* eggs serve as a fertility signal that advertises the queen's presence and reproductive capacity to her workers (Keller & Nonacs 1993; Beekman 2004; Endler et al. 2004; Endler et al. 2006). The pheromonal action of queen-laid eggs was first demonstrated by Endler et al. (Endler et al. 2004), who found that queen-laid eggs inhibit worker reproduction in orphaned worker groups. Endler et al. speculated that the pheromone's active

compounds are contained in the short-chained hydrocarbons found on eggs laid by queens of established colonies. Here we show queen-laid eggs possess a signal that protects the eggs from destruction. Previous work shows that egg surface hydrocarbons are used to discriminate between queen and worker-laid eggs (Endler et al. 2004). This suggests that egg surface hydrocarbons contain the signal that protects eggs from destruction. The same signal could also be responsible for inhibiting the activation of ovaries in workers.

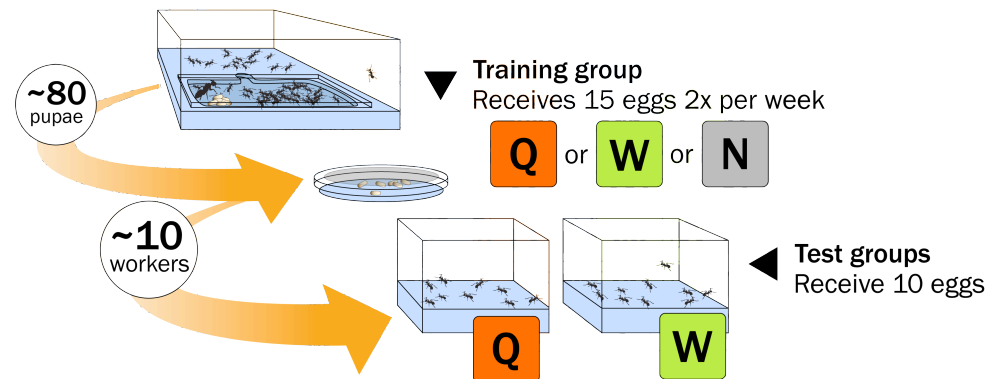
Conclusion

C. floridanus workers need to recognize queen-laid eggs throughout the lifespan of the colony, but the chemical profile of queen eggs is not constant across colony development. We find *C. floridanus* workers solve this recognition challenge through the use of both innate signals and learned cues. Innate recognition of eggs laid by established queens provides a means of recognizing queen-laid eggs that can be used by most workers. Learning allows the subset of workers that experience the queen's eggs during early stages of colony growth to recognize and care for these eggs, which workers from larger, older colonies would destroy. Recognition often involves a combination of innate and learned components. Innate and learned recognition can accomplish in combination what neither can do on its own.

A. Experiment 1



B. Experiment 2



C. Experiment 3

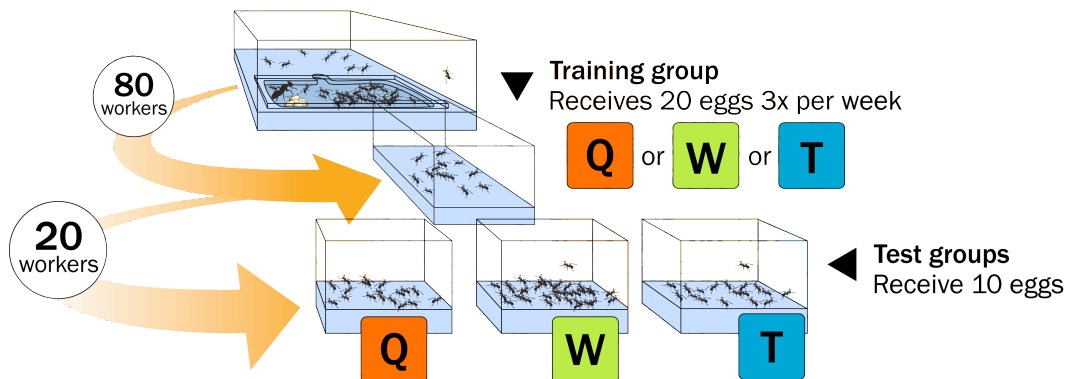
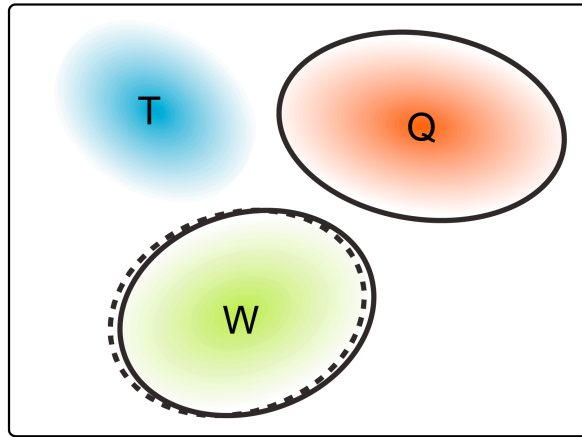


Figure 3.1. Schematic diagrams showing the experimental design of the three major experiments. For all diagrams, “Q,” “W,” “T,” and “N” denote established *C.-floridanus*-queen-laid eggs, *C.-floridanus* worker eggs, *C.-tortuganus*-established-queen-laid eggs, and no eggs, respectively. (A) In Experiment 1, 350

workers were isolated from each of 45 large, established colonies and provided approximately 15 queen-laid eggs, worker-laid eggs, or no eggs three times per week for nine weeks ($N=15$ for each group). At zero, two, five, and nine weeks, two test groups of 20 workers were isolated from the training group. One group received 10 queen-laid eggs; the other received 10 worker-laid eggs. The number of surviving eggs was counted after 24 hours. (B) In Experiment 2, approximately 80 late-stage pupae were isolated from each of 23 large, established colonies. Forty-eight hours later, all remaining pupae were removed, creating a cohort of naïve workers that eclosed within 60 days of one another. The Petri dish was kept in the parent colony and provided 15 queen-laid eggs, worker-laid eggs, or no eggs ($N=8, 7,$ and $8,$ respectively) two times per week until workers were 60 days of age. At that time, the surviving workers were split into two groups. One group received 10 queen-laid eggs; the other received 10 worker-laid eggs. The number of surviving eggs was counted after 24 hours. (C) In Experiment 3, 80 adult workers were isolated from each of 24 large, established colonies and provided 20 queen-laid eggs, worker-laid eggs, or *C.-tortuganus*-queen-laid eggs three times per week for two weeks ($N=8$ for each group). After two weeks, three groups of 20 workers were isolated from the training group. One group received 10 queen-laid eggs, one received 10 worker-laid eggs, and one received 10 *C.-tortuganus*-queen-laid eggs. The number of surviving eggs was counted after 24 hours.

A. Updating recognition template



B. Adjusting acceptance threshold

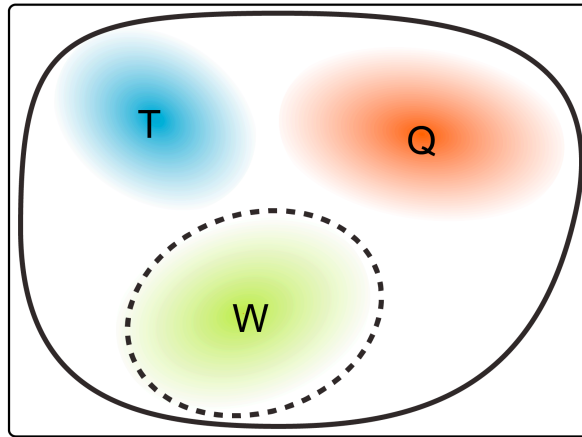


Figure 3.2. Acceptance of worker-laid eggs in Experiment 1 could be due to ants updating their recognition template to reflect the characteristics of the eggs they experience or to adjusting the permissiveness of their acceptance threshold. Colored clouds represent different egg profile phenotypes in a two-dimensional representation of the range of possible egg profiles. “Q,” “W,” and “T” represent *C.-floridanus*-queen-laid eggs, *C.-floridanus* worker eggs, and *C.-tortuganus*-established-queen-laid eggs, respectively. Dotted lines represent the workers’ recent egg experience. Solid lines enclose the range of eggs that are not destroyed by workers. (A) If workers update their recognition profile to reflect eggs they experience, then they should destroy unfamiliar eggs that are not laid by established queens. (B) If egg experience changes the egg acceptance threshold, then workers with more permissive acceptance thresholds would accept eggs other than established queen-laid eggs, even if they have no experience with such eggs.

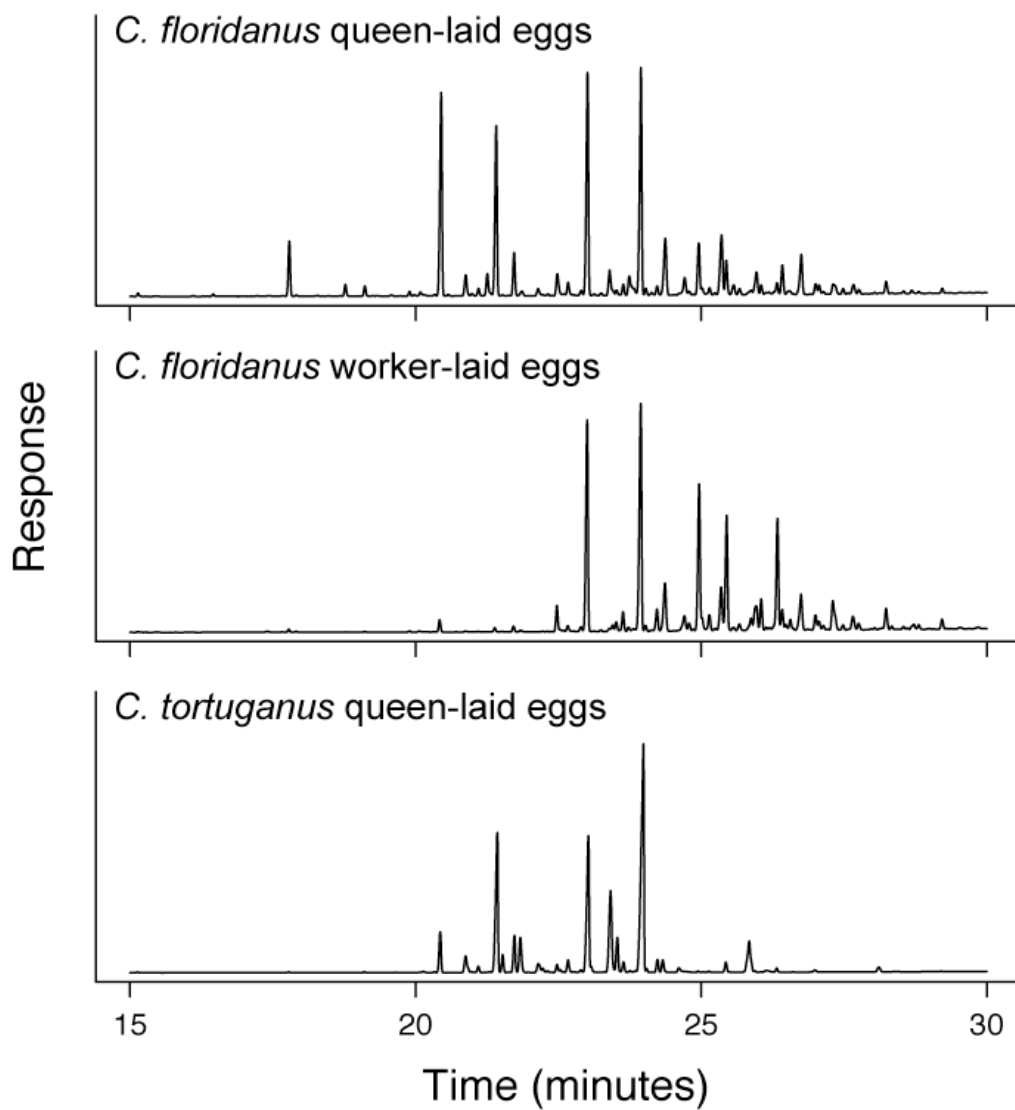


Figure 3.3. Representative chromatograms showing the non-polar compounds on the surface of *C. floridanus*-queen-laid eggs, *C. floridanus*-worker-laid eggs, and *C. tortuganus*-queen-laid-eggs.

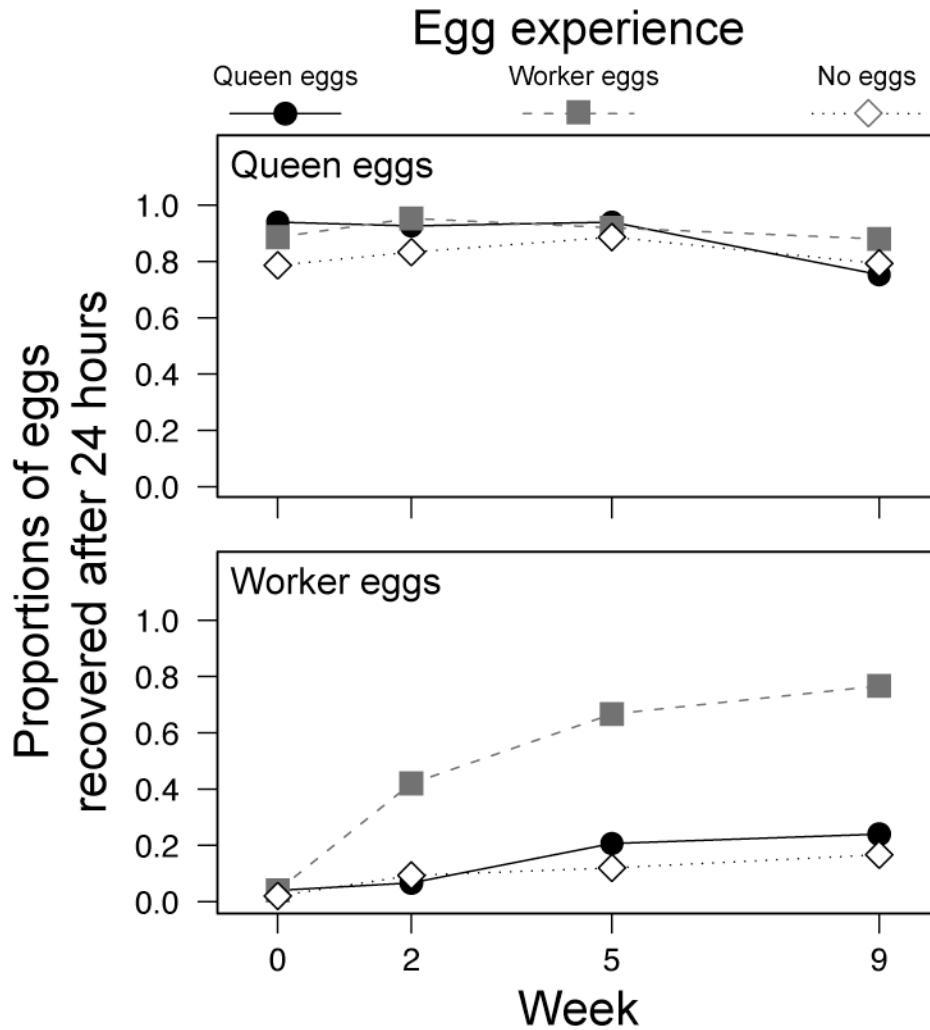


Figure 3.4. The proportion of queen-laid (top) and worker-laid (bottom) eggs surviving 24 hours after being presented to workers from queenless worker groups that have experienced queen-laid (circles), worker-laid (squares), or no eggs (diamonds) for zero, two, five, or nine weeks. Error bars are not pictured because standard error does not extend beyond the edges of the symbols.



Figure 3.5. The proportion of queen- (left) and worker-laid (right) eggs surviving after 24 hours when presented to workers from queenless worker groups that have experienced queen-laid (circles), worker-laid (squares), or no eggs (diamonds) during the first 60 days of their adult life. Error bars represent standard error. Where absent, the limits of the standard error do not extend beyond the edges of the symbol.

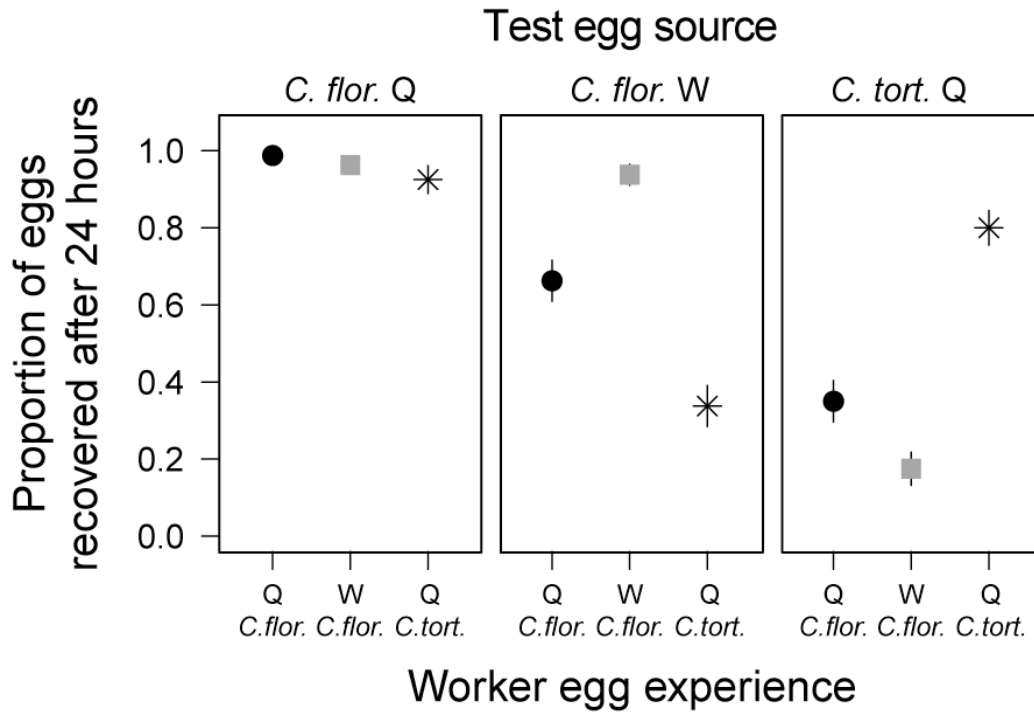


Figure 3.6. The proportion of *C.-floridanus*-queen- (left), *C.-floridanus*-worker-laid (center), and *C.-tortuganus*-queen-laid (right) eggs surviving after 24 hours when presented to workers from queenless worker groups that have experienced *C.-floridanus*-queen-laid (circles), *C.-floridanus*-worker-laid (squares), and *C.-tortuganus*-queen-laid (stars) for two weeks. Error bars represent standard error. Where missing, the limits of the standard error do not extend beyond the edges of the symbol.

Table 3.1. The effect of week (Z-transformed), worker egg experience, and test egg source on the survival of eggs presented to workers from queenless worker groups in Experiment 1.

Fixed effect	Estimate	Standard error
Intercept	3.2107	0.5749
Experience (queen)	1.2012	0.8546
Experience (worker)	1.0508	0.8293
Test (worker)	-8.1738	0.7715
Week	0.4880	0.4504
Experience (queen) × Test (worker)	-1.1007	1.1482
Experience (worker) × Test (worker)	3.4039	1.1095
Experience (queen) × Week	-1.2961	0.6708
Experience (worker) × Week	-0.8165	0.6476
Test (worker) × Week	0.5088	0.7427
Training (queen) × Test (worker) × Week	1.4574	1.0796
Training (worker) × Test (worker) × Week	2.8414	1.0052

Table 3.2. The effect of worker egg experience and test egg source on the survival of eggs presented to workers from queenless worker groups in Experiment 2.

Fixed effect	Estimate	Standard error
Intercept	7.0789	2.7526
Experience (queen)	-2.0101	3.1953
Experience (worker)	-0.9955	3.5043
Test (worker)	0.3256	2.7396
Experience (queen) × Test (worker)	-3.6932	3.1171
Experience (worker) × Test (worker)	14.6211	2205.7283

Table 3.3. The effect of worker egg experience and test egg source on the survival of eggs presented to workers from queenless worker groups in Experiment 3.

Fixed effect	Estimate	Standard error
Intercept	2.900	1.027
Experience (queen)	-4.366	1.391
Experience (worker)	-5.587	1.409
Test (queen)	1.066	1.511
Test (worker)	-4.375	1.377
Experience (queen) × Test (queen)	6.460	2.646
Experience (worker) × Test (queen)	6.516	2.238
Experience (queen) × Test (worker)	6.705	1.878
Experience (worker) × Test (worker)	11.166	2.023

Table 3.4. The effect of test egg source on the survival of eggs presented to workers from incipient colonies.

Fixed effect	Estimate	Standard error
Intercept	5.241	1.074
Test (worker)	-2.982	1.291
Test (<i>C. tortuganus</i> queen)	-9.384	1.398

Table 3.5. The effect of worker age (greater or less than 30 days) on the survival of worker-laid egg presented to workers from incipient colonies.

Fixed effect	Estimate	Standard error
Intercept	2.4527	0.9029
< 30 days of age	1.4679	0.9939

Chapter 4

CONCLUSIONS AND PERSPECTIVES

This work radiates from the observation that incipient queens of the ant *Camponotus floridanus* do not have the distinct hydrocarbons that characterize established queens, but its impact extends beyond the biology of this single species.

At the time I started graduate school, the scientific literature teemed with theory papers that argued that policing is an essential ultimate explanation for worker sterility (Wenseleers et al. 2004a; Wenseleers et al. 2004b; Ratnieks & Wenseleers 2005; Ratnieks et al. 2006; Wenseleers & Ratnieks 2006a; Ratnieks & Wenseleers 2008; Ratnieks & Helanterä 2009). These papers were accompanied by a drive to understand the origin of policing behavior, and this devolved into a debate over whether the relatedness hypothesis or the colony efficiency hypothesis better explained the pattern of policing (Hammond & Keller 2004; Wenseleers & Ratnieks 2006b). Of course, the two hypotheses are not mutually exclusive; both costs and kinship contributed to the evolution of policing behavior. Nevertheless, the relatedness hypothesis dominated the debate, and there was a strong need for empirical evidence that established the importance of factors other than relatedness in the evolution of policing behavior. The existence of policing behavior in a clonal ant species was one strong argument for the importance of factors other than relatedness (Hartmann et al. 2003). The results of the study reported in Appendix A provide more evidence that relatedness alone

does not always explain the expression of policing behavior. I showed that the expression of policing behavior changes across colony development in the ant *C. floridanus*, even though relatedness does not.

Appendix A was the first investigation of the expression of policing behavior across colony development. In 2009, Ohtsuki & Tsuji published a model that predicted that the costs of worker reproduction are the highest early in a colony's development, and therefore policing should be strongest in the earliest stages of colony growth. In my first study (Appendix A), I found no evidence of policing by egg-eating in incipient colonies of *C. floridanus*. In Chapter 2, I investigated the possibility that a policing mechanism other than egg-eating is used in incipient *C. floridanus* colonies. I found no evidence of another mechanism, and instead suggested another possibility: the costs of worker reproduction in an incipient colony could be so high that it is in the worker's self-interest to refrain from reproducing.

In addition to contributing a new idea about how worker-policing behavior might change with colony age, Chapter 2 serves as a reminder that extant social insect species are imperfect systems in which to study the origins of eusociality. The evolution of policing behavior can be evaluated in modern social insects only if the factors that modulate the expression of policing behavior in contemporary species are taken into account. The colony's developmental stage is one factor that influences the expression of policing behavior (Appendix A; Bonckaert et al. 2011; Walter et al. 2011)

Policing, defined as any trait in a worker that biases male production toward queens' sons, is a widespread phenomenon in social insects. Its theoretical importance in the evolution of near-sterile worker castes, as well as its connotation-laden term, has potentially limited our understanding of this common behavior. For example, it is not clear that the purpose of policing behavior in *C. floridanus* colonies is to limit selfish reproduction by workers. As shown in Chapter 2 and Endler et al. (2004), worker-laid eggs do not appear until workers have been separated from their queen for at least 60 days, and many worker groups show no evidence of producing eggs even after half a year of isolation. Nevertheless, policing behavior is strongly expressed in this species. To better understand the role of policing in contemporary insect studies, it is essential to study the expression of policing behavior outside the silos of particular theoretical predictions. Studies that originate from an intriguing observation (i.e., the absence of queen hydrocarbons on queen-laid eggs in incipient colonies) are one way to illuminate the full spectrum of explanations for policing behavior, and not just those favored by a single theory.

The interpretation of queen-specific hydrocarbons as an egg-marking signal is strongly upheld in my research. In Chapter 3, I find that eggs laid by established queens are always accepted by *C. floridanus* workers, regardless of their previous egg experience. There is also weak evidence that exposure to such eggs increases discrimination against worker-laid eggs. Nurturing eggs that bear queen hydrocarbons appears to be an innate response fashioned by natural selection. The flexibility to care for other eggs is achieved through learning. My

results demonstrate how learned and innate recognition can be combined to create a system of recognition that more closely matches an animal's ecological needs. I also provide a rare, insect example of learning in the context of recognizing targets of brood care.

My research contributes to a small but growing body of research that investigates how the organization of a social insect colony changes with colony ontogeny. It has long been recognized that a social insect colony follows a stereotyped trajectory of development (Wilson 1985). As it grows, the colony faces different ecological challenges and contends with different constraints. Until recently, very few studies explored how the biology of a social insect colony changes according to colony stage (Tschinkel 1999; Bonckaert et al. 2011; Clark 2011; Holbrook et al. 2011; Walter et al. 2011). Social insect colonies may represent an opportunity to extract general principles of developmental biology (Yang 2007), but progress cannot be made without empirical data.

Finally, the ability to recognize and respond appropriately to social information is a challenge faced by all group-living animals. The challenge is made even more difficult when multiple pieces of information are conveyed in the same modality. In Appendix B, I investigate how workers from established colonies integrate information about colony membership and fertility status, which are both encoded in an ant's cuticular hydrocarbons. I find fertility status overrides colony membership information in *C. floridanus*.

Among all animals, social insects are especially dependent on communication because the exchange of information is necessary to coordinate

the behavior of colony members. The absence of queen pheromones in incipient social insect colonies is therefore striking. How does a social insect colony accommodate the dramatic change in the quantity of queen pheromone that occurs during colony growth? The pursuit of this question has contributed new information to the fields of social evolution, sociogenesis, animal cognition, and animal communication.

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APPENDIX A
MECHANISMS OF SOCIAL REGULATION CHANGE ACROSS
COLONY DEVELOPMENT IN AN ANT

RESEARCH ARTICLE

Open Access

Mechanisms of social regulation change across colony development in an ant

Dani Moore^{*}, Jürgen Liebig

Abstract

Background: Mutual policing is an important mechanism for reducing conflict in cooperative groups. In societies of ants, bees, and wasps, mutual policing of worker reproduction can evolve when workers are more closely related to the queen's sons than to the sons of workers or when the costs of worker reproduction lower the inclusive fitness of workers. During colony growth, relatedness within the colony remains the same, but the costs of worker reproduction may change. The costs of worker reproduction are predicted to be greatest in incipient colonies. If the costs associated with worker reproduction outweigh the individual direct benefits to workers, policing mechanisms as found in larger colonies may be absent in incipient colonies.

Results: We investigated policing behaviour across colony growth in the ant *Camponotus floridanus*. In large colonies of this species, worker reproduction is policed by the destruction of worker-laid eggs. We found workers from incipient colonies do not exhibit policing behaviour, and instead tolerate all conspecific eggs. The change in policing behaviour is consistent with changes in egg surface hydrocarbons, which provide the informational basis for policing; eggs laid by queens from incipient colonies lack the characteristic hydrocarbons on the surface of eggs laid by queens from large colonies, making them chemically indistinguishable from worker-laid eggs. We also tested the response to fertility information in the context of queen tolerance. Workers from incipient colonies attacked foreign queens from large colonies; whereas workers from large colonies tolerated such queens. Workers from both incipient and large colonies attacked foreign queens from incipient colonies.

Conclusions: Our results provide novel insights into the regulation of worker reproduction in social insects at both the proximate and ultimate levels. At the proximate level, our results show that mechanisms of social regulation, such as the response to fertility signals, change dramatically over a colony's life cycle. At the ultimate level, our results emphasize the importance of factors besides relatedness in predicting the level of conflict within a colony. Our results also suggest policing may not be an important regulatory force at every stage of colony development. Changes relating to the life cycle of the colony are sufficient to account for major differences in social regulation in an insect colony. Mechanisms of conflict mediation observed in one phase of a social group's development cannot be generalized to all stages.

Background

Kin selection theory can explain the evolution of cooperation within groups of related individuals, but unless group members are clones, there is also potential for conflict [1]. Because relatedness establishes the basis for cooperation and conflict within family groups, it has overshadowed other factors that affect the degree of conflict [2-4]. Variations in relatedness are not necessarily the most important force determining the extent of conflict in a social group; costs and benefits of altruism

can be the dominant predictors of social behaviour [2,5]. In cooperative groups with a predetermined life cycle, such as a social insect colony, the group's developmental stage is one factor that may affect the degree of conflict within the group [6].

In the social Hymenoptera (ants, bees, and wasps), conflict may exist over male production [7]. In most species, workers retain functional ovaries and are capable of laying viable, male-destined eggs. Because workers are more closely related to their own sons than the queen's sons, workers are predicted to prefer producing their own sons over rearing the queen's sons. In reality,

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male production by workers is often absent or repressed under queenright conditions [4,8,9]. Worker sterility is hypothesized to be enforced by mutual policing. Worker policing occurs in two forms: (1) physical policing, in which workers with activated ovaries are attacked by nestmates [10-13], and (2) egg policing, in which workers detect and destroy worker-laid eggs [14-20]. The importance of policing in maintaining worker sterility has been emphasized in several recent papers [21-26].

Mutual worker policing can evolve if workers are more closely related to the queen's sons than to the sons of other workers (relatedness hypothesis). This is the case in polygynous or polyandrous species [14,16,27,28], but policing behaviour has also been described in species that are monogynous and monoandrous [19,29-31], and even in a clonal species [13]. In these species, workers are more related to other worker's sons than the sons of the queen, and thus worker policing cannot be explained solely on the grounds of relatedness. Instead, policing behaviour may have evolved in these species because the costs of worker reproduction reduce the inclusive fitness of workers (cost hypothesis) [4,32,33].

The costs of worker reproduction are not constant across colony development [5,6]. Incipient social insect colonies undergo a period of ergonomic growth in which new workers are added to the colony but no sexuals are produced [34]. Presumably, this reflects the relative value of colony growth over reproduction in the early stages of a colony's development. Worker reproduction is necessarily an investment in reproduction because worker-laid eggs can only develop into males, which do not work [35]. Worker reproduction further undermines ergonomic growth because reproductive workers are less productive than their non-reproductive counterparts [4,32,33,36-38]. Loss of worker productivity is especially costly to incipient colonies because the relative contribution of each worker is greatest when the colony is small [5], and colony mortality is highest in incipient colonies [39]. Because worker reproduction is more costly in incipient colonies than large colonies, worker policing is predicted to be strongest when the colony is young [6]. However, workers from incipient colonies have less incentive to reproduce, so the policing mechanisms present in large colonies may not be the same as in incipient colonies.

Effective policing requires that workers be able to identify cheaters or their eggs. Much evidence indicates hydrocarbons on the cuticles of adults or on the surfaces of eggs provide the information workers use to recognize the presence of accepted reproductives and to target reproductive cheaters. Hydrocarbons correlate reliably with fertility in more than 28 genera of ants, wasps, bees, and termites [40-44]. Physical policing can

occur when reproductive workers exhibit fertility-related hydrocarbons on their cuticle [12,45-47]. Egg policing can occur when the surface hydrocarbons of worker-laid eggs lack the fertility-related hydrocarbons present on the eggs of the queen [18,19,48].

Fertility information may not be available as an informational basis for policing in incipient colonies. As predicted by the hypothesis that hydrocarbons are an honest indicator of reproductive capacity [40,49], the concentration of fertility compounds on both the cuticle and egg surface increases with egg-laying rate [50-52]. Queen egg-laying rate is positively correlated with colony size; queens of small colonies lay very few eggs per day [52-54]. Therefore, founding queens and their eggs are expected to lack the hydrocarbons characteristic of highly productive reproductives. This prediction has been tested in two species, *Camponotus floridanus* [52] and *Lasius niger* [55]. As predicted, the abundance of fertility-related hydrocarbons on a queen's cuticle increases as the colony grows and the queen becomes more productive. Incipient queens of *C. floridanus* lack the shorter-chained compounds present on the cuticles of established queens. Eggs laid by founding queens are chemically indistinguishable from worker-laid eggs, and workers from large colonies destroy incipient-queen-laid eggs as frequently as worker-laid eggs [52]. This presents an interesting discrepancy. Worker policing is theoretically predicted to be strongest in growing colonies, but the informational basis for policing used in large colonies is not available in incipient colonies.

We explore worker-policing behaviour across colony development. Because relatedness within a colony is constant across development, variations in relatedness cannot explain any change in policing behaviour we observe between incipient and large colonies. We also address the proximate mechanisms of worker policing to understand how workers accommodate the changes in fertility signalling that accompany colony growth.

Fertility signalling and worker policing have been studied extensively in the monogynous carpenter ant *C. floridanus* [19,38,52], in which queens are only single-mated [56]. In this species, qualitative and quantitative differences exist between the cuticular hydrocarbons of workers and established queens. Approximately half of the total amount of hydrocarbons present on an established queen's cuticle represent compounds that correlate with fertility [19,52]. Queens bearing these fertility-related hydrocarbons can be transferred between established colonies without aggression [57]. The eggs of established queens are coated with a blend of hydrocarbons similar to the hydrocarbons on the queen's cuticle [19,52]. Worker-laid eggs lack the shorter-chained, fertility-related hydrocarbons present on queen-laid eggs. When worker-laid eggs are introduced into a large, queenright

colony, they are destroyed. Worker-laid eggs coated in queen hydrocarbons are destroyed less often than unmanipulated eggs, strongly suggesting hydrocarbons are responsible for the recognition of queen- and worker-laid eggs [19]. Physical policing does not occur in *C. floridanus* [38].

In this study, we tested the egg-policing behaviour of *C. floridanus* workers at three points in colony development. At each of the three points, we tested the response of workers to eggs laid by their own queen, eggs laid by an established queen (i.e., a queen at least one year of age with a colony of more than 1000 workers), and worker-laid eggs. We collected egg surface hydrocarbon data to correlate our behavioural results with the availability of relevant fertility information. In a second experiment, we explored the response to fertility information further by contrasting the response of workers from small colonies and large colonies to the introduction of foreign queens.

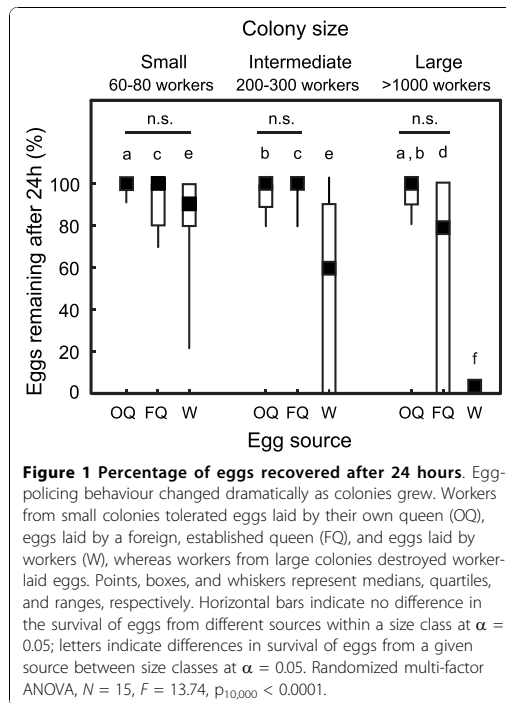
Results

The egg-policing behaviour of *C. floridanus* workers changed dramatically during colony growth (Randomized multi-factor ANOVA, $F = 13.74$, $p_{10,000} < 0.0001$, Table 1, Figure 1). In small colonies (60-80 workers), workers tolerated all eggs, regardless of origin. There was no significant difference in the median percentage of eggs recovered after 24 hours from workers receiving eggs laid by their own queen (median = 100%, range = 90-100%, $N = 15$), an established queen (median = 100%, range = 70-100%, $N = 15$), or foreign workers (median = 90%, range = 20-100%, $N = 15$). Egg-policing behaviour emerged only with colony growth. In large colonies (>1000 workers), the percentage of eggs surviving the 24-hour discrimination assay remained high for workers receiving their own queen eggs (median = 100%, range = 80-100%, $N = 15$), but dropped to zero for eggs laid by workers (median = 0%, range = 0%, $N = 15$). Acceptance of eggs laid by foreign, established queens by workers from large colonies was highly variable (median = 60%, range = 0-100%, $N = 15$).

The proportion of fertility-related compounds on the surface of queen eggs increased with colony size, as

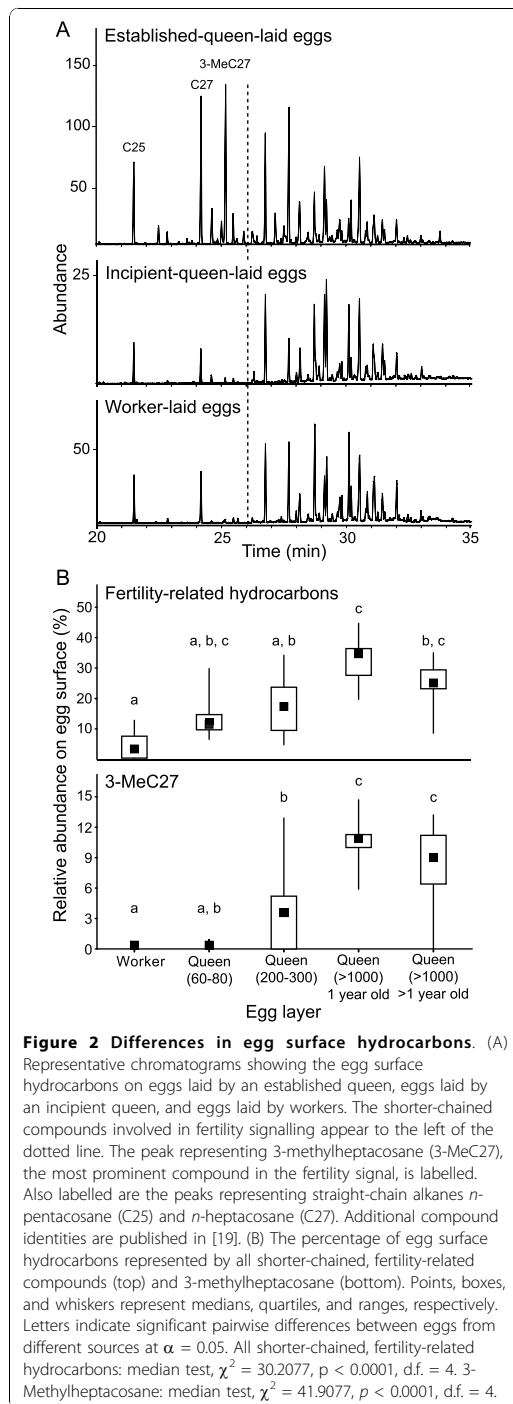
Table 1 Randomized multi-factor ANOVA with colony size and egg source as fixed factors and colony as a random factor

source	ss	df	ms	F	$P_{10,000}$
colony	49.526	14	3.538	0.80	0.6711
colony size	387.393	2	193.696	43.61	< 0.0001
egg source	623.348	2	311.674	70.18	< 0.0001
colony size × egg source	282.074	4	70.519	15.88	< 0.0001
error	497.407	112	4.441		



reported in Endler *et al.* [52]. Shorter-chained, fertility-related compounds (*n*-pentacosane to 10-methyl-, 12-methyl-, 14-methyloctacosane) comprised a greater percentage of surface hydrocarbons on eggs laid by queens of large experimental colonies (median = 34.7%, range = 19.7-44.7%, $N = 13$), than by established-queen-egg donors (median = 25.1%, range = 8.6-35.0%, $N = 15$), intermediate queens (median = 16.6%, range = 4.7-34.2%, $N = 12$), incipient queens (median = 11.5%, range = 6.6-29.8%, $N = 8$), and workers (median = 3.4%, range = 19.7-44.7%, $N = 8$; median test, $\chi^2 = 30.2077$, $p < 0.0001$, d.f. = 4; Figure 2). Straight-chain alkanes *n*-pentacosane (C25) and *n*-heptacosane (C27) comprise the majority of the fertility-related compounds on the eggs of workers and incipient queens.

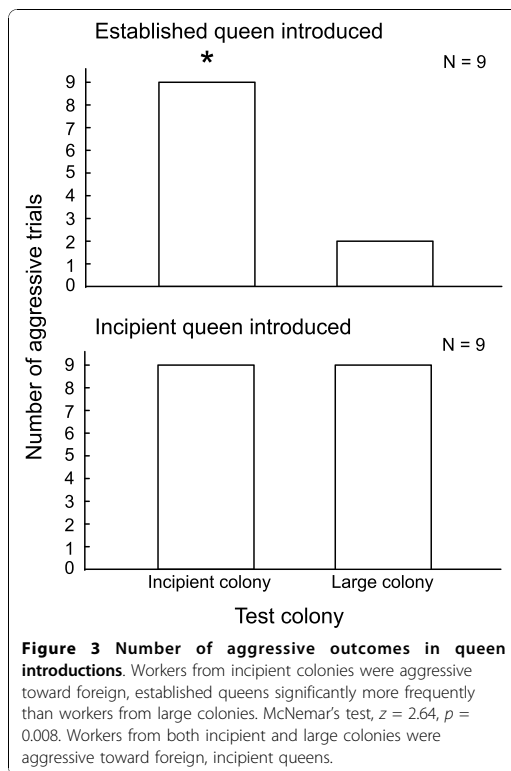
Although it is not yet known which of the fertility-related hydrocarbons *C. floridanus* workers use to distinguish eggs laid by established queens from eggs laid by incipient queens and workers, 3-methylheptacosane is the most prominent of the fertility-related hydrocarbons in the profile of an established-queen-laid egg. Another 3-methyl alkane, 3-methylhentricontane, was recently identified as the queen fertility pheromone in the ant *Lasius niger* [44], and 3-methyl alkanes correlate with fertility in a number of species [12,51,58-60].



The median percentage of 3-methylheptacosane was greatest for eggs laid by the queen of large experimental colonies (median = 10.9%, range = 5.9–14.7%, $N = 13$), followed by eggs laid by established-queen-laid-egg donors (median = 9.0%, range = 0.0–13.2%, $N = 15$), queens of intermediate experimental colonies (median = 3.2%, range = 0.0–12.9%, $N = 12$), queens of small experimental colonies (median = 0.0%, range = 0.0–0.9%, $N = 8$), and workers (median = 0.0%, range = 0.0–0.0%, $N = 8$; median test, $\chi^2 = 41.9077$, $p < 0.0001$, d.f. = 4; Figure 2).

To show that workers from small colonies can perceive and respond to complex hydrocarbon blends, we conducted nestmate recognition bioassays within a week of the egg-discrimination bioassay. Cuticular hydrocarbons are used to discriminate nestmates from non-nestmates [61–63]. In *C. floridanus*, the difference between hydrocarbons of workers from different colonies is much more subtle than the difference in the surface hydrocarbons between eggs laid by individuals of high (i.e., established queens) and low fertility (i.e., workers and incipient queens) [57]. We thus reasoned that ants that can detect the subtle differences between nestmates and non-nestmates can also detect the dramatic differences between eggs laid by workers and established queens [19]. Workers from small colonies were highly effective at recognizing and attacking non-nestmates. In 15 replicates, ants never attacked their own nestmate, but they attacked workers from foreign colonies of the same size in all 15 trials and workers from large foreign colonies in 14 out of 15 trials (Cochran Q test, $Q = 28.13$, $p < 0.0001$).

In a previous study, we showed workers from established colonies tolerate established queens from foreign colonies [57]. Tolerance of established queens is thought to occur because the queen's fertility overrides information regarding colony membership. To test if workers from incipient colonies responded to fertility information on established queens in the same manner as workers from large colonies, we presented established queens from large colonies (>1000 workers) to workers from incipient colonies (< 40 workers, $N = 9$). The same queens were also introduced to workers from large colonies as a control ($N = 9$). Workers from incipient colonies were highly aggressive toward foreign, established queens. Workers from incipient colonies attacked foreign, high-fertility queens in all 9 replicates. In contrast, workers from large colonies were rarely aggressive toward foreign, high-fertility queens, attacking the introduced queen in only 2 of the 9 trials (McNemar's test, $z = 2.64$, $p = 0.008$; Figure 3). We then performed the study using foreign queens from incipient colonies. Workers from both incipient and large colonies were highly aggressive to foreign, incipient queens. Foreign,



incipient queens were attacked in all 9 introductions to workers from incipient colonies and in all 9 introductions to workers from large colonies. In both treatments, queens were attacked significantly more often than expected by chance (binomial test, $p < 0.002$ for each case).

Discussion

Our study investigates worker policing behaviour across colony development and addresses both the proximate and ultimate causes of worker policing in social insect colonies. We find a dramatic change in the response to worker-laid eggs between workers from incipient and established colonies. Specifically, we find workers from incipient colonies do not police worker-laid eggs; egg-policing behaviour emerges only when colonies grew sufficiently large (Figure 1). At the ultimate level, our results suggest that the level of conflict within a colony changes across colony development, despite constant relatedness. At the proximate level, our results show the response to fertility information is facultative and changes across colony development.

Our results underscore the importance of factors besides relatedness as the ultimate explanation for patterns of policing behaviour. Relatedness is constant across colony growth in *C. floridanus*, but policing behaviour is absent in incipient colonies and present in large colonies (Figure 1). Relatedness alone cannot account for the pattern of policing behaviour observed in *C. floridanus*.

Mutual worker policing can also evolve when the costs of worker reproduction are high [32,33]. Surprisingly, our results show worker policing is absent in incipient colonies, when the costs of worker reproduction are greatest [6]. Nevertheless, worker reproduction seems not to occur in incipient colonies, since males are not produced and workers do not have developed ovaries (Moore, unpublished data). Why is egg policing unnecessary in incipient colonies? While we cannot exclude coercion by alternate mechanisms, we hypothesize policing behaviour is not expressed in incipient colonies because the costs of worker reproduction are so high that there is no incentive for workers to reproduce [5]. Worker reproduction diverts resources to reproduction at the expense of somatic growth [4,32,33,36-38], and incipient colonies are especially vulnerable to failure [39]. The number of sexuals that can be produced by an incipient colony is negligible compared to the number of sexuals that can be produced if the colony survives to maturity. It is in the workers' self-interest to channel their efforts toward colony growth (i.e., future reproduction) rather than divert resources for immediate male production. Mechanisms for enforcing sterility, including egg policing, are unnecessary if worker sterility is voluntary. Early in colony development, cooperation can occur without external enforcement.

A second, non-mutually exclusive hypothesis for the absence of worker policing in incipient colonies is the cost of recognition errors or informational constraints [64,65]. As our chemical data indicate, eggs laid by workers are very similar to eggs laid by incipient queens in the composition of their surface hydrocarbons, which may be an information constraint or provide insufficient information for a sufficiently large number of correct decisions (Figure 2). To avoid cannibalizing the queen's eggs, workers may be selected to have permissive acceptance thresholds for conspecific eggs in incipient colonies. This is consistent with the general egg tolerance we observed in incipient colonies (Figure 1). In a previous study, we showed that physical policing of reproductive workers is absent in larger colonies of *C. floridanus* [38]. The lack of informational differences potentially encoded in cuticular hydrocarbons between reproductive and non-reproductive workers may be involved here as well.

Our results show that the response of a worker to a conspecific egg changes with the developmental stage of the worker's colony. Workers from incipient colonies tolerated all conspecific eggs, regardless of origin, whereas workers from large colonies destroyed worker-laid eggs. Egg destruction by workers from large colonies is mediated by fertility-related hydrocarbons on the egg surface [19]; workers from large colonies destroy eggs laid by incipient queens and workers, which lack the shorter-chained, fertility-related hydrocarbons found on the surface of eggs laid by established queens [19,52]. Our results show the absence of fertility-related hydrocarbons does not trigger egg-policing behaviour in workers from incipient colonies. The response of workers to fertility information on eggs changes with colony growth.

Interestingly, workers from the largest colony size class tested in the present study showed a trend toward the destruction of eggs laid by foreign, established queens (Figure 1). Although the trend was non-significant, it seemingly conflicts with earlier studies, which show high survival of eggs laid by foreign, established queens [19,52]. One potential explanation for the discrepancy between the current results and those reported previously is that the source colony of the established, queen-laid eggs used in the current study was 1 to 6 years older than the discriminator colony. In previous experiments, the source colony was the same age or younger than the discriminator colony. Our chemical data indicate the median abundance of fertility-related hydrocarbons on the eggs of the older queens was less than the median abundance of fertility-related hydrocarbons on the eggs of yearling queens (Figure 2), potentially as a consequence of limited growth under laboratory conditions [48]. Workers from 1-year-old colonies may have destroyed eggs from older queens when the strength of the foreign queen's fertility signal was weaker than that of their own queen.

Workers from incipient and established colonies also differed in their response to fertility information in the context of queen introductions. Workers from incipient colonies attacked foreign, established queens in every trial, whereas workers from large colonies tolerated foreign, established queens in all but two trials (Figure 3). Workers from both incipient and large colonies attacked foreign, incipient queens (Figure 3). Tolerance of foreign, established queens by workers from large colonies is thought to occur because the queen's fertility status overrides colony membership information [57]. Incipient queens lack a strong fertility signal, and thus workers from both incipient and large colonies attack them. The rejection of established queens by workers from incipient colonies shows that a strong fertility signal does not guarantee acceptance in every context. It also contrasts

with our findings from the egg-policing assay, in which workers from incipient colonies tolerated eggs laid by foreign, established queens. This indicates a worker's response to fertility information depends on the recognition context: eggs or adults. Together, our egg-policing and queen-tolerance assays demonstrate the response to fertility information is not fixed, but changes across colony development.

This paper is the first to show that a worker's response to fertility signals changes with colony life stage, but we expect it is a widespread phenomenon. If queen fertility pheromones indeed serve as an honest indication of reproductive capacity [40,49], then changes in fertility signals corresponding with colony size should be a common feature of social insect colonies. When the queen's fertility signal changes throughout colony development, we predict workers' response to fertility information also changes [48].

Further research is necessary to identify the proximate mechanisms that account for the change in workers' responses to fertility signals. The change in behaviour may be the result of physiological differences between workers from incipient and large colonies (e.g., maternal effects), or the behaviour may be triggered by environmental cues. In particular, experience with fertility signals may change workers' acceptance thresholds [48,66,67]. It is also possible that the egg-tolerance observed in small colonies is due to age-dependent expression of policing behaviour; for example, if ants do not show policing behaviour until they reach a certain age, then egg policing may be absent from small colonies because the workers are not sufficiently old to demonstrate policing behaviour [34,68]. Similarly, if worker sterility in incipient colonies is voluntary, then it is necessary to determine the mechanism that induces worker sterility in incipient colonies. In large colonies, queen-laid eggs with fertility-related hydrocarbons have been shown to induce worker sterility [19], but no such eggs exist in incipient colonies. Determining the proximate mechanisms that generate the behavioural change we report here is critical to understanding the regulation of reproduction across colony development.

Although the regulation of worker reproduction has been studied intensively in social insects, very few studies have investigated the regulation of worker reproduction across the colony life cycle. This is problematic at two levels. First, the proximate mechanisms regulating worker reproduction can change across colony ontogeny [69], and these changes will only be apparent in studies that consider a range of developmental stages. Second, the intensity of regulation may change during colony development, according to the ultimate explanations for the evolution of policing behaviour. Ohtsuki & Tsuji were the first to predict that the level of policing

behaviour in a colony depends on the colony's developmental stage [6]. Specifically, their model predicts policing behaviour will be expressed in growing colonies because male production early in the colony life cycle reduces the future inclusive fitness of colony members. Our results do not match this prediction; we find workers from incipient colonies do not destroy worker-laid eggs, and policing behaviour emerges only with colony growth. However, our results are consistent with the broader prediction of Ohtsuki & Tsuji's model, which is that the expression of policing behaviour depends on the stage of colony growth. Perhaps the absence of egg policing in very small colonies, such as the incipient colonies tested here, represents an unanticipated phase of colony growth in which worker sterility is self-imposed and policing is not necessary. It is also possible that the self-restraint of workers from incipient colonies is the result of strong policing in the past, which has subsequently resulted in reproductive acquiescence in *C. floridanus* workers from small colonies.

Another prediction of Ohtsuki & Tsuji's model is that worker policing should subside in monogynous, monandrous species once the colony reaches reproductive maturity [6]. The largest colonies tested in the current study contained only 1000 to 2000 workers, whereas field colonies can grow up to 10,000 workers in size. Just as our current results show that the behaviour of a colony with 80 workers cannot be extrapolated from the behaviour of a colony with 1000 workers, the behaviour of a colony with 10,000 workers cannot be extrapolated from the behaviour of a colony one-tenth its size. To understand the ultimate causes of social regulations in social insects, it is essential to test regulatory behaviour across the life cycle of the colony.

Conclusions

The response to fertility information in an ant colony changes radically during colony ontogeny. We found workers from incipient colonies tolerate all conspecific eggs, but are aggressive toward foreign, established queens. In contrast, workers from large colonies discriminate against worker-laid eggs, but tolerate foreign, established queens. Together, these results show the response of workers to the presence or absence of fertility information changes over the course of a colony's life cycle. At the ultimate level, our results stress the importance of factors other than relatedness for understanding the regulation of reproduction. In particular, we suggest levels of intracolony conflict change across colony development, and as a consequence, mechanisms for managing conflict also change.

Nearly everything we know about social regulation in social insects comes from colonies beyond the earliest phases of colony growth. This study shows that regulatory mechanisms observed in one stage of colony

development cannot be generalized to all stages of a social insect colony's life cycle. We are missing critical parts of the colony's life cycle in our understanding of social regulation of reproduction. Further research is necessary to understand how social insect colonies are organized across the colony life cycle.

Methods

Animals and culturing conditions

Founding queens were collected after mating flights in the Florida Keys, USA, in August 2001, July 2002, November 2006, August and October 2007, August 2008 and August and November 2009. The queens were transferred to the lab and cultured as described in [19,52,57]. Queens were cultured singly because *C. floridanus* is haplo-diploid.

Egg discrimination bioassay

To determine how egg-policing behaviour changes with colony size, we tested the egg-policing behaviour of workers from 15 colonies collected in August and October 2007 at three points during the colony's development: when they contained 60 to 80 workers (small colony size), 200 to 300 workers (intermediate colony size) and more than a 1000 workers (large colony size). These sizes correspond to groups B, C, and D in [52] and were reached 4 to 6, 7 to 9, and 10 to 14 months after being collected as foundations in the field, respectively. At each size, we tested the response of workers to eggs laid by their own queen; eggs laid by a non-nestmate, established queen; and to eggs laid by non-sister workers. Three groups of twenty ants each were isolated from the experimental colonies with water and sugar-water and allowed to habituate for 30 minutes before receiving 10 eggs from one of the three egg sources. After 24 hours, we counted the number of eggs remaining. The study was not performed blind, but because *C. floridanus* eggs are relatively large (0.1 cm) and easily visible to the naked eye, the egg counts should be robust to observer bias. Eggs laid by non-nestmate, established queens came from large (>1000 workers), healthy colonies collected in August 2001, July 2002, and November 2006. Worker-laid eggs came from non-sister workers in worker groups originating from colonies collected in August 2001 and July 2002 and orphaned 4 to 18 months before testing. Due to logistical limitations, worker-laid eggs and established-queen-laid eggs for our egg discrimination bioassay had to come from non-nestmates. In other ant species, workers discriminate against eggs originating from non-nestmates [67]. However, two lines of evidence suggest the egg-layer's colony membership is less important than the egg-layer's fertility status in determining a worker's response to an egg in *C. floridanus*: (1) workers from large colonies destroy eggs laid by sister workers

[19] and (2) workers from large colonies do not destroy eggs laid by foreign, established queens [19,52].

Statistical analysis

Due to extreme heteroscedasticity of the egg survival data, parametric approaches were not appropriate. We used a randomization analysis of a multifactor ANOVA with colony size and egg source as fixed factors and recipient colony as a random factor; we used 10,000 rearrangements of the data to calculate the p-value [70]. Randomization analyses are robust to heteroscedasticity when sample sizes are equal [71]. Analysis was done in SAS (SAS Institute, Inc.) with the randomization wrapper written by Cassell [72]. A randomization analysis of a paired t-test was used for post-hoc comparisons. P-values calculated by randomization analyses are denoted by a subscript indicating the number of permutations.

Chemical analysis

We analyzed the egg surface hydrocarbons of a subset of the eggs laid by workers ($N = 8$) and by foreign, established queens ($N = 15$), as well as eggs laid by the experimental colony queens when their colonies were small ($N = 8$), intermediate ($N = 12$), and large ($N = 13$) in size. Sample sizes reflect the number of colonies from which we removed 10 eggs and extracted the eggs' surface hydrocarbons in 100 μ l of hexane for 2 minutes. The hexane was transferred to a clean vial and allowed to evaporate. We reconstituted the extract in 5 μ l of hexane and injected 1 μ l of the resulting suspension in the injection port of an Agilent 6980N series gas chromatograph (GC). Further details are described in [19]. Peak areas were measured in Enhanced ChemStation (Agilent Technologies 2005). We divided the egg surface hydrocarbon profiles into two parts as in [52]: the shorter-chained compounds characteristic of high-fertility queen eggs (*n*-pentacosane to 10-methyl-, 12-methyl, and 14-methyloctacosane), and the longer-chained compounds common to all eggs (12,16-dimethyloctacosane to 5,9,13,17-tetramethyltrtriacontane) [19,52,57]. We summed the peak areas of each part of the profile and compared the proportion of the overall profile represented by the shorter-chained fertility hydrocarbons using the median test in Statistica 7.1 (StatSoft Inc.). Two-sample median tests were used for the post-hoc comparisons; the Bonferroni correction was applied to account for the number of comparisons. We analyzed the proportion of the entire hydrocarbon profile represented by 3-methylheptacosane, the most prominent compound in the fertility signal, using the same approach. The 3-methylheptacosane peak of one exceptional established queen was below the determined detection threshold.

Nestmate recognition bioassay

We tested the reaction of the ants in the small colonies to individuals from their own colony, individuals from

another small colony (60 to 80 workers), and individuals from a large colony (>1000 workers) [73]. The introduced ants were removed from their colony and painted with a single dot of white Testor's enamel paint and allowed to dry for one hour. Meanwhile, we opened the lid of the experimental colony and allowed the ants to settle for at least 20 minutes. We then gently lowered one of the introduced ants into the nestbox with clean forceps (Figure 4). We observed the reaction of the experimental ants to the introduced ants for 5 minutes or until aggression was observed. An introduction was classified as "aggressive" if the experimental ants bit and held the focal ant or sprayed it with formic acid. The order of the introductions was random and the experimenter was blind to the identities of the individuals. We analyzed the aggression data using Cochran's Q test.

Tolerance of foreign queens by workers from incipient colonies

We tested the response of workers from incipient colonies (< 40 workers, $N = 9$) to established queens from large colonies (>1000 workers). The same queens were also introduced to workers from large colonies as a

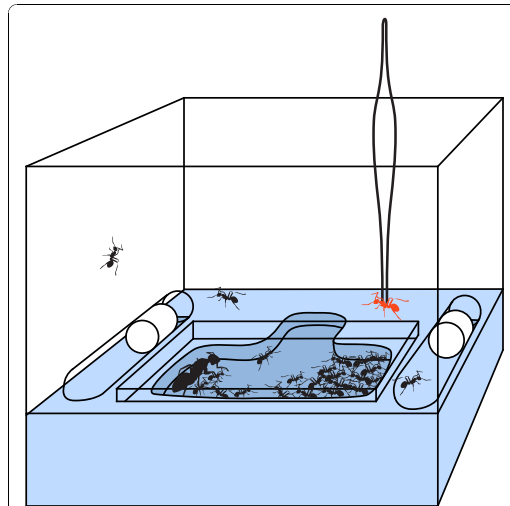


Figure 4 Experimental set-up for nestmate recognition assay.

For the nestmate recognition assay, focal ants were introduced directly into the incipient colony's nest box. Each colony is housed in a transparent plastic box (10 cm wide \times 7 cm high) with a dental plaster floor and a molded nest chamber. The nest chamber is covered with a plate of glass and has only one entrance. In incipient colonies, nearly all the workers are in the nest chamber; very few workers are outside the nest. With clean forceps, we lowered the focal ant (shown in red) into an unoccupied area of the nest box and the observed the reaction of the colony members to the introduced ant for 5 minutes.

control. The order of introductions for each queen (small or large colony first) was random, and we waited at least 24 hours between introductions. The small colonies were reared from foundresses that had been collected in August 2008 and the large colonies were reared from foundresses collected in August and September of 2007.

Twenty workers from the experimental colony were removed and isolated in a circular arena (8.4 cm diameter × 3.5 cm height) lined with clean copy paper. The arena's wall was coated with Fluon (Northern Products Inc.) to prevent escape. The workers were allowed to habituate for 30 minutes before the focal queen was gently lowered into the arena using clean forceps. The queen had been removed from her own colony and isolated with 5 worker ants at least 30 minutes before the start of the trial.

The queen remained in the arena for 3 minutes after its first encounter with one of the experimental workers or until aggression was observed. If aggression was observed, the queen was removed immediately from the arena to minimize the damage inflicted by the workers. An introduction was classified as "aggressive" if the experimental ants bit and held the focal ant or sprayed her with formic acid. All introductions were recorded on HDV film. We analyzed the aggression data using McNemar's test.

The same procedure was used to contrast the response of workers from incipient and large colonies to foreign, incipient queens. Incipient queens and workers came from colonies collected in August and November 2009. Incipient colonies had 28 to 178 workers at the time of testing (median = 86, $N = 9$). Workers from large colonies came from colonies collected in August 2008.

Acknowledgements

We thank A. Endler for collecting founding queens in 2001-02; D. Young for his help with the statistical analysis; and T. Bloss, B. Hölldobler, K. Haight, N. Mutti, C. Penick, A. Smith and four anonymous referees for their comments on the manuscript. DM was supported by a National Science Foundation Graduate Research Fellowship during the preparation of this manuscript.

Authors' contributions

DM and JL designed the experiments and wrote the manuscript. DM conducted the experiments and statistical analyses. All authors read and approved the final draft.

Received: 1 June 2010 Accepted: 27 October 2010
Published: 27 October 2010

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doi:10.1186/1471-2148-10-328

Cite this article as: Moore and Liebig: Mechanisms of social regulation change across colony development in an ant. *BMC Evolutionary Biology* 2010 **10**:328.

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APPENDIX B

MIXED MESSAGES: FERTILITY SIGNALING INTERFERES WITH
NESTMATE RECOGNITION IN THE MONOGYNOUS ANT *CAMPONOTUS*
FLORIDANUS

Mixed messages: fertility signaling interferes with nestmate recognition in the monogynous ant *Camponotus floridanus*

Dani Moore · Jürgen Liebig

Received: 29 September 2009 / Revised: 12 December 2009 / Accepted: 26 January 2010 / Published online: 24 February 2010
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Abstract Chemical communication is crucial for the organization of social insect colonies. However, with the heavy use of one communication modality, problems may arise such as the interference of different types of information. This study investigated how information about fertility and colony membership is integrated in the ant *Camponotus floridanus*. We introduced into mature, queen-right colonies (a) the nestmate queen, (b) a nestmate worker, (c) a foreign, high-fertility queen, (d) a foreign, low-fertility queen, and (e) a foreign worker. As expected, workers did not attack their nestmate queen or a nestmate worker but responded aggressively to foreign workers and foreign, low-fertility queens. Surprisingly, workers did not attack foreign, high-fertility queens. Chemical analysis demonstrated that the cuticular hydrocarbon profile of *C. floridanus* encodes information about fertility status in queens and workers and colony membership in workers. We suggest that ants respond to this information in the cuticular hydrocarbon profile: individuals with strong fertility signals are accepted regardless of their colony membership, but individuals without strong fertility signals are tolerated only if their cuticular hydrocarbon profile matches that of colony members. Learning how social insects respond to multiple types of information presented together is critical to our understanding of the recognition systems that permit the complex organization of social insect colonies.

Keywords Cuticular hydrocarbons · Nestmate recognition · Fertility signal · Queen pheromone · Formicidae

Introduction

The complex social behavior of ants and other social insects requires multiple levels of recognition. Ants can distinguish nestmates from non-nestmates, which allows them to limit altruism and cooperation to members of their own colony and protect their colony from exploitation by outsiders. Within a colony, ants may recognize the fertility status, caste, dominance rank, and/or task group of a nestmate and adjust their behavioral response to that individual accordingly (D'Ettorre 2008; Le Conte and Hefetz 2008). Understanding how ants process multiple levels of identity information simultaneously is a critical step toward understanding the recognition system that permits the extraordinary social coordination of an ant colony.

Cuticular hydrocarbons (CHCs) are involved in a diverse range of recognition functions in ants (reviewed in Howard and Blomquist 1982, 2005; Monnin 2006; Hefetz 2007; Le Conte and Hefetz 2008; Peeters and Liebig 2009). Experimental evidence shows that CHCs are involved in the recognition of nestmates (direct evidence: Lahav et al. 1999; Akino et al. 2004; Ozaki et al. 2005; Martin et al. 2008; Brandstaetter et al. 2008), dominance and fertility status (correlative evidence: Peeters et al. 1999; Dietemann et al. 2003; D'Ettorre et al. 2004; direct evidence: Smith et al. 2009), and task group membership (correlative evidence: Bonavita-Cougourdan et al. 1993; direct evidence: Greene and Gordon 2003). The use of CHCs in multiple recognition contexts implies that the CHC profile sometimes encodes at least two different types of information simultaneously (e.g., colony membership and fertility; Cuvillier-Hot et al. 2001;

Communicated by O. Rueppell

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Denis et al. 2006). This raises the possibility of interference of information.

The prevailing model of nestmate recognition suggests that ants detect variation in the relative abundance of the compounds in the CHC profile and respond antagonistically to individuals with CHC profiles that do not match that of their colony members (Vander Meer and Morel 1998). The use of CHCs in nestmate recognition and fertility signaling thus presents an intriguing paradox: to serve as reliable nestmate recognition cues, CHCs must be uniform throughout a colony; to serve as indicators of fertility, the CHC profile of a queen must be distinct from her non-reproductive nestmates. Although this paradox has received some theoretical attention (Dietemann et al. 2003; Hefetz 2007; Le Conte and Hefetz 2008), few studies have systematically tested the response of workers to different combinations of colony-membership and fertility information (Helanterä and Sundström 2007; Cournault and de Biseau 2009). Such experiments are the first step to understanding how this information paradox is resolved.

We explored how fertility and colony-membership information is integrated in the carpenter ant *Camponotus floridanus*. This highly aggressive species has long been a model system for the study of nestmate recognition in ants (e.g., Carlin and Hölldobler 1986, 1987). Studies of *C. floridanus* and congeners suggest that individuals of this species use colony-specific CHC profiles to identify nestmates (Morel et al. 1988; Lavine et al. 1990; Ozaki et al. 2005; Brandstaetter et al. 2008). *C. floridanus* is also one of the first species in which CHCs were directly linked to fertility signaling (Endler et al. 2004, 2006). Mature queens display a mixture of shorter-chained hydrocarbons on their cuticle in addition to the longer-chained hydrocarbons present on the cuticles of workers (Endler et al. 2004). The abundance of these queen-specific hydrocarbons in a queen's CHC profile correlates with her fertility status; queen-specific compounds are absent from the CHC profiles of founding queens, which lay very few eggs per day, but increase in abundance as colony size and queen egg-laying rate increase (Endler et al. 2006). The relationship between CHC profiles, colony size, and queen fertility is independent of queen age, since the queen's CHC profile will revert if colony size, and hence queen egg-laying rate, is experimentally reduced (Liebig, unpublished data).

We investigated whether *C. floridanus* workers retrieve and respond to both the fertility and colony membership of conspecifics introduced into their nest. We introduced into mature, queenright colonies (a) the nestmate queen, (b) a nestmate worker, (c) a foreign, high-fertility queen, (d) a foreign, low-fertility queen, and (e) a foreign worker. If workers respond to fertility information only after responding to an individual's colony-membership information, we

expect to see aggression against all foreign ants, regardless of their fertility status. If workers respond to colony membership only in the absence of a strong fertility signal, then foreign, high-fertility ants should not be attacked. We correlate the behavioral response with CHC patterns to provide a potential proximate mechanism for the behavioral pattern we observe.

Materials and methods

Study species and culturing conditions

C. floridanus is a common ant from the southeastern United States. Each colony has one single-mated queen (Gadau et al. 1996), and re-queening is unknown in this and most other obligately monogynous species (Hölldobler and Wilson 1990; Heinze and Keller 2000; Sanetra and Crozier 2002). Founding queens were collected from the Florida Keys, USA, in July 2002, November 2006, August and October 2007, and August 2008. The colonies originated between Cudjoe Key and Long Key, Florida, USA, a distance of 45 miles. The founding queens were cultured in dental-plaster nests with molded chambers. The ants were provided with sugar water, artificial diet (Bhatkar and Whitcomb 1970), and pieces of cricket (*Acheta domestica*) or beetle larvae (*Zophobas morio*) twice a week. The laboratory was maintained at 25°C on a 12 h:12 h light:dark cycle.

Aggression bioassays

We used 30 large (>1,000 workers) and 15 small (<60 workers), queenright colonies for the aggression bioassays. The large colonies were 1 to 2 years old (collected in 2006 and 2007), and the small colonies were less than 6 months old (collected in 2008). In December 2008, we introduced the following ants into 15 of the large colonies: the nestmate queen; a nestmate worker; a foreign, high-fertility queen (i.e., queen from a large colony); a foreign, low-fertility queen (i.e., queen from a small colony); and a foreign worker. For further details regarding the relationship between colony size and queen fertility, see Endler et al. (2006). The order of the introductions was random, and the experimenter was blind to the identities of the introduced ants. All focal ants were marked the previous day with a single dot of yellow Testor's enamel paint. At least 30 min before beginning the experiment, all focal ants were removed from their colonies and transferred to small, plastic holding pens with plaster floors.

Each focal ant was removed from its holding pen and gently lowered into a circular arena (8.4 cm diameter × 3.5 cm height) inside the experimental colony's nest box

using clean forceps. Both sides of the arena's wall were coated with Fluon (Northern Products Inc.) to prevent traffic between the experimental arena and the rest of the colony. Twenty minor workers were placed into the arena and allowed to habituate for 30 min before the introduction of each focal ant. These workers were replaced after each introduction and were not used more than once. The copy paper lining the bottom of the arena was exchanged after each introduction.

Each focal ant remained in the arena for 3 min after its first encounter with the experimental workers in the arena or until aggression was observed. Because we were exclusively interested in the perception and integration of information and not the possibility of long-term queen adoption, a 3-min assay was sufficiently long to meet the objective of this study. An introduction was classified as “aggressive” if the experimental ants bit and held the focal ant or sprayed her with formic acid (initial classification by DM). In two cases, the focal ant attacked the experimental ants but the experimental ants did not attack the focal ant. These trials were not counted toward the total number of “aggressive” encounters. In both cases, the focal ant was a foreign worker.

Because we used very distinctive behaviors to define aggression, the categorization of each encounter was unambiguous. Nevertheless, each introduction was recorded on HDV film and the classifications confirmed by another researcher (JL) who was also blind to the identities of the focal ants. Data were analyzed using Cochran's Q test; McNemar's test was used to detect pairwise differences. The Bonferroni correction was used to adjust the significance level for the pairwise comparisons.

Fertility signal

To determine the CHC profiles of the ants used in the aggression assays, we sampled the CHC profile of focal ants using solid-phase micro-extraction (Arthur and Pawliszyn 1990; Monnin et al. 1998; Liebig et al. 2000). The day before an ant was scheduled to be used in an aggression assay, a fiber (Supelco Inc.) coated with a 30- μ m polydimethylsiloxane film was drawn across her tergites 100 times and then inserted into the injection port of an Agilent 6980N series gas chromatograph (GC) equipped with a DB1-MS (J&W Scientific) non-polar capillary column (30 m \times 0.25 mm \times 25 μ m) and a flame ionization detector. The GC injection port was set to 250°C. The column temperature was held at 60°C for 2 min before increasing to 200°C at 20°C min⁻¹ and then to 320°C at 5°C min⁻¹. Helium was used as the carrier gas at 1 ml min⁻¹, and samples were injected in splitless mode. Peak areas were measured in Enhanced ChemStation (Agilent Technologies 2005).

We divided the CHC profiles into two parts as in Endler et al. (2006): the “fertility signal”, which includes the shorter-chained compounds observed only in queens (*n*-pentacosane to 12,16-dimethyloctacosane; Fig. 1a, part I), and the “colony profile,” which includes the longer-chained compounds present in both queens and workers (*n*-nonacosane to 5,9,13,17-tetramethyltrtriacontane; Fig. 1a, parts II and III; see Endler et al. 2004, 2006). This classification of fertility is very conservative, since the rest of the profile is also affected by differences in fertility increasing the actual differences between highly and weakly fertile individuals (see Fig. 1). The colony profile was further divided into two parts, part II (*n*-nonacosane to 4-methyltriacontane and 12,16-dimethyltriacontane) represents the portion of the profile that is more intense in high-fertility queens; part III (4,8-dimethyl-, 4,10-dimethyl-, 4,12-dimethyl-, and 4,14-dimethyltriacontane to 5,9,13,17-tetramethyltrtriacontane) represents the region that is more conserved between queens and workers.

To compare the relative abundance of the fertility signal portion of the profile, we summed the peak areas of each part of the profile and compared the proportion of the overall profile represented by the short-chained fertility signal (part I) in workers, low-fertility queens, and high-fertility queens using a Kruskal–Wallis ANOVA and post-hoc analysis in Statistica 7.1 (StatSoft Inc.). To see if fertility affects the colony profile, we performed non-parametric, multi-dimensional scaling on the relative proportions of the ten largest peaks in part III of the colony profile using Primer 6 (Primer-E Ltd.). Euclidean distances were used to calculate the distance matrices.

Colony signature

To determine if the CHCs of *C. floridanus* from our study population can encode information regarding colony membership in workers, we compared the CHC profiles of 24 major workers from three colonies. The colonies were selected at random, but the selection process was engineered so that two colonies originated from the same collection site (Long Key, Florida, USA; collected in Nov. 2006 and Oct. 2007) and the third colony from 45 miles away (Sugarloaf Key, Florida, USA; collected in July 2002) to better capture the range of inter-colony variation. The presence of colony information in the profiles of high-fertility queens was not tested because there is only one queen per colony, and multiple queens would be necessary to show this colony component.

Individual ants were placed in glass vials with 100 μ l of hexane. After 2 min, the ants were removed and the hexane was evaporated. The extracts were then reconstituted with 20 μ l of hexane, and 1 μ l of the resulting

Fig. 1 **a** Chromatograms of CHC profiles. We consider part I to be the fertility profile, since it is absent in workers and low-fertility queens. Parts II and III are present on all individuals and we term them the colony profile (Endler et al. 2006). Part III indicates the portion of the colony profile that is conserved between workers and queens, part II indicates the portion that is more intense in queens. The chromatograms are representative of the respective group; the high-fertility queen and worker are from the same colony to allow the comparison of within-colony similarity in the colony profile. Compound identities are reported in Endler et al. (2004). **b** The fertility signal compounds are a greater percentage of the total CHC profile in high-fertility queens ($n=30$) than in low-fertility queens ($n=14$) or workers ($n=29$). Bars, boxes, and points indicate the range, quartiles, and median, respectively. Different letters indicate significant pairwise differences. **c** Two-dimensional configuration of non-metric, multi-dimensional scaling of differences in part III of the colony profile among high-fertility queens, low-fertility queens, and workers. In addition to clustering by fertility status (high versus low), the data also cluster according to geography. Individuals marked with triangles and asterisks are from our southern collection sites (Little Torch Key to Cudjoe Key), and individuals marked with circles or X's are from our northern collection sites (Long Key to Grassy Key)

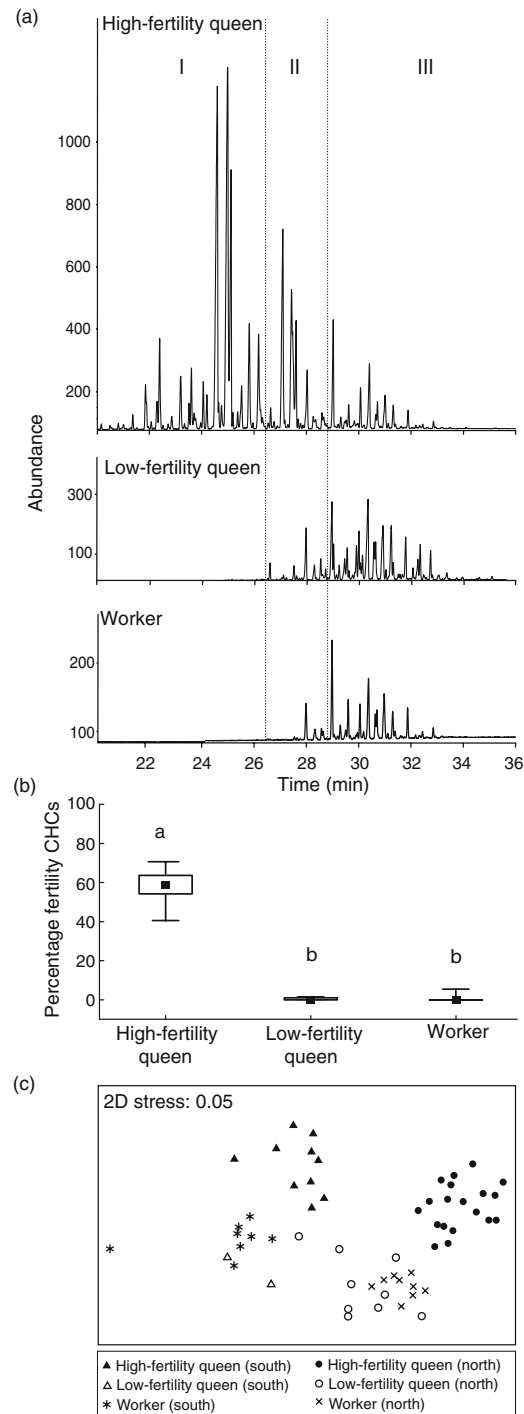
suspension was injected into a GC connected to an Agilent 5975 series mass selective detector. The GC injection port was set to 260°C; all other settings were as described above. Electron impact mass spectra were measured at 70 eV with a source temperature of 230°C. We confirmed that the compounds were hydrocarbons by comparing their mass spectra with published spectra (for compound identities, see Endler et al. 2004).

The relative proportions of the 11 largest peaks were used in the statistical analysis (the ten peaks used above, plus an additional peak from part II which was larger than the smallest of the peaks used in the previous analysis). We performed non-parametric, multi-dimensional scaling to analyze the similarity of CHC profiles of the three colonies using Primer 6 (Primer-E Ltd.). Euclidean distances were used to calculate the distance matrices.

Results

Aggression bioassays

In the aggression bioassay, we tested how ants react to individuals of varying fertility and colony membership. Foreign, low-fertility queens were attacked in all 15 trials, and foreign workers were attacked in 12 of 15 cases. In the 15 replicates, nestmate queens and nestmate workers were never attacked, and a foreign, high-fertility queen was attacked in only one trial. Nestmate queens, nestmate workers, and foreign, high-fertility queens all received significantly less aggression than foreign workers and foreign, low-fertility queens (Cochran's Q test, $Q_4=50.762$, $p<0.001$; Fig. 2). In aggressive trials, the median time between the introduction of the focal ant and aggression was 13 s (range=2–93 s).



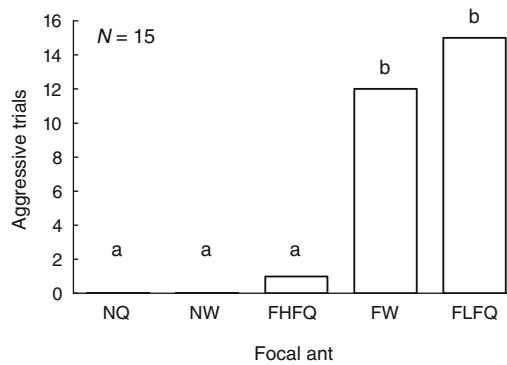


Fig. 2 Introductions of foreign workers (*FW*) and foreign, low-fertility queens (*FLFQ*) were significantly more likely to end in aggression than introductions of nestmate queens (*NQ*), nestmate workers (*NW*) or foreign, high-fertility queens (*FHFQ*). Different letters indicate significant pairwise differences

Fertility signal

The CHC profiles of mature queens were clearly distinguishable from those of founding queens and workers (Fig. 1a). Fertility signal compounds were a significantly greater percentage of the total CHC profile of high-fertility queens (median=58.6%, range=40.5–70.6%, $n=30$) than of low-fertility queens (median=0.3%, range=0.0–2.5%, $n=14$) or workers (median=0.0% range=0.0–6.3%, $n=29$; Kruskal–Wallis ANOVA, $H_2=59.153$, $p<0.0001$; Fig. 1b). This matches the pattern reported in Endler et al. (2006).

Non-parametric, multi-dimensional scaling of the ten largest peaks in part III of the CHC profile separated high-fertility queens from workers and incipient queens, which clustered together (Fig. 1c). The data also split according to geography; individuals from our southern collection sites (Little Torch Key to Cudjoe Key) clustered separately from individuals from our northern collection sites (Long Key to Grassy Key). The stress value of 0.05 indicates good graphical representation of the data structures (Clarke and Warwick 2001).

Colony membership

Non-parametric, multi-dimensional scaling of just the 11 largest peaks in the hydrocarbon profile of workers separated individuals into three distinct, colony-specific clusters with only one mismatch (Fig. 3). Including the additional 13 peaks present in the hydrocarbon profile of workers would probably result in even finer resolution of colony membership (Endler et al. 2004). The two colonies from Long Key (colonies 2 and 3) clustered more closely together than the colony (colony 1) from Sugarloaf Key.

The stress value of 0.03 indicates good graphical representation of the data structures (Clarke and Warwick 2001).

Discussion

The purpose of our study was to determine how information about colony membership and fertility is integrated in the ant *C. floridanus*. We found that *C. floridanus* can recognize both fertility status and colony membership, but strong fertility interferes with nestmate recognition. In our aggression bioassay, workers reliably attacked foreign workers and foreign, low-fertility queens but did not attack their nestmate queen or nestmate workers, as expected. Surprisingly, workers did not attack foreign, high-fertility queens (Fig. 2).

Morphology cannot be the factor that determines tolerance or rejection because foreign, low-fertility queens were attacked as frequently as foreign workers (Fig. 2), while high-fertility queens were not attacked. The principle difference between the attacked and tolerated individuals is their fertility status. The median egg-laying rate of queens from colonies the size of our small colonies is less than half the median egg-laying of queens from colonies the size of our large colonies (Endler et al. 2006). We conclude that fertility information interferes with the expected rejection of foreign individuals.

It is well established that CHC profiles correlate with fertility status (Peeters and Liebig 2009; Liebig 2010). In fact, CHCs are the only potential fertility signals identified in ants so far (except for the unique case of *Solenopsis*; Fletcher and Blum 1981; Vargo 1992). Direct evidence of the use of CHCs as a fertility signal has been demonstrated by Smith et al. (2009). In *C. floridanus*, the CHC profiles of



Fig. 3 Two-dimensional configuration of non-metric, multi-dimensional scaling of cuticular hydrocarbon blend differences among three colonies. Colony 1 originated from Sugarloaf Key (southern collection site) and colonies 2 and 3 originated from Long Key (northern collection site)

low- and high-fertility queens differ dramatically (Fig. 1a). More than 50% of the hydrocarbons found on the cuticle of a high-fertility queen belong to the queen-specific fertility signal. These compounds make up less than 1% of the total abundance of hydrocarbons on the cuticle of low-fertility queens, comparable to their abundance on workers (Fig. 1b). The CHC profile also reliably indicates colony membership in workers (Fig. 3), but potentially not in high-fertility queens (Fig. 1c). Therefore, the CHC profile contains all the necessary information to explain the pattern of discrimination we observed. This, coupled with the extensive evidence connecting CHCs to fertility signaling and nestmate recognition (Howard and Blomquist 1982, 2005; Monnin 2006; Hefetz 2007; Le Conte and Hefetz 2008; Peeters and Liebig 2009), strongly suggests that CHCs underlie the behaviors we report here.

Altogether, the response of *C. floridanus* workers from mature colonies to introduced ants of varying fertility and origin suggests fertility status as the major factor determining the response to foreign individuals in our experimental setup. High-fertility individuals were tolerated regardless of their colony membership, even when colonies originated from up to 40 miles apart and thus potentially have dramatically different colony-specific profiles (Fig. 1c; Fig. 3). Low-fertility individuals, in contrast, were attacked unless they originated from the experimental colony. A similar pattern is observed in studies of egg policing in *C. floridanus*. Eggs with a strong fertility signal are accepted by workers, even when they are laid by a foreign queen, whereas eggs lacking a strong fertility signal (i.e., eggs laid by workers or queens from small colonies) are destroyed (Endler et al. 2004, 2006). Endler et al. (2004) provide direct evidence for the involvement of hydrocarbons in the response of *C. floridanus* workers to eggs: eggs with a weak fertility signal can be rescued from destruction by applying queen hydrocarbons to their surface. Since adult ants exhibit a similar pattern of surface hydrocarbons as eggs (Endler et al. 2006), this further supports the possible role of hydrocarbon profiles as the information medium that determines the tolerance or rejection of adult ants by conspecifics. It also is consistent with the conclusion that fertility information is more important than colony-membership information in determining the response to adults and eggs in *C. floridanus*.

Nestmate recognition is essential for maintaining colony integrity in social insects. One of the potential costs of accepting non-nestmates is selfish reproduction by the intruder (Lopez-Vaamonde et al. 2004). Accepting an unrelated queen is even more costly than accepting an unrelated worker, since a queen has greater reproductive potential (Bourke and Franks 1995). For this reason, we expect workers to discriminate against foreign queens if they can detect them (Kikuchi et al. 2007). Surprisingly, we

found that ants tolerate foreign, high-fertility queens without aggression in our 3-min trials. We propose three potential proximate explanations for the tolerance of non-nestmate queens in *C. floridanus*: (1) high-fertility queens may lack reliable colony-recognition cues, (2) workers may fail to perceive colony-membership cues in the presence of a strong fertility signal, or (3) workers detect both fertility and colony-membership information, but prioritize fertility information over colony-membership information. All three scenarios are evolutionarily plausible, since mature queens do not leave the nest except in the most extreme circumstances (e.g., colony migration, which are rare in *Camponotus*; Hölldobler and Wilson 1990), and they are always surrounded by a protective retinue of workers. This means that mature *C. floridanus* queens are unlikely to encounter any workers besides their own daughters, and conversely, workers are unlikely to encounter a mature queen besides their own mother. Consequently, there is little selective pressure for queens to display colony-specific information, and there is little selective pressure for workers to perceive or respond to colony-specific information in an interaction with a mature queen. At this time, we cannot exclude any of the three hypotheses, but the third hypothesis seems the least likely of the three, since in one trial workers attacked a foreign queen. This is inconsistent with the idea that a strong fertility signal in and of itself triggers tolerance. It is more probable that a strong fertility signal interferes with the production or perception of colony level cues. This is consistent with our finding that the colony profile region of high-fertility queens differs systematically from the colony profiles of workers and incipient queens (Fig. 1c).

Although CHC profiles are known to be involved in many recognition contexts in ants, we still know very little about how information is encoded and extracted from CHC blends (Ozaki et al. 2005; Greene and Gordon 2007; Martin and Drijfhout 2009; Guerrieri et al. 2009). How the CHC profile can encode multiple pieces of information is one of the many unresolved questions. Le Conte and Hefetz (2008) propose a hierarchical recognition model to explain how workers extract multiple pieces of information from a single CHC profile. They suggest large profile differences in the CHC profile trigger antagonism against non-nestmates, and smaller differences are used to recognize specific individuals or classes of individuals within the colony. If it holds that CHCs underlie the recognition of colony membership and fertility in *C. floridanus*, our results do not match the predictions of Le Conte and Hefetz's model. In our system, the difference between the profile of a high- and low-fertility member of the same colony is far more dramatic than the difference between low-fertility members of two different colonies, and high-fertility queens are accepted regardless of colony membership. Le Conte and Hefetz's model may apply to other species of social insects (Courmault and de

Biseau 2009). We expect that the response of ants to fertility and colony-membership information when presented together will vary with the ecology of the species and their respective selective pressures.

The only other studies explicitly designed to test the perception of colony and fertility information when presented together both indicate that colony information overrides fertility information. Helanterä and Sundström (2007) report that *Formica fusca* workers accept more eggs laid by their own queen than eggs laid by a foreign queen or by sister workers. Cournault and de Biseau (2009) show that *Linepithema humile* and *Tapinoma erraticum* recruit nestmate, egg-laying queens but not nestmate, non-egg-laying queens or foreign, egg-laying queens. It is not clear why the response pattern of *C. floridanus* differs from that of *F. fusca*, *L. humile*, and *T. erraticum*. *C. floridanus* is the only monogynous species investigated thus far. The transition from monogyny to polygyny is accompanied by a suite of life-history changes, and these may include changes that affect recognition systems (Bourke and Franks 1995). Another possibility is that the recognition pattern reported for *F. fusca*, *L. humile*, and *T. erraticum* is not connected to polygyny, but to particular details of their life history. More species must be tested before we can determine if tolerance of foreign reproductives is the norm or exceptional and begin to piece together the selective forces that lead to the prioritization of colony-membership or fertility information.

In this study, we demonstrate that *C. floridanus* workers attack foreign workers and foreign, low-fertility queens but do not attack their own queen, nestmate workers, or foreign, high-fertility queens. We further demonstrate the CHC profile correlates with fertility and colony membership in *C. floridanus*. We suggest that ants retrieve information regarding fertility and colony membership from the CHC profile, and that fertility information can interfere with nestmate recognition. Our study demonstrates the importance of exploring how different species of ants and other social insects integrate multiple chemical cues and signals. Such investigations are particularly important for information encoded in the CHC profile because the CHC profile contains multiple types of information in a single blend of structurally similar molecules. Learning how social insects respond to these types of information when presented together is critical to our understanding of the recognition systems that permit the complex organization of social insect colonies.

Acknowledgments We thank A. Ender for collecting founding queens in 2002 and A. B. Roddy, A. G. Dolezal, P. D'Ettorre, and three anonymous reviewers for their comments on the manuscript. DM was supported by a National Science Foundation predoctoral fellowship. All experiments were conducted in accordance with American statutes governing research. The authors declare that they have no conflict of interest.

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APPENDIX C
APPENDIX A ERRATUM

The median percentage of eggs laid by foreign, established queens recovered from groups of 20 workers from large colonies was 80 percent, not 60 as reported in the text.

APPENDIX D

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